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Program and Abstracts

Twenty-Fourth International Conference on Antiviral Research

Sponsored by

The International Society For Antiviral Research

Kempinski Hotel Zografski Sofia

Sofia, Bulgaria

May 8 – May 11, 2011

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Susan Cox
Avexa, Ltd.
576 Swan Street
Richmond, VIC 3121
Australia

Phone +(61 3) 9208 4066
Fax: +(61 3) 9208 4004
E-mail: scox@avexa.com.au

The International Society For Antiviral Research (ISAR)
The Society was organized in 1987 as a non-profit scientific organization for the purpose of advancing and disseminating knowledge in all areas of antiviral research. To achieve this objective, the Society organizes an annual meeting. The Society is now in its twenty fourth year of existence, and has about 550 members representing 30 countries. For membership application forms or further information, please contact Dr. Susan Cox, Secretary, ISAR; Senior Vice President, Drug Development, Avexa Ltd., 576 Swan Street, Richmond, VIC 3121, Australia, Telephone +(61 3) 9208 4066; Fax +(61 3) 9208 4004; E-mail: scox@avexa.com.au. Membership application forms will also be available at the Conference Registration desk, or from our website www.isar-icar.com.

International Society For Antiviral Research

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KEYNOTE ADDRESS

“Emerging Virus Infections and Intervention Strategies”

Albert (ADME) Osterhaus, Ph.D.

Sunday, May 8, 2011

5:00 – 6:00 PM

MINI-SYMPOSIA

"Emerging Diseases and Antiviral Therapy"

Monday, May 9, 2011

8:00 A.M. – 12:00 P.M.

“Clinical Update on Antiviral Drugs”

Tuesday, May 10, 2011

2:00 – 4:00 P.M.

“Medicinal Chemistry and Drug Discovery

Wednesday, May 11, 2011

8:00 A.M. – 12:00 PM.

SOCIAL EVENTS

Opening Reception

with light hors d’oeuvres

Sunday, May 8, 2011

6:00 – 8:00 PM

Conference Banquet

Wednesday May 11, 2011

Reception 7:15 PM

Dinner and Program 7:45 – 10:00 PM

All Scientific and Social Events will be held in the Kempinski Hotel Zografski Sofia, Sofia, Bulgaria

Final Program

Twenty-Fourth International Conference on Antiviral Research

Sponsored by the

International Society for Antiviral Research

Kempinski Hotel Zograski Sofia
Sofia, Bulgaria

May 8 – May 11, 2011

Sunday, May 8, 2011

Interactive Workshop: Drug Discovery and Development 101
Chair(s): Angel Galabov, Ph.D., Joseph Colacino, Ph.D., and Phillip Furman, Ph.D.
Sofia 1 and 2

02:00 PM - 04:00 PM

Opening Greetings
Sofia 1 and 2

14:00 PLENARY: Raina Fichorova, M.D., Ph.D. Brigham and Women's Hospital, Boston, USA
Vaginal Microbicides - Key Points of a Winning Strategy and Impact on the Mucosal Barrier

04:30 PM - 05:00 PM
16:30 Welcome to the 24th ICAR: Joseph Colacino, Ph.D., President-ISAR.
16:40 Welcome to Sofia: Angel Galabov, Ph.D., Local Host- 24th ICAR.
16:55 Introduction of the Keynote Speaker: Phillip Furman, Ph.D., President-Elect-ISAR.

Keynote Address
Chair(s): Phillip Furman, Ph.D.
Sofia 1 and 2

05:00 PM - 06:00 PM
1. Emerging Virus Infections and Intervention Strategies.
Albert (ADME) Osterhaus
Erasmus Medical Center, The Netherlands

Opening Reception
Sofia 1 and 2

06:00 PM - 08:00 PM

Monday, May 9, 2011

Oral Session 1: Mini-Symposium - Emerging Diseases and Antiviral Therapy
Chair(s): Mike Bray, Ph.D.
Sofia 1 and 2

08:00 AM - 12:00 PM
08:00 2. Does Antiviral Therapy Have a Role in the Control of Japanese Encephalitis.
Ernest Gould, Ph.D.
Université de La Méditerraneé, France
08:40 3. Opportunities to Test New Therapies Against Crimean-Congo Hemorrhagic Fever.
Onder Ergonul, Ph.D.
Marmara University, Medical School, Turkey

09:20 BREAK.

09:50 4. Structural and Functional Elements to Consider RNA Capping as a Target for Antivirals in (+) RNA Viruses.
Bruno Canard, Ph.D.
CNRS-Université de la Méditerranée, France
10:30 5. Filoviral iVLPs Systems: From Basic Research to Antiviral Drug Screening.
Stephan Becker, Ph.D.
Philipps-Universität, Germany
11:10 6. The Challenge of Emerging Viruses.
Mike Bray, Ph.D.
NIAID, NIH, USA

Oral Session 2: Hepatitis Viruses
Chair(s): Johan Neyts, Ph.D. and Angela Lam, Ph.D.
Sofia 1 and 2

02:00 PM - 04:00 PM
14:00 7. PLENARY: New Insights into the Hepatitis C Virus Replication Cycle and Impact for Known and Novel Drug Targets.
Ralf Bartenschlager, Ph.D
University of Heidelberg, Germany

14:45 8. IDX375, A Novel Allosteric HCV Polymerase Inhibitor: *In Vitro* Antiviral Activity and Preclinical Profile.
C.B. Dousson[1], S.S. Good[2], M. La Colla[2], J. Bilello[2], M. Seifer[2], D.N. Standring[2], J.-L. Paparin[1], D. Surleraux [1]
[1]Idenix Pharmaceuticals, Parc Euromedecine , Montpellier, France, [2]Idenix Pharmaceuticals, Inc., Cambridge, MA, United States

15:00 9. Study of NS5B Oligomerization by FRET: Characterization and Inhibition.
Itxaso Bellón-Echeverría[1], Alberto J. López-Jiménez[1,2], Pilar Clemente-Casares[1], Jose A. Encinar[4], Elisa Martínez-Alfaro[2], Ricardo Pérez-Flores[3], Antonio Mas[1]
[1]Centro Regional de Investigaciones Biomédicas(CRIB), Albacete, Spain, [2]Disease Infectious Unit, Complejo Hospitalario Universitario de Albacete, Albacete, Spain, [3]Digestive Department, Complejo Hospitalario Universitario de Albacete, Albacete, Spain, [4]Instituto de Biología Molecular y Celular. Universidad Miguel Hernandez, Elche, Spain

15:15 10. *In vitro* Selection of HCV Replicons with Reduced Sensitivity to PSI-352938, a Cyclicphosphate Prodrug of β-D-2′-α-F-2′-β-C-METHYLGUANOSINE.
Angela M Lam, Christine Espiritu, Shalini Bansal, Holly M. Micolochick Steuer, Veronique Zennou, Michael J. Otto, Phillip A Furman
Pharmasset, Inc, Princeton, New Jersey, United States

15:30 11. Human subtilase Site-1 Protease (S1P): an emerging host cell target for hepatitis C virus (HCV) infection and HCV-associated steatogenesis.
Andrea D. Olmstead, François Jean
University of British Columbia, Vancouver, BC, Canada

15:45 12. DMPK and Metabolism Studies of Nucleoside Phosphoramidates Including INX-08189, a Novel Double Pro-drug and Clinical Candidate for Hepatitis C Virus Therapy.
Jeff T. Hutchins[1], Christopher McGuigan[2], Stanley D. Chamberlain[1], Karolina W. Madela[2], Mohamed Aljarah[2], John Vernachio[1], Joseph M. Patti[1], Geoffrey Henson[1]
[1]Inhibitex, Alpharetta, GA, United States, [2]Cardiff University, Cardiff, United Kingdom

Tuesday, May 10, 2011

William Prusoff Young Investigator Award
Sofia 1 and 2

08:00 AM - 08:45 AM
08:00 Presentation of the William Prusoff Young Investigator Award: Joseph Colacino, Ph.D., President-ISAR.
08:10 13. Development of Countermeasures Against Pathogenic Arenaviruses.
Brian Gowen, Ph.D.
University of Utah, USA

Oral Session 3: Retroviruses and Herpesviruses
Chair(s): Rhonda Cardin, Ph.D. and Masanori Baba, Ph.D.
Sofia 1 and 2

08:45 AM - 11:45 AM
08:45 14. Mechanism of HIV-1 Neutralization by an Antibody: Reversible Binding Stalls Entry.
David Kabat, Emily Platt
OHSU, Portland, OR, United States

09:00 15. Beta5 integrin is the major contributor to the alphaV integrin-mediated blockade of HIV-1 replication.
Ester Ballana[1], Eduardo Pauls[1], Bonaventura Clotet[1], Françoise Perron-Sierra[2], Gordon C Tucker[2], José A Esté[1]
[1]Irsicaixa - AIDS Research Institute, Badalona, Barcelona, Spain, [2]Institut de Recherches Servier, Departments of Medicinal Chemistry and Cancer Research and Drug Discovery, Croissy sur Seine, France

09:15 BREAK.

09:45 ICAR Business Meeting.
10:00 Invitation to the 25th ICAR - Sapporo, Japan: Amy Patick, Ph.D., Past President-ISAR and Masanori Baba, Ph.D., Local Host-25th ICAR.

10:15 16. Amido tyrosine esters: a promising new approach to antiviral nucleoside phosphonate prodrugs.
Charles E. McKenna[1], Boris A. Kashemirov[1], Valeria M. Zakharova[1], Ivan S. Krylov[1], Melissa Williams[1], Marcela KreĐmerová[2], John C. Drach[3], John M. Hilfinger[4]
[1]Department of Chemistry, University of Southern California, Los Angeles, CA, United States, [2]IOCB Research Centre, Academy of Sciences of the Czech Republic, Prague, Czech Republic, [3]School of Dentistry, University of Michigan, Ann Arbor, MI, United States, [4]TSRL, Inc., Ann Arbor, MI, United States

10:30 17. Cyclopropavir Inhibits the Normal Function of the Human Cytomegalovirus UL97 Kinase.
Mark Prichard[1], Caroll Hartline[1], Rachel Gill[1], Terry Bowlin[2], Scott James[1]
[1]University of Alabama at Birmingham, Birmingham, AL, United States, [2]Microbiotix Inc., Worchester, MA, United States

10:45 18. Structural and functional characterization of human cytomegalovirus terminase leads to a new antiviral target.
Marta Nadal[1,2], Zuzanna Kaczmarska[1,2], Philippe J Mas[3], Alexandre G. Blanco[1,2], Carme Arnan[1,2], Maria Solà[2], Darren J. Hart[3], Miquel Coll[1,2]

[1]Institute for Research in Biomedicine (IRB Barcelona), Barcelona, Barcelona, Spain, [2]Institut de Biologia Molecular de Barcelona (CSIC), Barcelona, Barcelona, Spain, [3]European Molecular Biology Laboratory, Grenoble Outstation, Grenoble, Cedex, France

11:00 19. *In Vivo* Efficacy of N-methanocarbathymidine (N-MCT) against Herpes simplex Virus Type 2 in Neonatal Guinea Pigs.
Rhonda Cardin[1], Fernando Bravo[1], Clark Jennifer[1], Earwood Julie[1], Robert Glazer[2], Aquilur Rahman[3], David Bernstein[1]
[1]Cincinnati Children's Hospital Medical Center, Cincinnati, OH, United States, [2]Georgetown University, Washington, D.C., District of Columbia, United States, [3]N & N Scientific, Inc., Rockville, MD, United States

11:15 20. CMX001 Potentiates the Efficacy of Acyclovir in Herpes Simplex Virus Infections.
Mark Prichard[1], Earl Kern[1], Caroll Hartline[1], Emma Harden[1], Randall Lanier[2], Debra Quenelle[1]
[1]University of Alabama at Birmingham, Birmingham, AL, United States, [2]Chimerix Inc., Durham, NC, United States

11:30 21. Safety and Human Pharmacokinetics of AIC316, a Potent Helicase-Primase Inhibitor of Herpes Simplex Virus (HSV).
Alexander Birkmann, Dirk Kropeit, David McCormick, Holger Zimmermann, Helga Ruebsamen-Schaeff
AiCuris GmbH & Co. KG, Wuppertal, Germany

Clinical Symposium
Chair(s): Richard Whitley, M.D. and Paul Griffiths, M.D.
Sofia 1 and 2

02:00 PM - 04:00 PM

Wednesday, May 11, 2011

Oral Session 4: Mini-Symposium - Medicinal Chemistry and Drug Discovery
Chair(s): Chris Meier, Ph.D., University of Heidelberg, Germany
Sofia 1 and 2

08:00 AM - 12:00 PM

08:00 22. Discovery of 2'-Deoxy-2'-α-Fluoro-2'-β-C-Methyl Purine Nucleotide Prodrugs for the Treatment of Hepatitis C Virus Infection.
Michael Sofia, Ph.D.
Pharmasset, Inc., USA

08:40 23. Structure- and Fragment-based Discovery of Antivirals Against Emerging and Neglected RNA Viruses.
Rolf Hilgenfeld, Ph.D.
University of Luebeck, Germany

09:20 BREAK.

09:50 24. The Discovery of Tegobuvir: From a BVDV Screening Hit to an Efficacious HCV Antiviral.
Gerhard Puerstinger, Ph.D.
Universitat Innsbruck, Austria

10:30 25. The Discovery of Potent HCV NS5A Inhibitors.
John Link, Ph.D.
Gilead Sciences, Inc., USA

11:10 26. Carbocyclic Nucleosides as Potential HIV Antivirals.
Chris Meier, Ph.D.
University of Heidelberg, Germany

Oral Session 5: Respiratory Viruses, Emerging Viruses and Biodefense
Chair(s): Brian Gowen, Ph.D. and Graciela Andrei, Ph.D.
Sofia 1 and 2

01:00 PM - 03:30 PM

13:00 27. Plenary: Molecular Mechanisms of Viral Resistance to Nucleotide Analogues, and Implications for Lethal Mutagenesis Strategies.
Esteban Domingo, Ph.D.
Campus de Cantoblanco, Spain

13:45 28. Important Role For Protein Kinase C-α In Combined Pneumolysin/Influenza A Virus-Induced Pulmonary Endothelial Hyperpermeability.
Júlia Vergara-Alert[1], Supriya Sridmar[2], Guang Yang[2], Ian Davis[3], Trinad Chakraborty[4], Ayub Darji[1], Rudolf Lucas[2]
[1]Centre de Recerca en Sanitat Animal (CReSA), UAB-IRTA, Bellaterra, Barcelona, Spain, [2]Vascular Biology Center, Medical College of Georgia, Augusta, Georgia, United States, [3]Veterinary Biosciences, Ohio State University, Columbus, Ohio, United States, [4]Institute of Medical Microbiology, Justus-Liebig University Giessen, Giessen, Germany

14:00 29. 2'- and 4'- Modified Ribonucleoside Analogs Can Inhibit All Four Serotypes of Dengue Virus In Human Primary Dendritic Cells as Competitive Inhibitors and Non-Obligatory Chain Terminators.
Hassan Javanbakht[1], Vincent Leveque[1], Zhinan Jin[1], Jerome Deval[2], Andreas Jekle[2], Gabrielle Heilek[2], Han Ma[1], Klaus Klumpp[1]
[1]Hoffman-La Roche, Nutley, NJ, United States, [2]Roche, Palo Alto, CA, United States

14:15 30. 2009 Pandemic Influenza Virus: What Special for Its HA and NA?
George F. Gao[1,2], Christopher J. Vavricka[1]
[1]CAS Key Laboratory of Pathogenic Microbiology and Immunology, Chaoyang, Beijing, China, [2]Beijing Institutes of Life Science Chinese Academy of Sciences, Chaoyang, Beijing, China

14:30 31. Identification of New Druggable Antiviral Targets by Chemical Genetics.
Richard Y Kao, Kwok-Yung Yuen
Research Center of Infection and Immunology, State Key Laboratory of Emerging Infectious Diseases, Department of Microbiology, LKS Faculty of Medicine, The University of Hong Kong , Hong Kong, Hong Kong

14:45 32. Activities of Viral M2 Channel, Neuraminidase, and RNA Polymerase Inhibitors on Oseltamivir-Resistant H275Y Influenza A (H_1N_1) Virus Infections in Mice .
Donald F. Smee[1], Min-Hui Wong[1], E. Bart Tarbet[1], Justin Julander[1], Matthew Gross[2], Jack Nguyen[2]
[1]Utah State University, Logan, Utah, United States, [2]Adamas Pharmaceuticals, Emeryville, California, United States

15:00 33. CMX001 (Hexadecyloxypropyl Cidofovir) Antiviral Activity against Adenovirus in Patients Correlates with Drug Levels and Viral Sensitivity.
ER Lanier, W Painter, TK Tippin, M Anderson, BM Lampert, H Mommeja-Marin, LC Trost
Chimerix Inc, Durham, NC, United States

15:15 92. Avoidance of Coxsackievirus Drug Resistance by Using a Novel Scheme of Combining Anti-Enteroviral Inhibitors *In Vivo*.
Ralitsa Vassileva-Pencheva, Angel S. Galabov
Department of Virology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Sofia, Bulgaria

ICAR Banquet Reception
Sofia 1 and 2

07:15 PM - 07:45 PM

ICAR Banquet and Program
Sofia 1 and 2

07:45 PM - 10:00 PM

Gertrude Elion Award Lecture
Sofia 1 and 2

08:30 PM - 09:30 PM
20:30 Presentation of the Gertrude Elion Award: Joseph Colacino, Ph.D., President-ISAR.
20:45 34. Why Develop a Drug for Smallpox, a Disease Which has Already Been Eradicated?
Earl Kern, Ph.D.
University of Alabama at Birmingham, USA

Monday, May 9, 2011

Poster Session 1: Retroviruses, Respiratory Viruses, Emerging Viruses and Antiviral Methods
Sofia 3 and Kyota

04:00 PM - 06:00 PM

35. Design, Synthesis and West Nile Protease Inhibitory Activity of Novel Isatin Derivatives.
Periyasamy Selvam[1], Priya Srinivasan[2], Tanvi Khot[2], R Padmanaban[2]
[1]Devaki Amma Memorial College of Pharmacy,, Malapuram 673634 , Kerala, India, [2]Microbiology and Immunology, Georgetown School of Medicine, Washington DC, United States

36. Effects of the Addition of Hiltonol® (Poly-ICLC) to a SARS-CoV S Protein Vaccine in Lethal SARS-CoV Mouse Model.
Dale L. Barnard[1], Miles Wandersee[1], Kevin Bailey[1], Aaron Smith[1], Zach Vest[1], John D. Morrey[1], Andres M. Salazar[2]
[1]Utah State University, Logan, UT, United States, [2]Oncovir, Inc., Washington, DC, United States

37. Synthesis and Anti-Hiv Activity of D-Peptide Analogs As HIV Fusion Inhibitors.
Alice Baron[1,2], Christine Kreuz[3], Gilles Gosselin[1], Frederic Lamaty[2], Jean Martinez[2], Dominique Surleraux[1], Claire Pierra[1], Pascal Clayette[3]
[1]Laboratoires Idenix, Montpellier, France, [2]Institut des Biomolécules Max Mousseron, Montpellier, France, [3]Bertin Pharma, Fontenay aux Roses, France

38. Activity of Novel Cyclophilin Inhibitors Based on The Polyketide, Sanglifehrin A, Against HIV.
Michael Bobardt[1], Steven Moss[2], Udayan Chatterji[1], Mohammad Nur-E-Alam[2], Tony Warneck[2], Barrie Wilkinson[2], Philippe Gallay[1], Matthew Gregory[2]
[1]The Scripps Research Institute, La Jolla, California, United States, [2]Biotica Technology Ltd, Cambridge, England, United Kingdom

39. Obr-5-340 – A Novel Pyrazolo-Pyrimidine Derivative With Strong Antiviral Activity Against Coxsackievirus B3 *In Vitro* and *In Vivo*.

Heike Braun[1], Vadim A. Makarov[2], Olga B. Riabova[2], Elena S. Komarova[2], Martina Richter[1], Peter Wutzler[1], Michaela Schmidtke[1]
[1]Institute of Virology and Antiviral Therapy, Jena University Hospital, Jena, Germany, [2] Institute of Biochemistry, Russian Academy of Science, Moscow, Russia

40. Occurrence of Opportunistic Infections in People Living with HIV/AIDS Following Antiretroviral Therapy in West Bengal, India.
Sayan Chakraborty, Mehebubar Rahman, Bibhuti Saha
Calcutta School of Tropical Medicine, Kolkata, West Bengal, India

41. Crimean Congo Haemorrhagic Fever Virus: An Emerging Concern For Iran's Public Health.
Sadegh Chinikar, Ramin Mirahmadi, Maryam Moradi, Mojtaba Ghiasi, Akram Sadeh, Sahar Khakifirouz, Fereshteh Sadat Varai, Maisam Asl Solaimani
Pasteur Institute of iran, Tehran, Tehran, Iran

42. Antiviral Drug-Resistant Influenza Viruses in Gyeonggi Province, South Korea, From 2005 to 2010.
Hangil Cho, Haekun Hong, Sukyoung Mun, Woonho Kim, Hyunkyung Lee, Mihye Yoon, Jongbok Lee
Gyeonggi-do Institute of Health and Environment, Suwon, Gyeonggi, South Korea

43. Rigid Amphipathic Fusion Inhibitors (RAFIs) Inhibit Infectivity of Enveloped Viruses by Targeting Envelope Lipids to Prevent Fusion With Cellular Membranes.
Che C. Colpitts[1], Alexey V. Ustinov[2], Vladimir A. Korshun[2], Luis M. Schang[1]
[1]University of Alberta, Edmonton, Alberta, Canada, [2]Russian Academy of Sciences, Moscow, Russia

44. Combine Action Rimantadine and Amizon on Flu Occasion.
G. Danilenko[1], S. Rybalko[2], T. Bukhtiarova[3], V. Danilenko[3], S. Guzhova[4], O. Esipenko[1]
[1]Institute of Organic Chemistry, Kyiv, 02094, Ukraine, [2]Institute of Epidemiology and Infectional Diseases, Kyiv, 03680, Ukraine, [3]Institute of Pharmacology and Toxicology, Kyiv, 03380, Ukraine, [4]Institute of Bioorganic Chemistry and Petrochemistry, Kyiv, 02094, Ukraine

45. An Analogue of the Antibiotic Teicoplanin Inhibits Dengue Virus Entry *in Vitro*.
Tine De Burghgraeve[1], Suzanne JF Kaptein[1], Jan Paeshuyse[1], Vanesa Ayala-Nunez[2], Maria Preobrazhenskaya[3], Andrea Gamarnik[4], Jolanda Smit[2], Johan Neyts[1]
[1]Rega Institute, KULeuven, Leuven, Belgium, [2]University of Groningen, Groningen, Netherlands, [3]Gause Institute of New Antibiotics, Moscow, Russia, [4]Fundacion Instituto Leloir, Buenos Aires, Argentina

46. Identification of HIV-1 Reverse Transcriptase Dual Inhibitors by A Combined Shape-, 2D-Fingerprint- and Pharmacophore-Based Virtual Screening Approach.
Simona Distinto[1], Francesca Esposito[2], Johannes Kirchmair[3], Cristina M. Cardia[4], Elias Maccioni[4], Stefano Alcaro[1], Luca Zinzula[2], Enzo Tramontano[2]
[1]Dip. Scienze Farmaco Biologiche, University of Catanzaro, Catanzaro, Italy, [2]Dipartimento di Scienze Applicate ai Biosistemi, University of Cagliari, Cagliari, Italy, [3]Department of Pharmaceutical Chemistry, University of Innsbruck, Innsbruck, Austria, [4]Dipartimento Farmaco Chimico Tecnologico, University of Cagliari, Cagliari, Italy

47. Antiviral Activity of The Mek-Inhibitor U0126 Against Pandemic H1N1V and Highly Pathogenic Avian Influenza Virus In Vitro and *In Vivo*.
Karoline Droebner[1], Stephan Ludwig[2], Stephan Pleschka[3], Oliver Planz[1]
[1]Friedrich-Loeffler-Institut, Institute of Immunology, Tübingen, BW, Germany, [2]Justus-liebig Universität, Giessen, Hessen, Germany, [3]Institute of Molecular Virology , Münster, NRW, Germany

48. Targeted Elimination of HIV Infected Cells: Synergistic Combination of Dexamethasone and DEAE As a Paradigm.
Najoua Elbourkadi, Lijun Zhao, Ethan Will Taylor
Laboratory for Molecular Medicine,University of North Carolina, Greensboro, NC, United States

49. Design and Synthesis of New Isatin Derivatives As HIV-1 Reverse Transcriptase Associated Ribonuclease H Inhibitors.
Francesca Esposito[1], Rita Meleddu[2], Maria Luisa Sanna[2], Simona Distinto[3], Angela Corona[1], Valeria Cannas[1], Enzo Tramontano[1], Maria Cristina Cardia[2]
[1]Dipartimento di Scienze Applicate ai Biosistemi, University of Cagliari, Cagliari, Italy, [2]Dipartimento Farmaco Chimico Tecnologico, University of Cagliari, Cagliari, Italy, [3]Dip. Scienze Farmaco Biologiche, University of Catanzaro, Catanzaro, Italy

50. Antiviral Activity of The Proteasome Inhibitor Vl-01 Against Human and Avian Influenza A Viruses.
Emanuel Haasbach[1,2], Eva-Katharina Pauli[2], Robert Spranger[2], David Mitzner[2,3], Ulrich Schubert[2,3], Ralf Kircheis[2], Oliver Planz[2,4]
[1]Friedrich-Loeffler-Institut, Institute of Immunology, Tuebingen, Germany, [2]ViroLogik GmbH, Innovation Centre for Medical Technology and Pharmaceuticals, Erlangen, Germany, [3]Clinical and Molecular Virology, Friedrich-Alexander-University of Erlangen-Nuernberg, Erlangen, Germany, [4]Interfaculty Institute for Cell Biology, Department of Immunology, Eberhard-Karl-University of Tuebingen , Tuebingen, Germany

51. *In Silico* Screening of Compounds Targeting Human Cyclin T1 and *In Vitro* Evaluation of Their Anti-HIV-1 Activity.
Takayuki Hamasaki, Mika Okamoto, Masanori Baba
Division of Antiviral Chemotherapy, Center for Chronic Viral Diseases, Kagoshima University, Sakuragaoka, Kagoshima, Japan

52. Novel 2-Styryl-8-Hydroxyquinolines (8sqs) Derivatives With Anti-HIV-1 Activity Targeting Viral Integrase and Protease.
Anton V. Hinkov[1], Kamelia R. Stanoeva[1,2], Sevdalina H. Raleva[3], Vasil G. Atanasov[4], Petya D. Genova-Kalou[3], Radka M. Argirova[3]
[1]Faculty of Biology, Sofia University “St.Kliment Ohridski”, Sofia, Bulgaria, [2]Medical Faculty, Medical University – Sofia, Sofia, Bulgaria, [3]Dept. of Virology, National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria, [4]Faculty of Chemistry, Sofia University “St. Kliment Ohridski”, Sofia, Bulgaria

53. A Novel Method - Amenable For High-Throughput Screening Purposes - To Quantify Antiviral Activity Against Viruses That Induce Limited CPE.
Dirk Jochmans[1], Bernadette G. van den Hoogen[2], Pieter Leyssen[1], Ron A. Fouchier[2], Johan Neyts[1]
[1]Rega Institute for Medical Research, University of Leuven (KULeuven), Leuven, Belgium, [2]Department of Virology, Erasmus MC, Rotterdam, Netherlands

54. Effective Prophylactic and Therapeutic Treatment of Yellow Fever Virus With An Adenovirus-Vectored Interferon, Def201, In a Hamster Model.
Justin G. Julander[1], Jane Ennis[2], John Morrey[1], Jeff Turner[2]
[1]Institute for Antiviral Research, Utah State University, Logan, UT, United States, [2]Defyrus Inc., Toronto, ON, Canada

55. RNA Interference Mediated Gene Silencing of Influenza A Virus: A Tool for Potent Antiviral Therapy.
Madhu Khanna[1] , Prashant Kumar[1], Vikas sood[2], Roopali Rajput[1], Akhil Banerjea[2]
[1]VP Chest Institute,Uni of Delhi, Delhi, Delhi, India, [2]VP Chest Institute,Uni of Delhi, Delhi, Delhi, India, [3]National Institute of Immunology, New Delhi, Delhi, India, [4]VP Chest Institute,Uni of Delhi, Delhi, Delhi, India, 52National Institute of Immunology, New Delhi, Delhi, India, 6 India

56. Antiviral Effect of The Sulfated Polysaccharide, P-KG03, Against Influenza A Virus.
Meehyein Kim[1], So-Yeon Kim[1], Hae Soo Kim[1], Joung Han Yim[2], Woo Ghil Lee[1], Chong-Kyo Lee[1]
[1]Korea Research Institute of Chemical Technology, Daejeon, South Korea, [2]Korea Polar Research Institute, Incheon, South Korea

57. Excision of AZT and d[4]T Modulated by Deletions in the β3-β4 Hairpin Loop of HIV-1 Reverse Transcriptase.
Mónica Kisic[1], Tania Matamoros[1], María Nevot[2], Jesús Mendieta[1], Javier Martinez-Picado[2,3], Miguel A Martínez[2], Luis Menéndez-Arias[1]
[1]Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Madrid, Madrid, Spain, [2]Fundació irsiCaixa, Hospital Univ. Germans Trias i Pujol, Badalona, Barcelona, Spain, [3]Institució Catalana de Recerca i Estudis Avançats, Barcelona, Barcelona, Spain

58. Doxycycline in Tick-Borne Encephalitis Virus Infection.
Liubov Kozlovskaya, Yulia Rogova, Galina Karganova
Chumakov Institute of poliomyelitis and viral encephalitides Russian academy of medical sciences, Institut poliomyelita, Moscow region, Russia

59. Antiarboviral Efficacy of Combined Application of Interferon Inducers and Proteolysis Inhibitor.
Mykhajloik Kozlovsky[1], Vikor Lozitsky[2], Igor Benzel[3], Ihor Lozynsky[1], Sergij Lyakhov[4], Lyudmyla Litvinova[4]
[1]Lviv Research Institute of Epidemiology and Hygiene, Lviv, Lviv, Ukrenia, [2]Ukrainian I.I.Mechnikov Research Anti-Plague Institute, Odesa, Odesa, Ukrenia, [3]Danylo Halytskyi Lviv National Medical University, Lviv, Lviv, Ukrenia, [4]A.V.Bogatsky Physico-chemical Institute, Odesa, Odesa, Ukrenia

60. HIV Full-Replication Technology For Identification of Novel HIV Inhibitors From Multiple Sources.
Stephan Kremb[1], Markus Helfer[1], Horst Wolff[1], Andrea Kleinschmidt[1], Jörg Durner[2], Philippe Schmitt-Kopplin[3], Ruth Brack-Werner[1]
[1]Helmholtz Zentrum München, VIRO, Neuherberg, Germany, [2]Helmholtz Zentrum München, BIOP, Neuherberg, Germany, [3]Helmholtz Zentrum München, IÖC, Neuherberg, Germany

61. An Antiviral Assay to Identify Inhibitors of The Human Metapneumovirus That Is Amenable for High-Throughput Screening Purposes.
Pieter Leyssen[1], Bernadette G. van den Hoogen[2], Dirk Jochmans[1], Ron A. Fouchier[2], Johan Neyts[1]
[1]Rega Institute for Medical Research, University of Leuven (KULeuven), Leuven, Belgium, [2]Department of Virology, Erasmus MC, Rotterdam, Netherlands

62. PHYTOCHIK: Biodiversity As a Source of Selective Inhibitors of CHIKV Replication.
Pieter Leyssen [1], Marc Litaudon[2], Jean-Claude Guillemot[3], Philippe Rasoanaivo[4], Jacqueline Smadja[5], Ameenah Gurib-Fakim[6], Bruno Canard[3], Françoise Guéritte[2]
[1]Rega Institute for Medical Research, University of Leuven, Leuven, Belgium, [2]Centre de recherche de Gif, Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France, [3]AFMB, UMR 6098, Campus de Luminy, Marseille, France, [4]Institut Malgache de Recherches Appliquées, Antananarivo, Madagascar, [5]Laboratoire de Chimie des Substances Naturelles et des Sciences des Aliments, Faculté des Sciences et Technologies-Université de La Réunion, Saint-Denis, Reunion, [6]Centre for Phytotherapy Research, Ebene, Mauritius

63. Synthesis, Influence of Polymer Molecular Weight on Drug Release and Anti-HIV Activity of PEGylated AZT Conjugates.
Wenjun Li[1], Peng Zhan[1], Jingde Wu[1], Yu Chang[1], Christophe Pannecouque[2], Erik De Clercq[2], Xinyong Liu[1]
[11] Shandong University, Jinan, Shandong, China, 22 Katholieke Universiteit Leuven, Leuven, Belgium

64. The Protective Action Arbivirin and Aminocaproic Acid During The Experimental Influenza.
V Lozitsky[1], A Fedchuk[1], T Grydina[1], S Rybalko[2], V Cherednichenko[3]
[1]I.I. Mechnikov Ukrainian Anti-Plague Research Institute , Odesa, Odesa, Ukrenia, [2]L.V. Gromashevsky Institute of Epidemiology and Infectious Diseases, Kyiv, Kyiv, Ukrenia, [3]Zdorovya Pharmaceutical Company, Kharkiv, Kharkiv, Ukrenia

65. Oseltamivir Influences Hepatic Cytochrome P-450 Dependent Oxidative Metabolism In Influenza Virus Infected Mice.
Milka M. Mileva, Lora S. Simeonova, Galya G. Genova, Angel S. Galabov
Department of Virology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

66. Antiviral Activity of Hemocyanin Isolated From Marine Snail *Rapana Venosa*.
Nadiya V. Nesterova[1], Svitlana D. Zagorodnya[1], Vesela Moshtanska[2], Pavlina Dolashka[2], Galina V. Baranova[1], Anna V. Golovan[1], Anna O. Kurova[1]
[1]Institute of Microbiology and Virology of NASU, Kyiv, Ukrenia, [2]Institute of Organic Chemistry, BAS , Sofia, Bulgaria

67. Long-Term Inhibition of HIV-1 Replication In CD4+ T Cells Transduced With A Retroviral Vector Conditionally Expressing The *Escherichia Coli* Endoribonuclease MazF.
Mika Okamoto[1], Hideto Chono[2], Hiroshi Tsuda[2], Koichi Inoue[2], Junichi Mineno[2], Masanori Baba[1]
[1]Center for Chronic Viral Diseases, Kagoshima University, Kagoshima, Kagoshima, Japan, [2]Center for Cell and Gene Therapy, Takara Bio Inc., Otsu, Shiga, Japan

68. SP600125 Inhibits Orthopoxviruses Replication on A JNK1/2-Independent Manner - Implication As A Potential Anti-Poxviral .
Anna C.P Pereira[2], Jamaria A.P. Soares[3], Flavia G.G Leite[4], André F. da Cruz[1], Alice A. Torres[1], Erna G. Kroon[1], Paulo C. P. Ferreira[1], Cláudio A Bonjardim[1]
[1]Grupo de Transdução de Sinal, Laboratório de Vírus, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, [2]Universidade Federal do Piauí , Parnaíba, Piauí, Brazil, [3]Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, Wisconsin, Milwaukee, United States, [4]Institute of Science and Technology, Viena, Klosterneuburg, Austria

69. Anti-viral Properties and Mode of Action of Standardized Echinacea purpurea Extract Against Highly Pathogenic Avian Influenza Virus (H5N1, H7N7) and Swine-Origin H1N1 (S-OIV).
Stephan Pleschka[1], Roland Schoop[2], James B Hudson [3]
[1]Institute of Medical Virology, Jutus-Liebig-University, Giessen, Hessen, Germany, [2]Bioforce AG, Roggwil, Bern, Switzerland, [3]Department of Pathology & Laboratory Medicine, University of British Columbia, Vancouver, British Columbia, Canada

70. Novel Chemical Compounds as Potential Blockers to the Swine-Origin Influenza A H1N1 (2009) Virus Replication.
Roopali Rajput[1], Prashant Kumar[1], Binod Kumar[1], Madhu Khanna[1], Deepti Sharma[2], Divya Mathur[2], Ashok K Prasad[2]
[1]Department of Microbiology, Faculty of Medical Sciences, University of Delhi, Delhi, Delhi, India, [2]Bio organic Laboratory, Department of Chemistry, University of Delhi, Delhi, Delhi, India

71. Photodynamic Effect of Phthalocyanine-Zn (II) Complexes on Some Enveloped Viruses.
M. Remichkova[1], L. Mukova[1], L. Doumanova[1], V. Mantareva[2], I. Angelov[2], V. Kussovski[1], A. S. Galabov[1]
[1]The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria, [2]Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria

72. Classical Swine Fever Outbreak Containment – Antivirals As an Epidemiologically and Economically Viable Alternative to Emergency Vaccination and Culling.
Stefaan Ribbens[1], Nesya Goris[2], Johan Neyts[3], Jeroen Dewulf[1]
[1]Ghent University, Merelbeke, Belgium, [2]Okapi Sciences NV, Heverlee, Belgium, [3]KULeuven, Leuven, Belgium

73. Identification and Characterization of OBR-5-340 - A Novel Broad-Spectrum Anti-Human Rhinovirus (HRV) Inhibitor.
Martina Richter[1], Vadim A. Makarov[2], Olga B. Riabova[2], Peter Wutzler[1], Michaela Schmidtke[1]
[1]Institute of Virology and Antiviral Therapy, Jena University Hospital, Jena, Germany, [2]Institute of Biochemistry, Russian Academy of Science, Moscow, Russia

74. Relationship Between Homocysteine Serum Level and Other Blood Analyses Parameters in HIV-Infected Patients.
Bernardino Roca, José Antonio Ferrero, Maria Cruz del Monte, Elena Resino
Hospital General, University of Valencia, Castellon, Castellon, Spain

75. Control Of HIV-Infection With Visits Scheduled Every Four or Every Six Months. A Comparative Randomized Study.
Bernardno Roca, Elena Resino, Maria Cruz del Monte, Manuel Roca
Hospital General, University of Valencia, Castellon, Castellon, Spain

76. European Virus Archive.
Jean-Louis Romette
UNIVMED, Marseille, France

77. HLA-C -35C/T Variant: Genetic Association to HIV-1 Disease Progression and Functional Links.
Alba Ruiz[1], Ester Ballana[1], Beatriz Mothe[1,2], Eulalia Grau[1], Bonaventura Clotet[1], Christian Brander[1,3], José A. Esté[1]
[1]Irsicaixa AIDS Research Institute-HIVACAT, Badalona, Catalunya, Spain, [2]Lluita contra la SIDA Foundation, Badalona, Catalunya, Spain, [3]Institució Catalana de Recerca i Estudis Avançats (ICREA), Badalona, Catalunya, Spain

78. Discovery of Novel Natural Neuraminidase Inhibitors (NAI) Based on *In Silico* Screening and Antiviral Investigations.
Michaela Schmidtke[1], Judith M. Rollinger[2], Ulrike Grienke[2], Nora Seidel[1], Andi Krumbholz[1], Peter Wutzler[1], Klaus R. Liedl[3], Johannes Kirchmair[3]
[1]Jena University Hospital, Institute of Virology and Antiviral Therapy, Jena, Germany, [2]University Innsbruck, Institute of Pharmacy/Pharmacognosy, Innsbruck, Austria, [3]University Innsbruck, Institute of Theoretical Chemistry, Innsbruck, Austria

79. Synthesis, Anti-Hiv and Cytotoxic Activity of Some Novel Isatine-Sulfisomidine Derivatives.
Periyasamy Selvam[1], Markandavel Chandramohan[2], Christophe Pannecouque[3], E De Clercq[3]
[1]Devaki Amma Memorial college of Pharmacy, Malapuram, Kerala, India, [2]Kamarajar Liver Hospital and Research centre, Madurai, Tamilnadu, India, [3]Rega Institute for Medical Research, Katholieke Universiteit , Leuven, Leuven, Belgium

80. Studies of HIV Integrase Inhibitory Activity of Novel Isatine Derivatives.
Periyasamy Selvam[1], Kasthuraiah Maddali[2], Christophe Marchand[2], Yves Pommier[2]
[1]Devaki Amma Memorial College of Pharmacy, Malapuram 676364, Kerala, India, [2]Laboratory of Molecular Pharmacology, NCI, NIH, Bethesda, MD 20892, Bethesda, MD , United States

81. Combined Anti-Influenza Virus Effect of Natural and Synthetic Viral Inhibitors.
Julia Serkedjieva[1], Iskra Ivanova[2]
[1]Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria, [2]Department of Microbiology, Sofia University, Sofia, Bulgaria

82. A Catalytic 3D Model Development of HIV-Integrase and Drug Resistance Understanding By Molecular Dynamics Simulation.
Ashoke Sharon, Tuniki Balaraju, Chandralata Bal
Department of Applied Chemistry, Birla Insititute of Technology, Mesra, Ranchi, Jharkhand, India

83. Susceptibility to Neuraminidase Inhibitors and M2 Blockers of Some Seasonal Influenza Strains Isolated in Bulgaria 2004-2007.
Lora Simeonova[1], Galina Gegova[1], Lucia Mukova[1], Angel S. Galabov[1], Rositza Koceva[2], Sylvie van der Werf[3]
[1]Department of Virology, The Stephan Angeloff Institute of Microbiology, BAS, Sofia, Bulgaria, [2]National Reference Laboratory of Influenza and Acute Respiratory Infections, NCIPD, Sofia, Bulgaria, [3]Unité de Génétique Moléculaire des Virus à ARN, Institut Pasteur, Paris, France

84. Prophylactic Activity of mDEF20[1] Against Vaccinia Virus Respiratory Infections in Mice.
Donald F. Smee[1], Min-Hui Wong[1], Eric Sefing[1], Ramtin Rahbar[2], Jane Ennis[2], Jeffrey D. Turner[2]
[1]Utah State University, Logan, Utah, United States, [2]Defyrus Inc., Toronto, Ontario, Canada

85. Novel Derivatives of Abacavir - Synthesis and Activity against Human Immunodeficiency Virus-Type [1] in cell culture.
Kamelia R. Stanoeva[1,2], Ivanka G. Stankova[3], Anton V. Hinkov[2], Ivailo I. Alexiev[4], Petya D. Genova-Kalou[5], Radoslav I. Chayrov[3], Radka M. Argirova[6]
[1]Medical Faculty, Medical University – Sofia, Sofia, N/A, Bulgaria, [2]Faculty of Biology, Sofia University "St.Kliment Ohridski", Sofia, N/A, Bulgaria, [3]Department of Chemistry, South-West University "Neofit Rilski", Blagoevgrad, N/A, Bulgaria, [4]National HIV Confirmation Laboratory, National Center of Infectious and Parasitic Diseases, Sofia, N/A, Bulgaria, [5]Laboratory for Cell Cultures , National Center of Infectious and Parasitic Diseases, Sofia, N/A, Bulgaria, [6]Laboratory of Retroviruses, National Center of Infectious and Parasitic Diseases, Sofia, N/A, Bulgaria

86. Efficacy of Zanamivir Administered by Different Routes and at Different Times for Treatment of an Influenza A/CA/04/2009 (H1N1) Virus Infection in Mice.
Bart Tarbet, Deanna Larson, Min-Hui Wong, Donald Smee
Institute for Antiviral Research, Utah State University, Logan, Utah, United States

87. Inactivated Vaccine Against Tick-Borne Encephalitis Virus As Surrogate Vaccine Against OMSK Hemorrhagic Fever Virus.
Liubov Terekhina[1], Nataliya Pripuzova[2], Mikhail Vorovitch[2], Yulia Rogova[2], Lidiya Romanova[2], Nataliya Tereshkina[2], Andrey Timofeev[2], Galina Karganova[2]
[1]Federal State Unitary Enterprise on Manufacture of Bacterial & Viral Preparation of Chumakov Institute of Poliomyelitis and Viral Encephalitides of Russian Academy of Medical Sciences, Institut poliomyelita, Moscow region, Russia, [2]Institute of Poliomyelitis and Viral Encephalitides of Russian Academy of Medical Sciences, Institut poliomyelita, Moscow region, Russia

88. Efficacy of Aminocaproic Acid Use for Prevention of Some Infectious Diseases in Organized Collectives.
V Trihleb[1], V Lozitsky[2], V Smorgunova[1], S Pozdnyakov[2], A Voronkov[3]
[1]Main Military Medical Clinical Center, Kyiv, Kyiv, Ukrenia, [2]Ukrainian I.I. Mechnikov Anti-Plague Research Institute, Odesa, Odesa, Ukrenia, [3]Research and Design Institute of Chemical Technology "Chemtechnology", Severodonetsk, Severodonetsk, Ukrenia

89. Polymer-Coupled Systems for Blocking the Viral Fusion [1]. Modeling *in silico* the *in vitro* HIV-1 Entry Inhibitors.
V. Tsvetkov[1,2], A. Veselovski[2], A. Serbin[1,3]
[1]Biomodulators RC, Health RDF, Moscow, Russia, [2]Inst Biomedical Chemistry, RAMS, Moscow, Russia, [3]Inst Petrochemical Synthesis, RAS, Moscow, Russia

90. Discovery and Development of Orally Active Antivirals for the Treatment of RSV: Identification of BTA9881 and a 2nd Generation Candidate.
Simon Tucker[1], Alistair Draffan[1], Jennifer Fenner[1], Jega Iswaran[1], Angela Luttick[1], Mike McCarthy[2], Gary Pitt[1], Jane Ryan[1]
[1]Biota Holdings Limited, Melbourne, VIC, Australia, [2]MedImmune Inc, Gaithersburg, MD, United States

91. Mechanistic Studies on a Novel Hydrophobic Derivative of Aglycoristocetin With Potent and Broad Activity Against Influenza Viruses.
Evelien Vanderlinden[1], Els Vanstreels[1], Kurt Vermeire[1], Dirk Daelemans[1], Pal Herczegh[2], Ferenc Sztaricskai[2], Lieve Naesens[1]
[1]Rega Institute for Medical Research, Leuven, Belgium, [2]Department of Pharmaceutical Chemistry, University of Debrecen, Debrecen, Hungary

92. Avoidance of Coxsackievirus Drug Resistance by Using a Novel Scheme of Combining Anti-Enteroviral Inhibitors *In Vivo*.
Ralitsa Vassileva-Pencheva, Angel S. Galabov
Department of Virology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Sofia, Bulgaria

93. Inhibition of Enveloped Virus Infection of Cultured Cells by Valproic Acid .
Angela Vázquez-Calvo[1], Juan-Carlos Sáiz[2], Francisco Sobrino[1], Miguel A Martín-Acebes[2]
[1]CBMSO, (UAM-CSIC), Madrid, Spain, [2]Dpto, Biotecnología, INIA, Madrid, Spain

94. Antiviral Effect of Molluscan Haemocyanines.
Lyudmila Velkova[1], Lubomira Nikolaeva-Glomb[2], Lucia Mukova[2], Aleksander Dolashki[1], Pavlina Dolashka[1], Angel S. Galabov[2]
[1]Institute of Organic Chemistry with Centre of Phytochemistry 9, Sofia, Sofia, Bulgaria, [2]The Stephan Angeloff Institute of Microbiology, Sofia, Sofia, Bulgaria

95. Novel Inhibitors of Nuclear Translocation of HIV-1 Integrase.
Kylie Wagstaff, Stephen Rawlinson, Anna Hearps, David Jans
Monash Uni, Monash, VIc, Australia

96. Development of Antimicrobial Peptides as Topical Microbicides for the Prevention of HIV.
Karen M Watson[1], Ashlee D Boczar[1], Guangshun Wang[2], Robert W Buckheit Jr.[1]
[1]ImQuest BioSciences Inc., Frederick, MD, United States, [2]University of Nebraska, Omaha, NE, United States

97. dsRNA Binding Characterization of Full Length Recombinant Wild Type and Mutants Zaire Ebolavirus VP35.
Luca Zinzula, Francesca Esposito, Daniela Pala, Enzo Tramontano
Dipartimento di Scienze Applicate ai Biosistemi, University of Cagliari, Cagliari, Italy

98. Role Of Cathepsin A and Lysosomes in The Intracellular Activation of Novel Anti-Papillomavirus Agent GS-9191.
Gabriel Birkus[1], Nilima Kutty[1], Christian Frey[1], Riri Shribata[1], Tsuifen Chou[2], Carston Wagner[2], Martin McDermott[1], Tomas Cihlar[1]
[1]Biology Department, Gilead Sciences, Foster City, CA, United States, [2]Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, MN , United States

99. Dioxolane L-Nucleoside Analogs Prevent Varicella-Zoster Virus Replication in Fibroblasts and Skin Organ Culture.
Chandrav De[1], Satish Chavre[2], Chung. K. Chu[2], Jennifer Moffat[1]
[1]SUNY Upstate Medical University, Syracuse, NY, United States, [2]University of Georgia, Athens, GA, United States

100. Evaluation of Approved Antivirals for Inhibition of Xenotropic Murine Leukemia-Related Virus (XMRV) in Cell-Based Assays.
Tracy L Hartman, Robert W Buckheit Jr
ImQuest Biosciences, Frederick, MD, United States

101. The Design and Synthesis of Pyrolle-Carbaldehydes as HIV-1 Integrase Strand-Transfer Inhibitors.
Raymond Hewer[1], Telisha Traut[1,2], Bradley Williams[2], Judy Coates[1]
[1]Mintek , Johannesburg, Gauteng, South Africa, [2]University of Johannesburg, Johannesburg, Gauteng, South Africa

102. Identification and Characterization of Azolo-1,3-benzoxazines and Condensed Benzopyrans as Potent Non-nucleoside Inhibitors of Orthopoxviruses.
Yuri Klimochkin[1], Vitalij Osyanin[1], Natalia Sidorina[1], Marina Leonova[1], Evgeny Belanov[2], Olga Serova[2], Sergey Balakhin[2], Nikolay Bormotov[2]
[1]State Technical University, Samara, Samara rgn., Russia, [2]FSRI SRC VB "Vector", Koltsovo, Novosibirsk rgn., Russia

103. Differential Expression of Host Cellular Factors upon HIV-1 Reactivation.
Paula Ordonez Suarez, Takayuki Hamasaki, Masanori Baba, Mika Okamoto
Division of Antiviral Chemotherapy, Center for Chronic Viral Diseases, Kagoshima University, Kagoshima, Kagoshima, Japan

104. Recycling of HIV Particles is Required for Infection by Endocytosed Virus After Cell to Cell Transfer.
Marc Permanyer, Ester Ballana, José A. Esté
IrsiCaixa, AIDS Research Institut, Badalona, Barcelona, Spain

105. Inhibition of influenza virus-induced NF-kB and ERK activation can simultaneously reduce both, virus titres and cytokine expression *in vitro* and *in vivo*.
Ruth Pinto[1], Susanne Herold[2], Lidija Cakarova[2], Katrin Hoegner[2], Jürgen Lohmeyer[2], Oliver Planz[3], Stephan Pleschka[1]
[1]Institute of Medical Virology, Justus-Liebig-University, Giessen, Hessen, Germany, [2]Department of Internal Medicine, University of Giessen Lung Centre, Giessen, Hessen, Germany, [3]Friedrich-Loeffler-Institute (FLI), Tübingen, Baden-Würtenberg, Germany

106. A Viable Human Influenza A Virus Lacking Neuraminidase (NA) Activity – Isolation and Characterization.
Martina Richter[1], Sandor Nietzsche[2], Elke Bogner[3], Peter Wutzler[1], Michaela Schmidtke[1]
[1]Institute of Virology and Antiviral Therapy, Jena University Hospital, Jena, Germany, [2]Centre of Electron Microscopy, Jena University Hospital, Jena, Germany, [3]Institute of Virology, Charité University Hospital , Berlin, Germany

107. Favipiravir (T-705) Treatment of Experimental Arenaviral Infection Initiated after the Onset of Clinical Disease.
Brian B. Gowen[1], Michelle Mendenhall[1], Andrew Russell[1], Donald F. Smee[1], Yousuke Furuta[2]
[1]Utah State University, Logan, Utah, United States, [2]Toyama Chemical Company, Ltd., Toyama, Japan

Tuesday, May 10, 2011

Poster Session 2: Hepatitis Viruses, Herpes Viruses, Pox Viruses, Other Antiviral Agents and Medicinal Chemistry
Sofia 3 and Kyota

04:00 PM - 06:00 PM

108. Identification of Alphavirus Inhibitors by Using Virus-Based Assays and a Chikungunya Replicon Cell Line.
Tero Ahola[1], Leena Pohjala[1,2], Pasi Kaukinen[1], Age Utt[3], Margus Varjak[3], Andres Merits[3], Päivi Tammela[2]
[1]Institute of Biotechnology, University of Helsinki, Helsinki, Finland, [2]Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland, [3]Institute of Technology, University of Tartu, Tartu, Estonia

109. New Antiviral Substances of Indoloquinoxaline and Diphenyl Nature.
Georgiy V. Antonovych[1], Olena Bogorad-Kobelska[1], Nadia M. Zholobak[1], Sergiy A. Lyakhov [2], Maria O. Shibinska[2], Sergiy A. Andronati[2], Mykola Ya. Spivak[1]
[1]D.K. Zabolotny Institute of Microbiology and Virology NAS of Ukraine, Kyiv, N/A, Ukrenia, 2O.V. Bogatsky Physicochemical institute NAS of Ukraine , Odesa, N/A, Ukrenia

110. Pegasys As A Second Line of Effective Treatment Plan For G3 Non Responders of Conventional Therapy.
Binish G Arshad[1], Abida Raza[2], Samina Shakeel[1], Muhammad A Anwar[3]
[1]Quaid-i-Azam University, Islamabad, Pakistan, [2]Nuclear Medicines Oncology and Radiotherapy Institute, Islamabad, Pakistan, [3]PAEC General Hospital, Islamabad, Pakistan

111. Effective Treatment Plan For G3 Patients.
Binish G. Arshad[1], Samina Shakeel[1], Abida Raza[2], Muhammad A. Anwar[3]
[1]Quaid-i-Azam University, Islamabad, Pakistan, [2]Quaid-i-Azam University, Islamabad, Pakistan, [3]NORI, Islamabad, Pakistan, [4]PAEC,Hospital, Islamabad, Pakistan

112. Cost Effective Rapid Virological Response Guided Peginterferon Therapy Plan In HCV Genotype 3 Pakistani Population.
Hafsa Aziz[1], Abida Raza[1], Uzma Adeeb[2], Amin Athar[3], Muzaffar L Gill[2]
[1]Nuclear Medicines Oncology and Radiotherapy Institute, Islamabad, Pakistan, [2]Islamabad Specialist Clinic, Islamabad, Pakistan, [3]IBB Punjab University, Lahore, Pakistan

113. Antiviral Activity of *Cymbopogon nardus* (L.) Rendle Fractions Against HSV-1.
Adibah A. Bahtiar[1], Nazlina Ibrahim[1], Ismail Ahmad[2]
[1]Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia, [2]Universiti Malaysia Sarawak, Sarawak, Sarawak, Malaysia, 3 Malaysia

114. Amino Acid Substitutions at Residue 207 of Viral Capsid Protein 1 (VP1) Confer Pleconaril Resistance in Coxsackievirus B3 (CVB3).
Heike Braun[1], Vadim A. Makarov[2], Olga B. Riabova[2], Peter Wutzler[1], Michaela Schmidtke[1]
[1]Institute of Virology and Antiviral Therapy, Jena University Hospital, Jena, Germany, [2]Laboratory of Biochemistry of Stresses of Microorganisms, RAS Institute of Biochemistry, Moscow, Russia

115. Engineering Genetic Suppressor Elements against Hepatitis C Virus.
Zhilei Chen, Rudo Simeno
Texas A&M University, College Station, TX, United States

116. 3',5'di-O-Trityluridine Inhibits Flavivirus (Dengue and Yellow Fever Virus) Replication and Targets the Viral RNA Dependent RNA Polymerase.
Tine De Burghgraeve[1], Suzanne JF Kaptein[1], Kai Dallmeier[1], Barbara Selisko[2], Michael Jacobs[3], Bruno Canard[2], Arthur Van Aerschot[1], Johan Neyts[1]
[1]Rega Institute, KULeuven, Leuven, Belgium, [2]Université de la Méditerranée, Marseille, France, [3]Royal Free & University College Medical School, London, United Kingdom

117. Identification of a Novel Antiviral Drug Targeting at Host Apoptotic Responses.
Linlin Gu, Qianjun Li
University of Alabama at Birmingham, Birmingham, Alabama, United States

118. New Caledonian Plants As a Source of Dengue Virus Inhibitors.
Jean-Claude Guillemot[2], Pierre-Marie Allard[3], Paul Coulerie[1], Cecilia Eydoux[2], Françoise Gueritte[3], Edouard Hnawia[1], Canard Bruno[2], Litaudon Marc[3]
[1]Laboratoire de chimie des substances naturelles, LIVE-UNC EA 4243, Université de la Nouvelle-Calédonie, Nouméa, BP R4 98858 , New Caledonia, [2]AFMB-ESIL, UMR 6098, Universités d'Aix-Marseille I et II, Marseille, Cedex 09, France, [3]Centre de Recherche de Gif ICSN, CNRS, Gif-sur-Yvette, 91198, France

119. Silibinin Abolish the Enhanced Expression of Fibrosis-Related Molecules Caused by Hepatitis C Virus E2 Protein.
Ming-Ju Hsieh1, Yen-Chen Liu[3], Tzy-Yen Chen[2], Hui-Ling Chiou[3],[4]
[1]Institute of Biochemistry and Biotechnology, Chung Shan Medical University, Taichung, Taiwan, [2]Department of Internal Medicine, Chung Shan Medical University Hospital, Taichung, Taiwan, [3]School of Medical Laboratory and Biotechnology, Chung Shan Medical University, Taichung, Taiwan, [4]Department of Clinical Laboratory, Chung Shan Medical University Hospital, Taichung, Taiwan

120. Antiherpes Activies of Some Medical Plants From The Lamiaceae.
Kalina A. Kostova[1], Anton V. Hinkov[1], Stoyan A. Shishkov[1], Daniel G. Todorov[1], Milena A Dimitrova[2], Zhenya P. Yordanowa[2], Veneta M. Kapchina-Toteva[2]
[1]"St. Kliment Ohridski" University, Faculty of Biology, Laboratory of Virology, Sofia, Sofia, Bulgaria, [2]"St. Kliment Ohridski" University, Faculty of Biology, Department of Plant Physiology, Sofia, Sofia, Bulgaria

121. Antiviral Activity of A Thioglycoside Derivative Mimicking Tunicamycin Structure.
Ewelina Krol[1], Dawid Nidzworski[1], Grazyna Pastuch-Gawolek[2], Grzegorz Grynkiewicz[3], Wieslaw Szeja[2], Boguslaw Szewczyk[1]
[1]University of Gdansk, Gdansk, Poland, [2]Silesian Technical University, Gliwice, Poland, [3]Pharmaceutical Institute, Warsaw, Poland

122. Hepatitis E Virus (HEV) Proteome and RNA Silencing Suppressors (RSS): A Search.
Amit Kumar, Subrat K Panda, Hemlata Durgapal
All India Institute of Medical Sciences, New Delhi, Delhi, India

123. Investigation of the Effects on Early Secretory Pathway in Cultured Cells and Potential Application of Antiviral Substances.
V. Levakova, A. S. Galabov
Department of Virology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Sofia, Bulgaria

124. Virucidal Activity of Calluses' Extracts From Tobacco-Plants.
V Lozitsky, A Fedchuk, T Grydina, L Mudryk, L Shitikova, L Socheslo
Ukrainian I.I. Mechnikov Anti-Plague Research Institute, Odesa, Odesa, Ukrenia

125. Impact of HIV Coinfection On State of Immunology of Patients With Chronic HCV-Infection.
Natallia V. Matsieuskaya, Vladimir M. Tsyrkunov
Medical University, Grodno, GR, Belarus

126. Styrylpyrone Derivative of *Goniothalamus Umbrosus* Inhibit HSV-1 Infection During Viral Early Replication Cycle.
Norefrina S. Md Nor, Nazlina Ibrahim
Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia, Malaysia

127. Hepatitis C Virus Vaccine Candidates From Chimeric Hepatitis B Core Virus-Like Particles Carrying Different Fragments of HCV Non-Structural Protein 3.
Marija Mihailova[1], Anastasija Dovbenko[1], Janis Bogans[1], Andreas Walker[2], Velta Ose[1], Irina Sominskaya[1], Sergei Viazov[2], Pauls Pumpens[1]
[1]Latvian Biomedical Research and Study Centre, Riga, Riga, Latvia, [2]Institute of Virology, Essen University Hospital, Essen, Essen, Germany

128. The Crimean Congo Hemorrhagic Fever European Consortium: Modern Approaches to Diagnostics, Epidemiology, Prevention, Therapy and Preparedness.
Ali Mirazimi[1], Jerome Weinbach[2], Manfred Weidmann[3], Anna Papa-Konidari[4], Peter Leyssen[5], Gülay Korukluoğlu[6], Friedemann Weber[7]
[1]Swedish Institute for Infectious Disease Control, Stockholm, Sweden, [2]Inserm-Transfert, Paris, France, [3]Universitaetsmedizin Goettingen, Goettingen, Germany, [4]Aristotelio Panepistimio Thessalonikis, Thessalonikis, Greece, [5]Katholieke Universiteit Leuven , Leuven, Belgium, [6]Refik Saydam National Public Health Agency , Ankara, Turkey, [7]Philipps Universität Marburg, Marburg, Germany

129. Nitazoxanide is an Indirect Inhibitor of HCV Replication Through Modulation of Cellular Kinase CKI Alpha to Enhance HCV NS5A Hyperphosphorylation.
Abigail Montero, Prasanth Viswanathan, Chansuek Yon, Brent Korba
Georgetown University Medical Center, Washington, DC, United States

130. Adenosine Deaminase-Like Protein 1 (ADAL1) Catalyzes Removal of Different Alkyl Groups From N6- Or O6-Substituted Purine Or 2-Aminopurine Nucleoside Monophosphates.
Eisuke Murakami, Haiying Bao, Ralph Mosely, Jinfa Du, Michael J Sofia, Phillip A Furman
Pharmasset, Inc, Princeton, New Jersey, United States

131. Inhibition of Human Cytomegalovirus Replication by Tricin (4',5,7-Trihydroxy-3',5'-Dimethoxyflavone).
Tsugiya Murayama[1], Ying Li[1,2], Rie Yamada[1], Hidetaka Sadanari[1], Keiko Matsubara[1], Yuuzo Tuchida[2], Mamoru Koketsu[3], Kunitomo Watanabe[3]
[1]Hokuriku University, Kanazawa, Ishikawa, Japan, [2]Hououdou Co. Ltd., Shinagawa, Tokyo, Japan, [3]Gifu University, Gifu, Gifu, Japan

132. Identification of Bicyclic Sulfone Inhibitors of HHV-6 Targeting The HHV-6 U77 Helicase.
Lieve Naesens[1], Graciela Andrei[1], Robert Snoeck[1], Daniel Gerry[2], Joseph E. Banning[2], Phillip D. Wilkerson[2], Zoe M. Renew[2], Chad E. Stephens[2]
[1]Rega Institute for Medical Research, K.U.Leuven, Leuven, Belgium, [2]Department of Chemistry and Physics, Augusta State University, Augusta, GA, United States

133. Antiviral Effect of Oxoglaucine in Combination with Some Enterovirus Replication Inhibitors.
Lubomira Nikolaeva-Glomb, Adelina Stoyanova, Anna Metodieva, Angel S. Galabov
The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Sofia, Bulgaria

134. Antiadenoviral Assay, Based on The Quantitative Detection of Infected Cells Containing Virus-Induced Intranuclear Inclusion Bodies.
Lidiya N. Nosach
Institute of Microbiology and Virology NASU, Kyiv, Ukrenia

135. Triterpenoids From Platycodon Grandiflorum Inhibit Hepatitis C Virus Replication.
Sang Jin Park[1], Jong Hwan Lim[1], Jae Won Yang[1], Jung Cheul Shin[1], Eun-Joo Kim[4], Joo Won Suh[2], Soon B. Hwang[3], Jong Woo Kim[1,2]
[1]Laboratory of Infectious Disease, B&C Biopharm Co. Ltd., Suwon, Gyeonggi-do , Korea, [2]Division of Bioscience and Bioinformatics, Myongji University, Yongin, Gyeonggi-do , Korea, [3]Ilsong Institute of Life Science, Hallym University, Anyang, Gyeonggi-do , Korea, [4]Department of Pharmacology, Korea Institute of Toxicology, Korea Research Institute of Chemical Technology, Daejeon, Chungcheongnam-do, Korea

136. Combinatorial Anti-Arenaviral Therapy With The Small Molecule SKI-1/S1P Inhibitor PF-429242 and Ribavirin.
Antonella Pasquato[1], Cylia Rochat[1], Juan Carlos de la Torre[2], Stefan Kunz[1]
[1]University Hospital Center and University of Lausanne, Lausanne, Switzerland, Switzerland, [2]The Scripps Research Institute, La Jolla, CA, United States

137. Generation of dsRNAs Targeting VP1 and VP3 Gene Regions of Coxsackievirus B1 Utilizing Bacteriophage ϕ6 Polymerase Complex.
Nikolay M. Petrov[1], Dennis H. Bamford[2], Ralitsa Vassileva-Pencheva[1], Angel S. Galabov[1]
[1]Department of Virology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Sofia, Bulgaria, [2]Institute of Biotechnology, University of Helsinki, Helsinki, Helsinki, Finland

138. Inhibition of Herpes- and Adenovirus Replication by Extract of *Artemia Salina* Cysts From Crimean Hypersaline Lakes.
Olga Yu. Povnitsa[1], Lidiya N. Nosach[1], Irina I. Rudneva[2], Valentin G. Shayda[2], Nadiya V. Nesterova[1]
[1]Institute of Microbiology and Virology NASU, Kyiv, Ukrenia, [2]Institute of Biology of the Southern Seas NASU, Sevastopol, Ukrenia

139. European Consortium on antiviral Drug Development: SILVER.
Jean-Louis Romette, Ernest Gould
UNIVMED, Marseille, France

140. *In-vitro* Screening for Compounds Active Against Polyomavirus BK: a Seven Year Experience .
Parmjeet S
University of Pittsburgh, Pittsburgh, PA, United States

141. Studies on Anti-HSV Activity and Cytotoxicity of Morinda citrifolia L Noni Leaf.
Periyasamy Selvam[1], Julie M. Breitenbach[2], Katherine Z. Borysko[2], John C. John C. Drach[2]

[1]Devaki Amma Memorial College of Pharmacy, Chelembra, Malapuram, Kerala, India, [2]School of Dentistry and College of Pharmacy, University of Michigan, Michigan, Ann Arbor, MI, United States

142. Antiviral Activity of Carbohydrate-Containing Biopolymers of *Pseudomonas chlororaphis* Subsp. *Aureofaciens*.
Victoria V. Shepelevitch, Volodymyr V. Shubchynskyy, Ludmila D. Varbanets, Elena A. Kiprianova
Zabolotny Institute of microbiology and virology, Kiev, Ukraine,

143. European Training Network on (+)RNA Virus Replication and Antiviral Drug Development.
Frank van Kuppeveld[1], Eric Snijder[2], Alexander Gorbalenya[2], Bruno Canard[3], Ralf Bartenschlager[4], Johan Neyts[5], Andrea Brancale[6], Chris McGuigan[6]
[1]Radboud University Nijmegen , Nijmegen, Netherlands, [2]Leiden University Medical Center, Leiden, Netherlands, [3]Université de la Méditerranée , Marseille, France, [4]University Hospital of Heidelberg, Heildelberg, Germany, [5]Rega Institute KU Leuven, Leuven, Belgium, [6]Cardiff University, Cardiff, Wales

144. Ellagitannins as New Highly Efficient Inhibitors of Herpes Simplex Virus Replication and Synergists of Acyclovir.
Nelly Vilhelmova[1], Adelina Stoyanova[1], R. Jacquet[2], S. Quideau[2], Angel S. Galabov[1]
[1]Department of Virology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Sofia , Bulgaria, [2]Université de Bordeaux, Institut des Sciences Moléculaires (CNRS-UMR 5255), Institut Européen de Chimie et Biologie, Pessac Cedex, France, France

145. *In Vitro* Combination Therapy With Tegobuvir (GS-9190) Is Highly Efficient In Curing Cells From HCV Replicon and In Delaying/Preventing The Development of Antiviral Resistance.
Inge Vliegen[1], Jan Paeshuyse[1], I-hung Shih[2], Gerhard Pürstinger[3], Steven Bondy[2], William A. Lee[2], Weidong Zhong[2], Johan Neyts[1]
[1]Rega Institute, Leuven, Belgium, [2]Gilead Sciences, Inc., Foster City, CA, United States, [3]University of Innsbruck, Innsbruck, Austria

146. Human Papillomavirus Genotype Distribution In Women In Montenegro.
Danijela Vujoševic[1], Vineta Vuksanovic[1,2], Mario Poljak[3], Nebojša Joksimovic[4]
[1]Institute of Public Health, Podgorica, Montenegro, Yugoslavia, [2]University of Montenegro, Podgorica, Montenegro, Yugoslavia, [3]Institute of Microbiology and Immunology, Ljubljana, Slovenia, Slovenia, [4]Clinical Centre of Monetengro, Podgorica, Montenegro, Yugoslavia

147. Enhanced Cellular Penetration of ODE-(S)-MPMPA Accounts For Its Prolonged Post-Exposure Anti-HCV Activity.
David L. Wyles[1], Krysten A. Jones[1], Nadejda Valiaeva[1,2], James R. Beadle[1,2], Robert T. Schooley[1], Karl Y. Hostetler[1,2]
[1]University of California, San Diego, La Jolla, CA, United States, [2]Veterans Medical Research Foundation, San Diego, CA, United States

148. Anti-EBV Activity of Hemocyanin Isolated From *Helix Lucorum*.
Svitlana D. Zagorodnya[1], Pavlina Dolashka[2], Galina V. Baranova[1], Anna V. Golovan[1], Nadiya V. Nesterova[1]
[1]Institute Microbiology and virology NASU, Kyiv, Ukrenia, [2]Institute of Organic Chemistry, BAS, Sofia, Bulgaria

149. Investigation of Anti-EBV Activity of Ganciclovir In Combination With Antiflogistics.
Svitlana D. Zagorodnya[1], Anna O. Kurova[1], Galina V. Baranova[1], O. L. Chyuko [2], Georgiy I. Danilenko[2], Nadiya V. Nesterova[1]
[1]Institute Microbiology and virology NASU, Kyiv, Ukrenia, [2]Institute of Organic Chemistry NASU, Kyiv, Ukrenia

150. Thiazolylthioacetamides as a Novel Class of Potential Antiviral Agents.
Peng Zhan [1] , Xuwang Chen[1] , Xinyong Liu* [1], Christophe Pannecouque[2] , Lieve Naesens[2] , Erik De Clercq[2] , Ailin Liu[3], Guanhua Du[3]
[1]Department of Medicinal Chemistry, School of Pharmaceutical Sciences, ShanDong University, Ji'nan , ShanDong , China, [2]Rega Institute for Medical Research, Minderbroedersstraat, Leuven, Belgium, [3]Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing , Beijing , China

151. Antiviral Effectivity of Ceria Colloid Solutions.
Nadiya Zholobak[1], Alexander Shcherbakov[1], Vladimir Ivanov[2], Zoya Olevinskaya[1], Nikolay Spivak[1]
[1]Zabolotny Institute of Microbiology and Virology NASU, Kiyv, Ukrenia, [2]Kurnakov Institute of General and Inorganic Chemistry, Moskow, Russia

152. Computer-Aided Design and Evaluation of Novel Anti-Chikv Compounds.
Marcella Bassetto[1], Tine De Burghgraeve[2], Pieter Leyssen[2], Johan Neyts[2], Andrea Brancale[1]
[1]The Welsh School of Pharmacy, Cardiff Univeristy, Cardiff, Wales, United Kingdom, [2]Rega Institute for Medical Research, Leuven, Flemish Brabant, Belgium

153. Intravitreal Alkoxyalkyl Esters of Cyclic Cidofovir for Treatment of Ocular Viral Infections.
James R. Beadle[1], Lingyun Cheng[2], Karl Y. Hostetler[1], William R. Freeman[2]
[1]Univ. of California, San Diego, La Jolla, CA, United States, [2]Jacobs Retina Center, Univ. of California, San Diego, La Jolla, CA, United States

154. Effect of Molecular Symmetry on Potency in Novel Down-Modulators of the CD4 Receptor.
Thomas W. Bell[1], Violeta G. Demillo[1], Florian Goulinet-Mateo[1], Rameez Ali[1], Nicholas C. Pflug[1], Chiraphorn Khan[1], Kurt Vermeire[2], Dominique Schols[2]
[1]Department of Chemistry, University of Nevada, Reno, NV 89557, United States, [2]Department of Microbiology and Immunology, Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

155. Tripartate Prodrugs of Hydroxy-Containing Compounds.
María-José Camarasa[1], Alberto Diez-Torrubia[1], Silvia Cabrera[1], Graciela Andrei[2], Robert Snoeck[2], Jan Balzarini[2], Sonsoles Velázquez[1]
[1]Instituto de Química Médica (CSIC) , Madrid, Spain, [2]Rega Institute for Medical Research, K.U. Leuven , Leuven, Belgium

156. Synthesis and Evaluation of Biological Activity of New Aminoadamantane Amides Containing Hydroxycinnamoyl Moiety.
Maya G. Chochkova[1], Asya P. Georgieva[1], Galya I. Ivanova[2], Ivanka G. Stankova[1], Tsenka S. Milkova[1]
[1]South-West University "Neofit Rilski" Department of Organic Chemistry, 2700 , Blagoevgrad, Bulgaria, [2]Requimte, Polo da Universidade do Porto Departamento de Química Rua do Campo Alegre, 4169-007 , Porto, Portugal

157. Coumarins Hybridized with Heterocycles or Ribonucleosides for Eradication of Hepatitis C Virus.
Reuben Jih-Ru Hwu[1,2], Shu-Yu Lin[1], Shwu-Chen Tsay[1], R. Singha[1], J. Neyts[3]
[1]National Tsing Hua University, Hsinchu, Hsinchu, Taiwan, [2]National Central University, Jhongli, Jhongli, Taiwan, [3]Katholieke Universiteit Leuven, Leuven, Leuven, Belgium

158. HSV-1 and Alzheimer's Disease: The Case For Antiviral Treatment.
Ruth F Itzhaki[1], Alison L Frost[1], Matthew A Wozniak[1]
[1]University of Manchester, Manchester, Lancs, United Kingdom, [2]University of Manchester, Manchester, Lancs, United Kingdom, [3]University of Manchester, Manchester, Lancs, United Kingdom

159. Synthesis and Antiviral Evaluation of N9-[2-(Phosphonomethoxy)ethyl] (PME) Analogues Derived from 8-Substituted Purines.
Zlatko Janeba[1], Antonín Holý[1], Robert Snoeck[2], Graciela Andrei[2], Erik De Clercq[2], Jan Balzarini[2]
[1]Institute of Organic Chemistry and Biochemistry, AS CR, Prague, Czech Republic, [2]Rega Institute for Medical Research, KU Leuven, Leuven, Belgium

160. Acyclic Nucleoside Phosphonates: Past, Present and Future.
Zlatko Janeba, Antonín Holý
Institute of Organic Chemistry and Biochemistry, AS CR, Prague, Czech Republic

161. Design, Synthesis, and Anti-HCV Activity of 2'-Modified-4'-selenonucleosides.
Lak Shin Jeong[1], Jin Ha Yu[1], Hyuk Woo Lee[1], Jung Hee Choi[1], Won Jun Choi[1,2]
[1]Ewha Womans University, Seoul, Seoul, South Korea, [2]Dongguk University, Ilsan, Kyungki-do, South Korea

162. Genome Specific Diagnosis of Influenza Virus Strains by Hairpin-Type Peptide Nucleic Acid.
Kunihiro Kaihatsu, Nobuo Kato
Osaka University, Ibaraki, Osaka, Japan

163. Development of Novel Evaluation Method to Anti-influenza Drug Resistance using Docking Study.
Norihito Kawashita[1,2], Masashi Yasuda[1], Yu-Shi Tian[1], Kousuke Okamoto[1], Masaya Kawase[3], Teruo Yasunaga[2], Tatsuya Takagi[1,2]
[1]Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka, [2]Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, [3]Nagahama Institute of Bio-Science and Technology, Nagahama, Shiga,

164. Antiviral Properties of New Cage Compounds.
Yuri Klimochkin[1], Marat Baimuratov[1], Ekaterina Knyazeva[1], Nadezhda Baleeva[1], Marina Leonova[1], Michail Skomorokhov[1], Evgeny Belanov[2], Sergey Balakhnin[2]
[1]State Technical University, Samara, Samara rgn., Russia, [2]FSRI SRC VB "Vector", Koltsovo, Novosibirsk rgn., Russia, [3], Samara region, Russia, 4, Samara region, Russia, 5, Samara region, Russia, 6, Samara region, Russia

165. Indolylarylsulfones as HIV-1 Non-Nucleoside Reverse Transcriptase Inhibitors. New Cyclic Substituents at the Indole-2-carboxamide.
Giuseppe La Regina[1], Antonio Coluccia[1], Andrea Brancale[2], Giovanni Maga[3], Jan Balzarini[4], Ettore Novellino5, Romano Silvestri[1]
[1]Sapienza University, Roma, Italy, [2]Cardiff University, Cardiff, United Kingdom, [3]CNR, Pavia, Italy, [4]Katholieke Universiteit, Leuven, Belgium, 5Federico II University, Napoli, Italy

166. Anti-HCV Drug Development.
Shu-Yu Lin
National Tsing Hua University, Hsinchu, Taiwan, Taiwan

167. A Novel Family of Multivalent Compounds Able To Interact With GP120: Anti-Hiv Evaluation and Binding Analysis With Surface Plasmon Resonance.
Virginia Lozano[1], Leire Aguado[1], Bart Hoorelbeke[2], Marleen Renders[2], María-José Camarasa[1], Ana San-Félix[1], Jan Balzarini[2], María-Jesús Pérez-Pérez[1]
[1]Instituto de Química Médica (CSIC), Madrid, Madrid, Spain, [2]Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Leuven, Belgium

168. Targeting HCV (+) Strand RNA genome by a Novel PNA-neamine Conjugate.
Dineshkumar Manvar, Nootan Pandey, Virendra N Pandey
UMDNJ-New Jersey Medical School, Dept. of Biochemistry, Newark, NJ, United States

169. Targeting The Flavivirus Helicase.
Eloise Mastrangelo[1], Margherita Pezzullo[2], Martino Bolognesi[2], Suzanne Keptein[3], Johan Neyts[3], Boris Pastorino[4], Xavier de Lambellerie[4], Mario Milani[1]
[1]CNR- Istituto di Biofisica, Milano, Italy, [2]Università di Milano, Milano, Italy, [3]University of Leuven, Leuven, Belgium, [4]Université de la Méditerranée, Marseille, France

170. Stereoselective Synthesis of Different Types of Nucleotide Prodrugs.
Chris Meier[1], Rios Morales Edwuin Hander[1], Arbelo Román Cristina[1], Lindström Nadine[1], Balzarini Jan[2]
[1]Organic Chemistry, Department of Chemistry, Faculty of Sciences, University of Hamburg, Hamburg, Germany, [2]Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium

171. A Comparison of the ability of wild-type and S282T mutant HCV NS5B to incorporate 2'-a-F-2'-b-C-methylguanosine-5'-monophosphate and 2'-a-OH-2'-b-C-methylguanosine-5'-monophosphate
Eisuke Murakami, Haiying Bao, Angela M Lam, Christine Espiritu, Shalini Bansal, Phillip A F
Pharmasset, Inc, Princeton, New Jersey, United States

172. QSAR Analysis of Anti-Influenza (A/H1N1) Activity Of Azolo-Adamantanes.
Eugene Muratov[1,2], Ekaterina Varlamova[1], Anatoly Artemenko[1], Victor Kuz'min[1], Pavel Anfimov[3], Vladimir Zarubaev[3], Victor Saraev[4], Oleg Kiselev[3]
[1]A.V.Bogatsky Physical-Chemical Institute NAS of Ukraine, Odessa, Ukrenia, [2]University of North Carolina, Chapel Hill, NC, United States, [3]Influenza Research Institute, Saint Petersburg , Russia, [4]Saint Petersburg State Institute of Technology, Saint Petersburg , Russia

173. Viral Genome Dynamics during Antiviral Resistance Selection: A First Glimpse into Viral Evolution.
Simone Musiu, Jan Paeshuyse, Mathy Froeyen, Philippe Lemey, Johan Neyts
Katholieke Universiteit Leuven, Leuven, Vlaams Brabant, Belgium

174. Structure-Based Design of Small-Molecules That Selectively Inhibit Dengue Virus Methyltransferase.
Christian G. Noble
Novartis Institute for Tropical Diseases, 10 Biopolis Road, 05-01 Chromos, Singapore

175. Phosphoramidate Dinucleosides As Inhibitors of Hepatitis C Virus Subgenomic Replicon And NS5B Polymerase Activity.
S. Priet[1], I. Zlatev[2], I. Barvik[3], J. Neyts[4], H. Dutartre[1], B. Canard[1], F. Morvan[2], K. Alvarez[1]
[1]afmb, Cnrs Umr 6098, Marseille Cedex 09, France, [2]ibmm, Umr 5247 Cnr, Montpellier, France, [3] Faculty of Mathematics and Physics, PRAGUE, Czech Republic, [4]Rega Institute for Medical Research, LEUVEN, Belgium

176. Tick-Borne Flaviviruses Infection In Non-Human Primates.
Yulia Rogova, Natalia Pripuzova, Larissa Gmyl, Natalia Tereshkina, Liubov Terekhina, Mikhail Vorovich, Karina Grishina, Galina Karganova
Chumakov Institute of Polimyelitis and Viral Encephalitides Russian academy of medical sciences, Institut poliomyelita, Moscow region, Russia

177. Synthesis of Novel CADA Analog Prodrugs Designed as Down-Modulators of the CD[4] Receptor.
Emily D. Scarbrough[1], Kurt Vermeire[2], Dominique Schols[2], Thomas W. Bell[1]
[1]Department of Chemistry, University of Nevada, Reno, NV 89557, United States, [2]Department of Microbiology and Immunology, Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

178. Polymer-Cooperative Approach to multi-Blocking the Viruses.
A. Serbin[1,2], E. Karaseva[1,2], O. Alikhanova[1], V. Tsvetkov[1,3]
[1]Biomodulators RC, Health RDF, Moscow, Russia, [2]Topchiev Institute of Petrochemical Synthesis, RAS, Moscow, Russia, [3]Orehovich Institute of Biomedical Chemistry, RAMS, Moscow, Russia

179. Synthesis and *in vitro* Anti-influenza Activity of New Amino Acids and Peptidomimetics Derivatives of Oseltamivir and Rimantadine .
Ivanka Stankova[1], Kiril Chuchkov[1], Michaela Schmidtke [2], Angel Galabov[3]
[1]Department of Chemistry, South-West University "Neofit Rilski", Blagoevgrad, Bulgaria, [2]Jena University Hospital, School of Medicine, Department of Virology and Antiviral Therapy, Jena, Germany, [3]The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

180. A Computational Approach To Search Active Peptides As Membrane Fusion Inhibitors of HIV-1.
Yu-Shi Tian[1], Norihito Kawashita[1,2], Chris Verathamjamras[1], Kousuke Okamoto[2], Teruo Yasunaga[2], Masanori Kameoka[2], Tatsuya Takagi[1,2]
[1]Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka, Japan, [2]Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan

181. The 3D-Screen Technology, An Innovative Cell-Based Assay To Identify Modulators That Alter Target Protein Conformation: Example With HCV.
Isabelle Valarché, Amaya Berecibar, Mehdi Lahmar, Philippe Guedat, Majid Mehtali
Vivalis, Saint Herblain, LA, France

182. Synthesis and Antiviral Activity of 3-Methoxy-2-(phosphonomethoxy)propyl Nucleoside Esters Against HCV and HIV-1.
Nadejda Valiaeva[1,2], James R. Beadle[1,2], David L. Wyles[1], Robert T. Schooley[1], Karl Y. Hostetler[1,2]
[1]University of California, San Diego, La Jolla, CA, United States, [2]Veterans Medical Research Foundation, La Jolla, CA, United States

183. Herpes Simplex Virus Thymidine Kinase Inhibitor GLS122E and Its 6-Deoxy Prodrug GLS361B (Sacrovir™) - Potential for Preventing Viral Disease Recurrence *In Vivo*.
Ivan B. Yanachkov[1], Edward J. Dix[2], Milka I. Yanachkova[1], Sofya Dvoskin[1], Wei-Chu Xu[1], Bryan M. Gebhardt[3], George E. Wright[1]
[1]GLSynthesis, Inc., Worcester, MA, United States, [2]Assumption College, Worcester, MA, United States, [3]Louisiana State University Eye Center, New Orleans, LA, United States

184. Anti-HBV Activities of Novel [2´, 3´]--C-substituted beta-L-nucleoside Analogues.
Xiao-Xiong Zhou[1], Staffan Torssell[1], Olov Wallner[1], Piaoyang Sun[1], Tim Shaw[2], Zhuhui Huang[3], Charlotte Larsson[4], Bjorn Kull[4]
[1]Novadex Pharmaceuticals AB, Huddinge, N/A, Sweden, [2]Victorian Infectious Diseases Reference Laboratory, Melbourne, Victoria, Australia, [3]Southern Research Institute, Frederick, MD, United States, [4]Actar AB, Stockholm, N/A, Sweden

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Abstracts

24th ICAR Abstract Issue

Oral Session 2: Hepatitis Viruses *Chairs: Johan Neyts, Ph.D. and Angela Lam, Ph.D.* 1:30–4:00 pm Sofia 1 and 2

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IDX375, A Novel Allosteric HCV Polymerase Inhibitor: *In Vitro* Antiviral Activity and Preclinical Profile

C.B. Dousson[1,*], S.S. Good[2], M. La Colla[2], J. Bilello[2], M. Seifer[2], D.N. Standring[2], J.-L. Paparin[1], D. Surleraux[1]

[1] *Idenix Pharmaceuticals, Parc Euromedecine, France*
[2] *Idenix Pharmaceuticals, Inc., Cambridge, USA*

Background: The HCV NS5B polymerase is essential for HCV replication and has four distinct non-nucleoside allosteric binding sites that provide clinically validated HCV targets. This report presents the characterization of IDX375, a novel non-nucleoside inhibitor (NNI) that binds to the palm (NNI3) site domain of NS5B.

Methods: The antiviral activity and specificity of IDX375 were assessed in standard assays utilizing purified polymerases and HCV replicons. The pharmacokinetic profile of IDX375 was determined by standard methods in mice and cynomolgus monkeys.

Results: An extensive evaluation of five structurally diverse series of palm inhibitors led to the selection of IDX375 as the clinical candidate. IDX375 exhibited genotype 1b and 1a enzymatic potencies of 5 and 16 nM, respectively, with an EC_{50} of 2.3 nM in the 1b HCV replicon, and was inactive against human cellular polymerases. IDX375 generally showed additivity in combination with the HCV PI IDX320 or the nucleotide IDX184, but exhibited very strong synergy when combined with both classes of direct acting antiviral agents. Substantial plasma exposures (μM C_{max} levels) were attained following oral administration of IDX375 (15 mg/(kg day)) in the mouse and cynomolgus monkey, and IDX375 was substantially concentrated in the liver in the mouse.

Conclusions: IDX375 is a potent inhibitor of the HCV polymerase that has been dosed in healthy volunteers (de Bruijne et al., in press) and is currently in a 3-day proof of concept study in HCV-infected subjects. The current pharmacokinetic profile of IDX375 in human subjects supports a twice-daily dosing regimen.

Reference

de Bruijne, J., Wetering de Rooij, J.v.d., Leempoels, J., Zhou, X.J., Weegink, C.J., Molenkamp, R., Schinkel, C.J., Temam, M.F., Molles, J., Chen, J., Pietropaolo, K., Sullivan-Bólyai, J.Z., Mayers, D., Reesink, H.W., in press. Phase I Study in Healthy Volunteers and Patients with IDX375, a Novel Non-Nucleoside HCV Polymerase Inhibitor.

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Study of NS5B Oligomerization by FRET: Characterization and Inhibition

Itxaso Bellón-Echeverría[1,*], Alberto J. López-Jiménez[1,2], Pilar Clemente-Casares[1], Jose A. Encinar[4], Elisa Martínez-Alfaro[2], Ricardo Pérez-Flores[3], Antonio Mas[1]

[1] *Centro Regional de Investigaciones Biomédicas (CRIB), Albacete, Spain*
[2] *Disease Infectious Unit, Complejo Hospitalario Universitario de Albacete, Albacete, Spain*
[3] *Digestive Department, Complejo Hospitalario Universitario de Albacete, Albacete, Spain*
[4] *Instituto de Biología Molecular y Celular, Universidad Miguel Hernandez, Elche, Spain*

Hepatitis C virus (HCV) is a positive strand RNA virus ((+)RNA) that replicates its genome in replication complexes (RC) associated to endoplasmic reticulum (ER)-derived micro-vesicles. One key protein in these complexes is NS5B, the viral RNA-dependent RNA-polymerase (RdRp). Recently, it has been demonstrated that NS5B interacts itself forming oligomers, and mutations that disrupt these interactions are lethal for polymerase function. Therefore, NS5B oligomerization could be a new target for the design of anti-HCV compounds. We have developed a new accurate method to analyze NS5B–NS5B interactions by using Förster-resonance-energy-transfer (FRET) *in vitro* using recombinant proteins. This method has allowed us to analyze the conditions driving the interactions between NS5B polymerases, in our case the NS5B–cyan and NS5B–citrine constructs. The most important interactions among monomers are electrostatic because of the dependence on ionic strength. Both, NaCl and KCl lead to concentration-dependent changes in the oligomerization status of NS5B. We have also tested different combinations of point mutants affecting FRET values from zero to around 100%. Cooperativity in RNA synthesis activity has also been analyzed by determining the Hill coefficient and the results are consistent with those obtained for oligomerization. We have extended these studies to HCV RNA-polymerases from different genotypes (genotypes 1–5), including the analyses of reaction

conditions needed for *de novo* RNA-polymerase activity. Finally, oligomerization experiments in the presence of the non-nucleoside inhibitor (NNI) PF-254027 gave a statistically significant reduction in the FRET signal, suggesting a new connection between NS5B oligomerization and NNI binding. These results together with data reported by other groups have been used to *in silico* simulation docking studies that have allowed us to infer the fingers–thumb geometry of the interaction.

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In vitro Selection of HCV Replicons with Reduced Sensitivity to PSI-352938, A Cyclicphosphate Prodrug of β-D-2′-α-F-2′-β-*C*-Methylguanosine

Angela M. Lam*, Christine Espiritu, Shalini Bansal, Holly M. Micolochick Steuer, Veronique Zennou, Michael J. Otto, Phillip A. Furman

Pharmasset, Inc, Princeton, USA

PSI-352938 is a cyclicphosphate prodrug of β-D-2′-deoxy-2′-α-fluoro-2′- β-*C*-methylguanosine monophosphate. PSI-352938 inhibited HCV replication in genotype (GT) 1a, 1b and 2a replicon cells with EC_{50} values of 0.20 μM, 0.13 μM and 0.14 μM, respectively. Cross resistance studies showed replicon cells containing the NS5B S282T variant remained fully susceptible to PSI-352938. In this study, we describe the selection of HCV replicons with lowered susceptibility to PSI-352938. Selection studies were performed using GT 1a (H77), 1b (Con1), or 2a (J6/JFH-1) replicon cell lines. Selected HCV replicons with lowered susceptibility were analyzed for mutations within NS5B. Replicon mutants were constructed by site directed mutagenesis and examined for resistance to PSI-352938. After 158 days, HCV GT 2a replicon cells with a 19.2-fold decrease in sensitivity to PSI-352938 were selected Sequence analysis identified mutations within the HCV NS5B, including S15G, R222Q, C223Y/H, V321I, and L320I. Evaluation of these mutations indicated that combinations of at least three amino acid changes that included C223H was required for a significant loss of sensitivity to PSI-352938. With the exception of S15G, these mutations were located in close proximity to the conserved D220 and D318 residues at the active site. We were unable to select for resistant GT 1a or 1b replicons and subsequently determined that the C223Y/H mutation was lethal for the GT 1b replicon. Similar results have been obtained with a related guanosine analog monophosphate prodrug PSI-353661. The difficulty in selecting resistant variants to PSI-352938 compared to other inhibitors such as non-nucleoside analog inhibitors of NS5B, HCV NS3 protease inhibitors and NS5A inhibitors, which select for mutations rapidly both *in vitro* and *in vivo*, suggests that PSI-352938 has a very high barrier to resistance. The unique resistance profile of PSI-352938 makes it a promising compound for mono- and/or combination therapy with other HCV inhibitors, including other nucleoside/tide analogs.

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Human subtilase Site-1 Protease (S1P): An Emerging Host Cell Target for Hepatitis C Virus (HCV) Infection and HCV-associated Steatogenesis

Andrea D. Olmstead*, François Jean

University of British Columbia, Vancouver, Canada

In this study, we hypothesize that targeting cellular enzymes acting as master regulators of lipid homeostasis could represent a powerful approach to developing a novel class of broad-spectrum anti-virals against infection associated with human viruses such as HCV, Dengue virus, and rotaviruses, whose replication and pathogenesis depend on the interaction with lipid droplets (LDs). In the case of HCV, overstimulation of host lipid metabolism in the liver during viral infection promotes cholesterol intracellular storage in host LDs, a critical cellular event for HCV replication, assembly, and budding. One such master regulator of cholesterol metabolic pathways is the host subtilase S1P. Also known as SKI-1, S1P plays a critical role in the proteolytic activation of SREBPs, which control the expression of key enzymes of cholesterol and fatty-acid biosynthesis. Here, we report that strategic manipulation of cellular S1P activity levels by engineered serine protease inhibitors (serpins), Spn4A variants (Richer et al., 2004), provides a means of effectively inhibiting the S1P-dependent proteolytic cleavage of SREBPs in hepatoma cells, a critical step in the hijacking of the host cholesterol pathways by HCV. First, we describe the bioengineering, serpin functionality, and specificity studies of our novel S1P-directed Spn4A variant using a recombinant adenovirus (Ad) system. We demonstrated the anti-proteolytic and anti-HCV activities of our new Ad-expressing variant directed at S1P [reactive site loop: –RRKR– → –RRLL–]. Expression of the Ad_Spn4A_RRLL in Huh-7.5.1 cells results in inhibition of the S1P-mediated activation of SREBP-2 and down-regulation of the SREBP-2 target gene products as revealed by Western blotting. Using fluorescence microscopy, we found that specific inhibition of S1P reduces the abundance of LDs in Huh-7.5.1 cells. As hypothesized, inhibiting S1P activity blocked HCV infection (JFH-1 strain) of Huh-7.5.1 cells in a dose-dependent manner. The results of our studies contribute to our understanding of the HCV lifecycle and associated steatogenesis and to efforts in developing novel host-directed broad-spectrum anti-virals.

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Reference

Richer, M., Jean, F., et al., 2004. PNAS.

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12

DMPK and Metabolism Studies of Nucleoside Phosphoramidates Including INX-08189, A Novel Double Pro-drug and Clinical Candidate for Hepatitis C Virus Therapy

Jeff T. Hutchins[1,*], Christopher McGuigan[2], Stanley D. Chamberlain[1], Karolina W. Madela[2], Mohamed Aljarah[2], John Vernachio[1], Joseph M. Patti[1], Geoffrey Henson[1]

[1] *Inhibitex, Alpharetta, USA*
[2] *Cardiff University, Cardiff, United Kingdom*

Hepatitis C Virus (HCV) infection is a serious health problem that leads to chronic liver disease, such as cirrhosis and hepatocellular carcinoma, in an estimated 2–15% of the world's population. A collaboration between Inhibitex and the University of Caridiff in Wales has produced a novel double pro-drug approach to the

anti-HCV agent 2′-β-C-methylguanosine. A phosphoramidate (ProTide) motif and a C^6-methoxy base pro-drug moiety are combined to generate lipophilic prodrugs of the monophosphate of the guanine nucleoside. Extensive DMPK studies in multiple species which supported the selection of the lead compound will be discussed. Details of the pre-clinical development of INX-08189 including radiolabeled metabolism studies will be described. INX-08189 has completed investigational new drug enabling studies and has been progressed into human clinical trials for the treatment of chronic HCV infection.

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Oral Session 3: Retroviruses and Herpesviruses *Chairs: Rhonda Cardin, Ph.D. and Masanori Baba, Ph.D.* 8:45–11:45 am Sofia 1 and 2

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Mechanism of HIV-1 Neutralization by an Antibody: Reversible Binding Stalls Entry

David Kabat*, Emily Platt

OHSU, Portland, USA

Despite structural knowledge of broadly neutralizing monoclonal antibodies (NMAbs) complexed to the HIV-1 envelope glycoproteins gp120 or gp41 1 and tools to analyze the HIV-1 cell entry pathway, a mechanism for neutralization has never been proven. In part, this deficiency derives from ambiguities in neutralization assays, with different assays giving widely discrepant results using identical antibodies and viruses. We inferred from these discrepancies that NMAbs might impair virions rather than inactivate them, in which case their residual infectivities would depend on factors limiting specific assays. Using the most common neutralization system, which employs a HeLa-CD4/CCR5 cell clone made in our laboratory, we recently found that HIV-1 titers are determined by a race between entry of cell-attached virions and competing kinetic processes leading to inactivation. Here we show that the widely studied model NMAb, which efficiently inhibits infection after passive transfer into patients, neutralizes by slowing entry of adsorbed virions. Specifically, it slows the assembly and lowers the steady-state concentration of virus complexes with CD4 and coreceptors that control entry rates. Further analysis revealed the stoichiometry of the NMAb required for neutralization and the specific entry step that is slowed. Surprisingly, removing the NMAb from culture media caused its dissociation from virions coupled to accelerated entry and restored infectivity. We believe this is the first evidence that neutralization of viruses can be reversible. These results reveal a kinetic mechanism for HIV-1 neutralization and demonstrate that antibodies able to control infection in patients can function by reversibly slowing entry, a mechanism that has not been previously proposed for any virus.

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Beta5 integrin is the major contributor to the alphaV integrin-mediated blockade of HIV-1 replication

Ester Ballana[1,*], Eduardo Pauls[1], Bonaventura Clotet[1], Françoise Perron-Sierra[2], Gordon C. Tucker[2], José A. Esté[1]

[1] *Irsicaixa – AIDS Research Institute, Badalona, Spain*
[2] *Institut de Recherches Servier, Departments of Medicinal Chemistry and Cancer Research and Drug Discovery, Croissy sur Seine, France*

Monocytes and macrophages are targets of HIV-1 infection and play critical roles in multiple aspects of viral pathogenesis. During the differentiation of monocytes to macrophages, adhesion molecules such as integrins are upregulated, therefore providing signals that control the process and subsequently may render macrophages more susceptible to HIV-1 infection. Integrins are a family of transmembrane cell adhesion receptors that recognize cell-surface and extracellular matrix ligands. Previous work demonstrated that blocking alphaV containing integrins triggered a signal transduction pathway leading to the inhibition of NF-kappaB dependent HIV-1 transcription. However, very little is known regarding the contribution of the different beta integrins to HIV-1 infection in macrophages. Here, we show the influence of the different alphaV-coupled beta integrins in HIV-1 replication in macrophages, by evaluating the antiviral effect of anti-beta integrin antibodies, small RGD mimetic compounds and RNA interference. Expression of beta integrins was evaluated in monocyte derived macrophages (MDM) by flow cytometry. siRNAs specifically targeting beta integrins that dimerize with alphaV were used to transiently downregulate the corresponding integrin expression in MDMs. MDMs treated or not with siRNA were infected using R5-tropic virus. Inhibition of beta integrins either by specific monoclonal antibodies, small RGD mimetic compounds or RNA interference, showed that integrin beta5 was the major contributor to the integrin-mediated blockade of HIV-1 replication. Importantly, such inhibition did not induce changes in cell adhesion to the substrate. In conclusion, we demonstrate that HIV-1 infection in MDMs is influenced by integrin function, especially by the integrin dimer alphaVbeta5. In addition, these results highlight the use of RNA interference as a powerful tool to study and identify cellular factors associated to HIV replication and disease, the first step towards the identification and characterization of potential novel antiviral targets.

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Amido tyrosine esters: a promising new approach to antiviral nucleoside phosphonate prodrugs

Charles E. McKenna[1,*], Boris A. Kashemirov[1], Valeria M. Zakharova[1], Ivan S. Krylov[1], Melissa Williams[1], Marcela Krečmerová[2], John C. Drach[3], John M. Hilfinger[4]

[1] *Department of Chemistry, University of Southern California, Los Angeles, USA*
[2] *IOCB Research Centre, Academy of Sciences of the Czech Republic, Prague, Czech Republic*
[3] *School of Dentistry, University of Michigan, Ann Arbor, USA*
[4] *TSRL, Inc., Ann Arbor, USA*

Acyclic nucleoside phosphonates (ANPs) are effective antivirals, but their low permeability limits their therapeutic applications. In a continuing project to increase the oral bioavailability of ANPs, we have synthesized a series of water soluble amido tyrosine ester prodrugs of cyclic (*S*)-HPMPC, (*S*)-HPMPA and also of PMEA

and (*R*)-PMP-DAP, ANPs lacking a hydroxymethylene functionality. The new (*S*)-HPMPC and (*S*)-HPMPA prodrugs have IC_{50} values *vs* cowpox virus, vaccinia virus, HCMV and HSV-1 similar to or better than that of the parent drugs while exhibiting low cytotoxicity, good stability characteristics and efficient metabolic conversion pathways. *In vitro* antiviral evaluation of PMEA and (*R*)-PMP-DAP derivatives will be presented in comparison. The effect on pharmacokinetic properties and antiviral potency of phosphonate ester stereochemistry in diastereomeric prodrugs has been an intriguing question. To address it, a convenient synthetic procedure allowing preparation of the individual diastereomers was elaborated, allowing their absolute configurations to be unambiguously established based on X-ray diffractometry. *In vitro* antiviral and *in vivo* transport evaluation (murine model) of the individual diastereomers of L-Tyr-NH-*i*Bu cHPMPA were performed. The (S_p)-diastereomer demonstrated significant enhancement of prodrug oral bioavailability over the parent (*S*)-HPMPA (39% *vs* <5%). SAR studies exploring the effect of structural modifications in the amido tyrosine promoiety (C-terminal amide alkyl groups, amino acid stereochemistry) on *in vitro* antiviral activity, lipophilicity and solubility will also be presented.

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Cyclopropavir Inhibits the Normal Function of the Human Cytomegalovirus UL97 Kinase

Mark Prichard[1,*], Caroll Hartline[1], Rachel Gill[1], Terry Bowlin[2], Scott James[1]

[1] *University of Alabama at Birmingham, Birmingham, USA*
[2] *Microbiotix Inc., Worchester, USA*

Cyclopropavir (CPV, MBX400) is active against human cytomegalovirus (CMV) as well as both variants of human herpes virus 6 and human herpesvirus 8. The mechanism of action of CPV against CMV is similar to that of ganciclovir (GCV) in that it is phosphorylated initially by the CMV UL97 kinase and the triphosphate metabolite is thought to inhibit the viral polymerase. Resistance to CPV maps to the UL97 kinase, but is associated primarily with H520Q mutations and thus retains good antiviral activity against most GCV-resistant isolates. An examination of infected cultures treated with CPV revealed an unusual cell morphology associated with the absence of UL97 kinase activity. A surrogate assay for UL97 kinase activity confirmed that CPV inhibited UL97 kinase activity and its activity was similar to that of maribavir (MBV) in this assay. Deep sequencing of a CPV-resistant laboratory resistant isolate confirmed the H520Q mutations associated with resistance. In a subpopulation of viral genomes, a mutation in the active site of the UL97 kinase was also identified (V356G). This mutation is located near the active site of the enzyme and is in the same region as those that confer resistance to MBV. Since MBV inhibits the UL97 kinase, it is thought to reduce the activation of GCV and has been reported to antagonize its antiviral activity. Combination studies using real time PCR confirmed these results and indicated that CPV exhibited a similar level of antagonism against GCV. We conclude that the mechanism of action of CPV against CMV is complex and involves both the inhibition of DNA synthesis as well as the inhibition of the normal activity of the UL97 kinase.

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Structural and functional characterization of human cytomegalovirus terminase leads to a new antiviral target

Marta Nadal[1,2,*], Zuzanna Kaczmarska[1,2], Philippe J. Mas[3], Alexandre G. Blanco[1,2], Carme Arnan[1,2], Maria Solà[2], Darren J. Hart[3], Miquel Coll[1,2]

[1] *Institute for Research in Biomedicine (IRB Barcelona), Barcelona, Spain*
[2] *Institut de Biologia Molecular de Barcelona (CSIC), Barcelona, Spain*
[3] *European Molecular Biology Laboratory, Grenoble Outstation, Grenoble, France*

Human cytomegalovirus (HCMV) is one of the eight human herpesviruses. HCMV infection in the healthy host is usually silent but it can have serious consequences in immunocompromised individuals. Current anti-HCMV drugs (ganciclovir, foscarnet and cidofovir), which inhibit the viral DNA polymerase, have considerable side effects and some patients develop resistance to them. New antiviral compounds targeting other viral proteins may overcome these drawbacks.

HCMV replicates its DNA via concatamers, a long molecule of DNA with several copies of the genome, which has to be cut in single genome units. This packaging process is facilitated by the terminase complex, which is composed by main protein subunits UL89 and UL56. Viral encapsidation has no counterpart in mammalian cells, thus implying that the proteins involved might be selective antiviral targets.

Here we used the high-throughput screening method ESPRIT to identify a soluble domain of UL89 from a library of 18,432 randomly truncated constructs. The soluble domain, called UL89-C, corresponds to the C-terminus domain of UL89. UL89-C was purified and crystallized and its three-dimensional structure was solved. The structure showed that UL89-C has the RNase H fold. Other proteins with a similar fold are the E. coli RuvC resolvase, the HIV and ASV integrases and some transposases. We demonstrated that UL89-C corresponds to the nuclease domain of the terminase and that its function is strongly dependent on Mn^{2+} ions.

We tested the effect of various HIV integrase inhibitors on the function of UL89-C. Raltegravir – one of the most recently approved drugs for the treatment of AIDS – inhibited the nuclease function at micromolar levels. Our study opens the way for the development of new inhibitors against herpesvirus.

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In Vivo Efficacy of N-methanocarbathymidine (N-MCT) against Herpes simplex Virus Type 2 in Neonatal Guinea Pigs

Rhonda Cardin[1,*], Fernando Bravo[1], Clark Jennifer[1], Earwood Julie[1], Robert Glazer[2], Aquilur Rahman[3], David Bernstein[1]

[1] *Cincinnati Children's Hospital Medical Center, Cincinnati, USA*
[2] *Georgetown University, Washington, D.C., USA*
[3] *N & N Scientific, Inc., Rockville, USA*

The outcome of neonatal infections, even after therapy with high dose acyclovir (ACV), is not optimum. We therefore evaluated N-methanocarbathymidine [N-MCT], a nucleoside analogue with in vivo antiviral activity against herpesviruses and orthopoxviruses, in our guinea pig model of neonatal herpes as this model mimics many aspects of Herpes simplex virus type 2 (HSV-2) disease in newborn infants. Newborn guinea pigs were inoculated intranasally within 48 h of birth with 8.7×10^5 pfu of HSV-2, MS strain. Intraperitoneal treatment of ACV (60 mg/(kg day)) and N-

MCT (1, 5, and 25 mg/(kg day)) were compared when initiated 24–72 h post inoculation (hpi). Animals were evaluated for survival and symptoms of neonatal herpes infection. We had previously shown that high dose ACV (60 mg/(kg day)) is effective in this model, but only when treatment is initiated within 24 hpi. Therefore, in the first experiment, we evaluated therapy begun at 24 hpi. Both ACV and N-MCT significantly improved survival, but only 25 mg/(kg day) N-MCT significantly reduced the number of animals with symptoms. When therapy was begun at 48 hpi, N-MCT (25 mg/(kg day)) significantly increased survival to 91% compared to 27–30% for the untreated and ACV groups ($P < 0.01$). We next evaluated a lower dose of N-MCT (1 and 5 mg/(kg day)) begun at 48 hpi, as well as N-MCT (5 and 25 mg/(kg day)) begun at 72 hpi. In this study, 80–89% of animals treated with N-MCT at 48 hpi survived compared to 38% of untreated animals ($P < 0.01$). When therapy was initiated at 72 hpi, 80% of N-MCT (5 mg/(kg day)) and 100% of N-MCT (25 mg/(kg day)) treated animals survived. The number of animals with symptoms was also significantly reduced in the low dose treatment groups when therapy was initiated at both 48 and 72 hpi. In conclusion, N-MCT was highly effective and superior to high dose ACV therapy for the treatment of neonatal herpes in the guinea pig model.

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CMX001 Potentiates the Efficacy of Acyclovir in Herpes Simplex Virus Infections

Mark Prichard[1,*], Earl Kern[1], Caroll Hartline[1], Emma Harden[1], Randall Lanier[2], Debra Quenelle[1]

[1] *University of Alabama at Birmingham, Birmingham, USA*
[2] *Chimerix Inc., Durham, USA*

Although acyclovir (ACV), valacyclovir and famciclovir have proven to be effective in the therapy of certain herpes simplex virus (HSV) infections, there is a need for more effective therapies particularly for serious infections in neonates and immunocompromised individuals where resistance to these drugs can be problematic. CMX001 is an orally bioavailable lipid conjugate of cidofovir that is substantially less nephrotoxic than the parent drug and has excellent antiviral activity against all the human herpesviruses. Since this nucleotide analog does not require phosphorylation by the viral thymidine kinase it retains full antiviral activity against ACV-resistant clinical isolates. Further studies indicated that combinations of CMX001 and ACV synergistically inhibited the replication of both HSV-1 and HSV-2 in cell culture. Combined therapy with CMX001 and ACV was also highly effective in murine models of HSV infection and synergistically reduced mortality. These results suggest that CMX001 may be effective in the treatment of HSV infections and as an adjunct therapy in individuals with suboptimal responses to ACV.

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Safety and Human Pharmacokinetics of AIC316, a Potent Helicase-Primase Inhibitor of Herpes Simplex Virus (HSV)

Alexander Birkmann*, Dirk Kropeit, David McCormick, Holger Zimmermann, Helga Ruebsamen-Schaeff

AiCuris GmbH & Co. KG, Wuppertal, Germany

Background: AIC316 belongs to a novel class of anti-HSV compounds, the helicase-primase inhibitors, which have a mode of action that is distinct from that of nucleoside analogues currently in clinical use (e.g. acyclovir). AIC316 is in phase II clinical development and a comprehensive nonclinical and clinical program has been conducted. Here we report on the safety and pharmacokinetics (PK) of AIC316 in healthy volunteers within phase I clinical trials.

Methods: Safety and PK of AIC316 was investigated in two double-blinded, placebo controlled trials comprising single (trial A) and multiple (trial B) dose escalation as well as in an open-label food interaction cross-over trial (trial C). Single oral doses of 5–600 mg AIC316 in males and an oral dose of 80 mg AIC316 in females (to assess a potential gender effect) were administered in trial A. A single oral dose of 80 mg was administered in trial C with and without a high fat, high calorie breakfast. In trial B, subjects received once daily doses of 5 mg, 25 mg, and 100 mg for three weeks.

Results: In all three trials AIC316 was safe and well tolerated. No dose-dependent adverse events occurred, no effects on safety laboratory, vital signs and ECG parameters were detected. There was dose proportional increase in exposure after single doses of up to 400 mg (C_{max}) and 480 mg (AUC), respectively, and with daily doses up to 100 mg at steady state. Terminal elimination half-life varied between 52 and 85 h after single dose and at steady state resulting in about 5 times higher plasma concentrations at steady state compared to single dose administration. No clinically relevant gender-related difference in exposure was detected for the single dose of 80 mg. The rate of absorption was decreased by food intake, but both C_{max} and AUC showed a slight increase. Comparison of human exposures with the effective concentration of AIC316 in cell culture showed that plasma levels were maintained above the EC_{90} for 24 h after 40 mg single dose administration and at steady state with daily doses of 25 mg.

Conclusion: AIC316 was safe and well tolerated in healthy volunteers and has a favorable PK profile indicative of efficacy with once per day dose administration.

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Oral Session 5: Respiratory Viruses, Emerging Viruses and Biodefense Chairs: Brian Gowen, Ph.D. and Graciela Andrei, Ph.D. 1:30–3:30 pm Sofia 1 and 2

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Important Role for Protein Kinase C-α in Combined Pneumolysin/Influenza A Virus-induced Pulmonary Endothelial Hyperpermeability

Júlia Vergara-Alert[1,*], Supriya Sridhar[2], Guang Yang[2], Ian Davis[3], Trinad Chakraborty[4], Ayub Darji[1], Rudolf Lucas[2]

[1] *Centre de Recerca en Sanitat Animal (CReSA), UAB-IRTA, Bellaterra, Spain*
[2] *Vascular Biology Center, Medical College of Georgia, Augusta, USA*
[3] *Veterinary Biosciences, Ohio State University, Columbus, USA*
[4] *Institute of Medical Microbiology, Justus-Liebig University Giessen, Giessen, Germany*

Influenza viruses have posted an increasing threat of potential pandemics, as was shown in the past outbreaks involving H5N1 or H1N1 viruses. Influenza A virus (IAV) and *Streptococcus pneumoniae* represent important etiological agents of severe pneumonia, which is the main cause of death in children under 5 years of age worldwide. Mortality after influenza A infection has been suggested to be mainly due to secondary pneumoccocal infections. Death in pneumococcal-induced pneumonia can occur days after initiation of antibiotic therapy, when tissues are sterile and the pneumonia is clearing and correlates with the presence of virulence factors, the most important one of which is the pore-forming toxin pneumolysin (PLY). In this study, we report that co-treatment of monolayers of human microvascular endothelial cells (HL-MVEC) with both PLY (7.5 ng/ml) and UV-inactivated IAV (A/Wisconsin/33 (H1N1) strain, 1 IU/cell), induces a significant loss of barrier integrity (normalized transendothelial resistance drops from 1.0 to 0.4), as measured by means of using the electrical cell substrate impedance sensing technique (ECIS 1600R, Applied Biophysics, Troy, NY), whereas each treatment independently fails to do so. Since Protein Kinase C has been demonstrated to be involved in regulating endothelial permeability, we have therefore assessed its potential implication in the observed effects of IAV/PLY. As such, we could detect that both PLY and IAV induce PKC-α activation in HL-MVEC within 1 h. Moreover, a specific PKC-a inhibitor Ro-32-4032 (10 nM) significantly blunts the permeability-increasing effect of the combined IAV/PLY treatment in HL-MVEC by about 50%. In conclusion, these results indicate that PKC-α may represent an important therapeutic target in IAV infection-associated pulmonary endothelial hyperpermeability.

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2′- and 4′-Modified Ribonucleoside Analogs Can Inhibit All Four Serotypes of Dengue Virus in Human Primary Dendritic Cells as Competitive Inhibitors and Non-obligatory Chain Terminators

Hassan Javanbakht[1,*], Vincent Leveque[1], Zhinan Jin[1], Jerome Deval[2], Andreas Jekle[2], Gabrielle Heilek[2], Han Ma[1], Klaus Klumpp[1]

[1] *Hoffman-La Roche, Nutley, USA*
[2] *Roche, Palo Alto, USA*

Dengue virus (DENV), an emerging pathogen from the Flaviviridae family with neither vaccine nor antiviral treatment available, causes a serious worldwide public health threat. The RNA-dependent RNA polymerase NS5 of DENV is structurally related to NS5B of Hepatitis C Virus (HCV). We tested a number of 2′ and 4′-modified nucleoside analogs as inhibitors of either HCV or DENV, using hepatic and human primary dendritic cells as representative target cells, respectively. We identified a number of active nucleosides from these series, which inhibited all 4 serotypes of DENV in human transformed hepatocytes, as well as human primary dendritic cells. The biochemical profile of potent inhibitors of DENV replication was consistent with base-specific competitive inhibition of natural nucleoside substrate incorporation, and incorporation of 2′- or 4′-modified nucleosides into the nascent RNA was associated with immediate inhibition of RNA chain extension. Phosphoramidates could successfully circumvent a phosphorylation block of certain inactive nucleosides from the series in primary human dendritic cells. Among 4′-substituted nucleosides, Balapiravir (4′-azido-cytidine) was identified as a novel inhibitor of DENV replication. Based on these results, a randomized, double-blind, multiple-dose, placebo-controlled study was initiated to evaluate the safety, tolerability and efficacy of 5-day treatment with balapiravir in adult male patients with confirmed DENV infection and whose symptoms began within 48 h preceding the first administration of balapiravir. Further improvement in antiviral potency could be achieved with additional nucleoside modifications.

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2009 Pandemic Influenza Virus: What Special for its HA and NA?

George F. Gao[1,2,*], Christopher J. Vavricka[1]

[1] *CAS Key Laboratory of Pathogenic Microbiology and Immunology, Chaoyang, China*
[2] *Beijing Institutes of Life Science Chinese Academy of Sciences, Chaoyang, China*

The 2009 pandemic influenza seemingly spreads extremely quickly with worrisome mortalities and resembles some characteristics of the previous three pandemics (1918 Spanish-flu, 1957 Asian-flu and 1968 Hong Kong-flu). The virus was recognized as a new swine-origin H1N1 influenza A virus (S-OIV). Functional and structural characterization of both the haemagglutinin (HA) (09H1) and the neuraminidase (NA) (09N1) might give us some clues about its pathogenesis and directs the drug application. In our group both the 09H1 and the 09N1 were prepared in a baculovirus-based system and the 09N1 enzymatic activity was verified *in vitro*. The 09N1 crystal structure has been solved (1.9 Å) and the structure surprisingly shows a Group 2 active cavity, different from other known N1 structures which are all categorized into Group 1. The 09N1 structures in complex with substrate sialic acid, Oseltamivir (Tamiflu) or Zanamivir (Relanza) have also been solved at 1.8 Å, 1.7 Å and 1.9 Å respectively, showing typical binding modes and revealing the structural basis of the effectiveness of the NA-targeted drugs against the 2009 pandemic. More importantly, the newly defined Group-1 150-loop cavity proposed as a drug target should be reconsidered as it is not as common as we thought. This is the first solved NA structure derived from swine and the first complex structure with sialic acid for Group 1 members.

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Identification of New Druggable Antiviral Targets by Chemical Genetics

Richard Y. Kao*, Kwok-Yung Yuen

Research Center of Infection and Immunology, State Key Laboratory of Emerging Infectious Diseases, Department of Microbiology, LKS Faculty of Medicine, The University of Hong Kong, Hong Kong, Hong Kong

Viral infection is a continuing threat to mankind and drug resistance to available antivirals has appeared in an alarming rate. There is an urgent need to identify new druggable targets and find new antiviral drugs in light of rapid emerging and re-emerging viral pathogens. Our first success in applying forward chemical genetics in the identification of biologically active small molecule inhibitors of SARS-CoV in 2004 (Kao et al., Chemistry & Biology, 11:1293) has prompted us to tackle influenza A virus pathogenesis using this novel approach. Applying the concept of chemical genetics, after screening a validated high quality chemical library (50,240 structurally diverse small molecules) with an automated robotic platform for high-throughput screening (HTS), we have identified more than 1000 small-molecule inhibitors that will inhibit the infectivity of the viruses in Madin-Darby canine kidney (MDCK) cell-based HTS assay. Subsequent secondary screening and hit validation processes identified 39 potent antiviral compounds interfering with influenza A infection. We further identified compounds that perturbed intracellular trafficking of the viral nucleoprotein (NP) and characterized a compound (nucleozin) that apparently stopped the nuclear localization of the NP (Kao et al., Nature Biotechnology 28:600). The binding site of compound nucleozin was mapped to residue Y289 of NP. Balb-c mice treated by nucleozin were significantly protected after infection by a hypervirulent strain of influenza A H5N1/Vietnam/1194/04, illustrating the *in vivo* efficacy of nucleozin in inhibiting H5N1 infection. Further investigation using immunofluorescence techniques and gel-shift assays illustrated that nucleozin induced a time-dependent and RNA enhanced specific aggregation of NP. Our results demonstrated that chemical genetics is an attractive approach for the identification of druggable targets, new antiviral drugs, and novel antiviral mechanisms.

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Activities of Viral M2 Channel, Neuraminidase, and RNA Polymerase Inhibitors on Oseltamivir-Resistant H275Y Influenza A (H1N1) Virus Infections in Mice

Donald F. Smee[1,*], Min-Hui Wong[1], E. Bart Tarbet[1], Justin Julander[1], Matthew Gross[2], Jack Nguyen[2]

[1] *Utah State University, Logan, USA*
[2] *Adamas Pharmaceuticals, Emeryville, USA*

A novel influenza infection model was developed by serially passaging influenza A/Mississippi/3/2001 (H1N1) H275Y virus seven times in mice to increase its virulence. The viral neuraminidase was sensitive to inhibition by zanamivir, but had reduced susceptibility to peramivir, and was resistant to oseltamivir carboxylate, with IC_{50} values of 1, 39, and 100 nM, respectively. Several compounds were evaluated against lethal A/Mississippi H275Y virus infections in mice. Treatments with amantadine or rimantadine (viral M2 channel blockers), oseltamivir or zanamivir (viral neuraminidase inhibitors), or ribavirin (viral RNA polymerase inhibitor) were initiated 2 h before or 24 h after intranasal virus challenge, continuing twice daily for 5 days. Oral oseltamivir treatment at 1–30 mg/(kg day) was ineffective, whereas treatment with 100 and 300 mg/(kg day) gave 30 and 60% protection from death, respectively, starting at −2 h. All doses of oseltamivir were inactive starting at +24 h. Intraperitoneal treatments with zanamivir at 100 and 300 mg/(kg day) gave 60 and 90% protection, respectively, starting at −2 h. Zanamivir failed to protect mice when treatments were initiated at +24 h. The results with zanamivir were unexpected, based upon its potency in cell culture. Oral treatments with the other inhibitors were initiated at −2 h. Amantadine was effective at 10, 30, and 100 mg/(kg day), rimantadine was protective at 10 and 30 mg/(kg day) (100 mg/(kg day) was not tested), and ribavirin was active at 30 and 75 mg/(kg day), with survival ranging from 60 to 100%. Treatment with these agents was also effective when begun at +24 h. Here, amantadine activity was present at 30 and 100 mg/(kg day), rimantadine showed efficacy at 10 and 30 mg/(kg day), and ribavirin was protective at 75 mg/(kg day), with 60–100% survival for each group. These results are important in establishing this model for evaluation of drug combinations against this oseltamivir-resistant virus.

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CMX001 (Hexadecyloxypropyl Cidofovir) Antiviral Activity against Adenovirus in Patients Correlates with Drug Levels and Viral Sensitivity

E.R. Lanier*, W. Painter, T.K. Tippin, M. Anderson, B.M. Lampert, H. Mommeja-Marin, L.C. Trost

Chimerix Inc, Durham, USA

Background: Adenoviruses (AdV) are double-stranded DNA viruses; there are at least 52 distinct types. CMX001 is a lipid derivative of (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine (HPMPC, CDV) with potent antiviral activity against AdV in vitro. CMX001 has been used to treat patients with severe AdV infections under Emergency Investigational New Drug Applications. Notably, most patients had previous exposure to cidofovir (CDV). Here we investigate the effects of drug levels and AdV drug sensitivity on the activity of CMX001.

Methods: We analyzed 22 patients with AdV viremia who had received at least 2 doses of CMX001, had measurable plasma viremia at baseline, and had viral load data available at ≥2 weeks after the first dose of CMX001. Phenotypic resistance to CDV and CMX001 was determined using a cytoprotection assay in HeLa cells. Noncompartmental pharmacokinetic (PK) analysis was conducted using plasma concentration data obtained after the first dose of CMX001.

Results: There was a median $-2.5\ \log_{10}$ change in viremia from baseline by the last timepoint (range -6.0 to $+0.3\ \log_{10}$). Patients who had either sensitive virus or no proven resistance had a median decrease of almost 1000-fold ($-2.9\ \log_{10}$) from baseline while those with CDV resistance (all >10 fold relative to wild-type [$n = 6$]), attributed to prior suboptimal CDV therapy, had a median $-0.55\ \log_{10}$ decrease from baseline. Patients with both virologic and PK data (13 of 22) were evaluated by comparing those with ($n = 10$) or without ($n = 3$) $a > 1\ \log_{10}$ reduction in viremia at week 2. The median exposure (AUC0-inf) was 1.7-fold higher (3485 vs 2047 h ng/mL) and Cmax was more than 2-fold higher (387 ng/mL vs 153 ng/mL) in patients with $a > 1\ \log_{10}$ reduction in viremia at week 2.

Conclusions: These data demonstrate that the majority of CMX001 treated patients had $a > 99\%$ decrease in viral load compared to baseline after 2 weeks of therapy. Antiviral activity of CMX001 was reduced in patients with high level resistance to CDV, all of whom had received prior treatment with CDV. Finally, higher plasma exposure to CMX001 appeared to correlate with a more rapid response. CMX001 is a promising antiviral for severe adenovirus infection.

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Poster Session 1: Retroviruses, Respiratory Viruses, Emerging Viruses and Antiviral Methods Chairs 4:00–6:00 pm Sofia 3 and Kyota

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Design, Synthesis and West Nile Protease Inhibitory Activity of Novel Isatin Derivatives

Periyasamy Selvam[1,*], Priya Srinivasan[2], Tanvi Khot[2], R. Padmanaban[2]

[1] *Devaki Amma Memorial College of Pharmacy, Malapuram 673634, India*
[2] *Microbiology and Immunology, Georgetown School of Medicine, Washington, USA*

Background: The mosquito-borne viral pathogens of global significance include the members of flavivirus genus of Flaviviridae family. Two important human pathogens are dengue and West Nile viruses which cause considerable morbidity and mortality throughout tropical and subtropical regions of the world. No vaccines or antiviral therapeutics are available for these two pathogens. The overall goal of our study is to develop potent inhibitors of West Nile virus serine protease, which is a quintessential viral target as it is required for viral replication. In this study, we examined whether derivatives of isatin (2,3-dioxoindole) could be versatile lead compounds for structure–activity relationship (SAR) study.

Methods: Novel isatin-sulphadimidine derivative synthesized and screened for their inhibitory activities of WNV protease in vitro.

Results: The N-benzoyl derivative (SPIII-5H-BZ) and 5-chloro-N-acetyl derivative (SPIII-5Cl-AC) exhibited significant inhibitory activities against the WNV protease (IC_{50} values of 15 μM and 8.4 μM, respectively).

Conclusions: To our knowledge, this is the first report regarding the inhibitory activities of isatin derivatives against the WNV serine protease. Further work on SAR study for lead optimization is in progress.

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Effects of the Addition of Hiltonol® (Poly-ICLC) to a SARS-CoV S Protein Vaccine in Lethal SARS-CoV Mouse Model

Dale L. Barnard[1,*], Miles Wandersee[1], Kevin Bailey[1], Aaron Smith[1], Zach Vest[1], John D. Morrey[1], Andres M. Salazar[2]

[1] *Utah State University, Logan, USA*
[2] *Oncovir, Inc., Washington, USA*

The principal containment strategy for emerging diseases has been rapid diagnosis/isolation followed by immunization, but there is often a gap between isolation/identification and immunization. Host-targeted therapeutics that could provide immediate, broad-spectrum resistance to disease could fill this gap in protection to allow time for agent specific immunization to take effect. Hiltonol® is a stabilized dsRNA therapeutic viral mimic or pathogen-associated molecular pattern (PAMP) that activates 2′5′ OAS, PKR, RIG-I, mda-5, DCs, natural killer cells interferons and various cytokines and chemokines, while at the same time accelerating and enhancing the quality of adaptive cellular/humoral immunity. It induces a broad-spectrum innate-immune antiviral state lasting up to 3 weeks. The current study was done to see if Hiltonol® could be used to protect during the time between the isolation/identification of an agent and adaptive immunization. Mice immunized i.m. twice with S Protein vaccine in alum, 14 days apart, were protected against death from viral challenge 14 days after the last immunization. A one time Immunization with varying doses of vaccine (0.3, 1, 5 μg/mouse) along with simultaneous Hiltonol® (10 μg/mouse) intranasally also significantly protected mice against death ($P < 0.001$). Mice treated with 5 μg of vaccine and only 1 μg of Hiltonol®, showed diminished survival to only 60%. These data suggest that the co-administration of Hiltonol® at higher doses of the S protein vaccine enhanced the protection of mice against death. When mice were challenged with SARS-CoV 3 days after one immunization with vaccine (0.3, 1, 5 μg/mouse) plus Hiltonol® at 10 μg, both given i.n., then all mice survived the infectious challenge. Mice receiving one course of vaccine plus alum and challenged on day 3 all died; mice receiving Hiltonol® at 10 μg/mouse and challenged on day 3 all survived. Thus, it appears that Hiltonol® provided an immediate protection against disease that allowed time for the more specific vaccination strategy to take effect.

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Synthesis and Anti-HIV Activity of D-peptide Analogs as HIV Fusion Inhibitors

Alice Baron[1,2,*], Christine Kreuz[3], Gilles Gosselin[1], Frederic Lamaty[2], Jean Martinez[2], Dominique Surleraux[1], Claire Pierra[1], Pascal Clayette[3]

[1] *Laboratoires Idenix, Montpellier, France*
[2] *Institut des Biomolécules Max Mousseron, Montpellier, France*
[3] *Bertin Pharma, Fontenay aux Roses, France*

The identification of new anti-HIV molecules remains an important challenge. To date, HIV viral entry becomes a promising target for HIV drug development as illustrated for example by the approval of Enfurvitide by regulatory agencies as HIV fusion inhibitor, and more recently, Maraviroc as HIV entry inhibitor. Although highly effective, Enfurvirtide, a C-peptide of 36 amino acids mimicking the CHR region of gp41 has several serious

limitations including protease degradation, high dosing requirements, subcutaneous administration, and emergence of resistant HIV strains. Based on these data, we present herein the synthesis and the anti-HIV evaluation of new D-peptide analogs, expected to be resistant to proteolytic degradation and endowed with improved bioavailability, as specific inhibitors of the hydrophobe pocket of the gp41N trimer. Using a similar cyclic D-peptide approach, a consensual 8-mer sequence with 4 recurrent amino acids (D-Glu9, D-Trp10, D-Trp12, and D-Leu13) has been previously defined by other research groups. First, our efforts were devoted to understand the key elements responsible for their pharmacological activity using molecular modelisation/dynamic approaches. This initial study showed that D-Glu9, D-Trp10, D-Trp12, and D-Leu13 interact with residues in or on the edge (D-Glu9) of the hydrophobe pocket and, that C- and N-terminal terminations govern structural changes in cyclic peptides and probably their antiretroviral effects. Then, considering these data, more than 40 cyclic analogs were synthesized and evaluated against HIV-1-LAI by measuring virus-induced cytopathogen effects (CPE) and the production of the major HIV nucleocapsid p24 protein in acutely infected MT-4 cells. Retro and/or inverso peptides as well as 7-mer D-peptides showed no antiviral effects. Only the substitution of D-Trp12 and D-Leu13 residues by l-napthyl and/or cyclobutyl group(s) have improved antiretroviral efficacy.

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Activity of Novel Cyclophilin Inhibitors based on the Polyketide, Sanglifehrin A, against HIV

Michael Bobardt[1,*], Steven Moss[2], Udayan Chatterji[1], Mohammad Nur-E-Alam[2], Tony Warneck[2], Barrie Wilkinson[2], Philippe Gallay[1], Matthew Gregory[2]

[1] *The Scripps Research Institute, La Jolla, USA*
[2] *Biotica Technology Ltd, Cambridge, United Kingdom*

Cyclophilin inhibitors, such as Cyclosporine A (CsA) and analogues, such as DEBIO-025 (Alisporivir) have previously shown activity in *in vitro* antiviral assays against HIV isolates (Ptak et al., 2008), however, when tested clinically, DEBIO-025 only reduced HIV-1 RNA levels by $\geq$0.5 and >1 $\log^{10}$ copies/mL in nine and two patients respectively, whilst 27 of the treated patients showed no reduction in HIV-1 RNA levels (Steyn et al., 2006). DEBIO-025 was trialled in HCV/HIV coinfected patients, showed better efficacy against HCV (Flisiak et al., 2008), and was followed by a substantial focus in HCV treatment. Sanglifehrin A (SfA), a polyketide natural product, has been shown to bind Cyclophilins (CyPs), such as CyPA, and to have antiviral activity in HCV replicon assays. Novel analogues with improved drug like properties were generated through proprietary biosynthetic engineering technology and semi-synthetic methods. These analogues were tested *in vitro* against HIV, and were seen to have significantly improved potency, both in terms of EC_{50} and maximal antiviral effect. Novel SfA analogues offer additional advantages over other virus-targeting agents under development for HIV and detailed profiling of selected candidates is underway.

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OBR-5-340—A Novel Pyrazolo-Pyrimidine Derivative with Strong Antiviral Activity Against Coxsackievirus B3 *In Vitro* and *In Vivo*

Heike Braun[1,*], Vadim A. Makarov[2], Olga B. Riabova[2], Elena S. Komarova[2], Martina Richter[1], Peter Wutzler[1], Michaela Schmidtke[1]

[1] *Institute of Virology and Antiviral Therapy, Jena University Hospital, Jena, Germany*
[2] *Institute of Biochemistry, Russian Academy of Science, Moscow, Russia*

There is no specific medication to address diseases induced by CVB3, e.g. harmless respiratory infections, diarrhea, myocarditis, meningoenceophalitis, pneumonia, and pancreatitis until now.

In the present study, the toxicity and anti-CVB3 activity of OBR-5-340, a novel pyrazolo-pyrimidine derivative was determined *in vitro* and *in vivo*. OBR-5-340 is well tolerated (CC_{50} > 500 μM) and exhibits strong dose-dependent antiviral activity against the pleconaril-resistant CVB3 31-1-93 in HeLa cells. The IC_{50} determined for CVB3 31-1-93 was 0.03 μM. In contrast, pleconaril included as control was inactive. For the *in vivo* studies, adult male NMRI mice were infected intraperitoneally with 5^{-4} pfu of CVB3 31-1-93. OBR-340 and the control compound pleconaril were administered orally twice daily (100 mg/kg). In pleconaril- and placebo-treated CVB3 31-1-93-infected mice disease onset was observed at day 3 or 4 p.i. Between day 5 and 10 p.i. severity of disease, lost of body weight were maximal (−15% related to day 0 p.i.). OBR-5-340 treatment almost completely prevented CVB3 31-1-93-induced disease. No loss of body weight was observed and the histopathological scores in heart and pancreas tissue as well as the viral load in heart tissue were significantly reduced compared to pleconaril- or placebo-treated mice. *In conclusion*, OBR-5-340 exhibits a strong antiviral effect against a pleconaril-resistant CVB3 variant *in vitro* and *in vivo*. It represents a promising new drug candidate for the treatment of CVB3 infections.

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Occurrence of Opportunistic Infections in People Living with HIV/AIDS Following Antiretroviral Therapy in West Bengal, India

Sayan Chakraborty*, Mehebubar Rahman, Bibhuti Saha

Calcutta School of Tropical Medicine, Kolkata, India

As per the data of 2008, approximately 33.4 million people are living with HIV/AIDS worldwide and there were around 2.0 million AIDS related deaths in that year. Occurrence of opportunistic infections (OIs) is the main cause of morbidity and mortality in HIV infected patients. OIs encompass a wide variety of microorganisms that produce fulminant infections in immunocompromised HIV infected patients. HIV makes the infected person immunocompromised by destroying his CD4 T-lymphocytes. CD4 T-lymphocyte count (or CD4 count) is a standard laboratory marker to monitor disease progression in HIV infected patients. When the CD4 count of a HIV infected person decreases he or she becomes susceptible to OIs due to the compromised immune system. Antiretroviral Therapy (ART) decreases multiplication HIV and thereby decreases CD4 cell destruction. So there is increase in CD4 count and improvement in immunity which leads to less occurrence of OIs in HIV patients. We followed 88 patients for 3 years, who were getting first line ART at ART center, Calcutta School of Tropical Medicine. For analysis we divided 88 patients into 5 groups based on their initial CD4 count i.e.

0–50, 51–100, 101–150, 151–200 and 201 and above. In each group we monitored clinical improvement, occurrence of OIs, increment of CD4 count. We found that respiratory tract infection (RTI) was the most frequently occurring infection in our study groups. Next common OIs were tuberculosis and oral candidiasis (OC) followed by diarrheal diseases and herpes virus infections. After following up for 3 years we found a significant increase in CD4 counts in all groups. Incidence of total number of OIs decreased from 91% during the 1st year of ART to 36% during the 3rd year in our study population. Incidence of RTI decreased in our 5 study groups from 75%, 66%, 42%, 56.25% and 65.2% during the 1st year to 0%, 50%, 10.5%, 12.5%, and 17% respectively during the 3rd year. Similarly incidence of tuberculosis (pulmonary and extra-pulmonary combined) decreased from 41%, 50%, 32%, 6.25% and 26% to 8%, 5.6%, 0%, 0%, and 0% respectively. This study on OIs in people living with HIV/AIDS in West Bengal will help any further study in the said field.

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Crimean Congo Haemorrhagic Fever Virus: An Emerging Concern for Iran's Public Health

Sadegh Chinikar*, Ramin Mirahmadi, Maryam Moradi, Mojtaba Ghiasi, Akram Sadeh, Sahar Khakifirouz, Fereshteh Sadat Varai, Maisam Asl Solaimani

Pasteur Institute of Iran, Tehran, Iran

Introduction: Crimean Congo Haemorrhagic Fever (CCHF) is a tick borne viral haemorrhagic zoonosis with a mortality rate up to 50%, caused by CCHF virus (CCHFV), genus Nairovirus, family Bunyaviridae. In the transmission cycle of the disease, ticks play both vector and reservoir roles. CCHF is transmitted by tick bite, handling of infected livestock organs or blood and nosocomially.

Methods: Since the emergence of CCHF in Iran, in 1999, it is considered a major health problem and after the foundation of the laboratory of Arboviruses and Viral Haemorrhagic Fevers in the Pasteur Institute of Iran as a National Reference Laboratory, sera were collected from Iranian probable patients from June 2000 till now and tested by Elisa for anti CCHF antibodies (IgM and IgG) and by RT-PCR (Real time and gel based) for a fragment of the virus genome.

Results: Our data show that the disease has infected 23 out of 30 provinces of Iran and has been continuously seen in some provinces such as Sistan va Baluchestan through these last 11 years, while it was sporadically seen in the other provinces. During all these years, Sistan va Baluchestan has always been the first contaminated province in Iran and in the year 2010, it was followed by Khorasan and Yazd as the second and third most contaminated provinces respectively.

Conclusion: Our results demonstrate that CCHF is the most important viral haemorrhagic fever in Iran and a major public health problem. As the most infected province, Sistan-Baluchestan has faced the disease annually, because its neighbors with a large border in the east are Pakistan and Afghanistan where the disease is endemic. Moreover, the phylogenetic studies have confirmed the origin of the Iranian CCHF strain very similar to that of Pakistan (Matin strain).

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Antiviral Drug-Resistant Influenza Viruses in Gyeonggi Province, South Korea, from 2005 to 2010

Hangil Cho*, Haekun Hong, Sukyoung Mun, Woonho Kim, Hyunkyung Lee, Mihye Yoon, Jongbok Lee

Gyeonggi-do Institute of Health and Environment, Suwon, South Korea

Amantadine and oseltamivir are pharmaceutical options used to control influenza infections. To investigate the prevalence of antiviral resistance among influenza viruses in the Gyeonggi province of South Korea, genetic and phenotypic assays were conducted for 491 influenza viruses (86 A/H1N1, 152 A/H3N2, 121 pandemic A/H1N1 2009(pdm) and 132 B), isolated between 2005 and 2006 season and 2009–2010 season. To identify potentially resistant viruses to the amantadine and oseltamivir, the Matrix(M)2 and Neuraminidase(NA) gene were amplified by RT-PCR and followed by sequence analysis. The frequency of resistance to amantadine among A type influenza viruses was 30% ($n = 70$) A/H1N1, 76% ($n = 124$) A/H3N2 and 100% ($n = 114$) A/H1N1 pdm, respectively. The A/H1N1 isolates from 2007 to 2008 season, A/H3N2 from 2005 to 2006, 2007 to 2008, 2008 to 2009 season and all A/H1N1 pdm had resistance to amantadine, but A/H3N2 isolates in 2006–2007 season revealed 53% ($n = 62$) resistance. The S31N substitution in M2 protein mainly contributed to amantadine-resistance and only an A/H3N2 isolate had L26F/S31N substitution, confirmed by virus reduction assay. The resistant pattern to oseltamivir was also analysed. 56% ($n = 71$) A/H1N1 were resistant, but 117 A/H3N2, 80 B and 74 A/H1N1 pdm were susceptible to oseltamivir. Especially, A/H1N1 isolates in 2008–2009 season harbored H274Y mutation in NA protein and were found to be resistant using the fluorometric NA inhibition assay. The widespread resistance to amantadine and recent increase in oseltamivir-resistance among influenza viruses raises public health concerns. The close monitoring system for the antiviral resistance should be intensified and maintained to provide guideline for prophylaxis and treatment of influenza.

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Rigid Amphipathic Fusion Inhibitors (RAFIs) Inhibit Infectivity of Enveloped Viruses by Targeting Envelope Lipids to Prevent Fusion With Cellular Membranes

Che C. Colpitts[1,*], Alexey V. Ustinov[2], Vladimir A. Korshun[2], Luis M. Schang[1]

[1] *University of Alberta, Edmonton, Canada*
[2] *Russian Academy of Sciences, Moscow, Russia*

We have described a family of novel antiviral compounds, the rigid amphipathic fusion inhibitors (RAFIs). RAFIs are active against otherwise unrelated enveloped viruses, including HSV-1, HSV-2, HCV and VSV. They inhibit viral entry with no obvious effects on physiological cellular fusions. Our lead RAFIs, dUY11 and aUY11, are not cytotoxic. Amphipathicity, molecular shape (with hydrophilic heads larger than their hydrophobic tails), rigidity and planarity are all essential for their antiviral activity. Here, we show that RAFIs target the lipids in virion envelopes to inhibit fusion. We examined the spectra of the intrinsically fluorescent dUY11 in environments of different polarities. The spectra were most similar when dUY11 was mixed with VSV virions or liposomes. Both spectra were distinct from that in aqueous buffer but very similar to that in octanol. Confocal microscopy revealed that dUY11 also localized to cell membranes. We next tested fusion using fluorescence dequenching assays. VSV virions labeled at self-quenching concentrations

with the membrane dye octadecyl rhodamine chloride (R18) were exposed to dUY11, and then mixed with Vero cells on ice. Fusion, induced by raising the temperature to 37 °C and lowering the pH to 5.5, was monitored by fluorescence dequenching. Fluorescence was dequenched by 15% when virions were exposed to vehicle, but by only 2.5% when they were exposed to dUY11. Furthermore, inhibition of lipid mixing and infectivity were most consistent for several RAFIs (Table 1). In conclusion, RAFIs inhibit the infectivity of enveloped viruses by targeting virion envelope lipids to prevent fusion of viral and cellular membranes.

	dUY11 (μM)	aUY11 (μM)	aUY12 (μM)	dUY5 (μM)
Fusion IC_{50}	0.0111	0.0149	5.1	3.2
Plaquing IC_{50}	0.0087	0.0096	3.6	3.2

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Combine Action Rimantadine and Amizon on Flu Occasion

G. Danilenko[1,*], S. Rybalko[2], T. Bukhtiarova[3], V. Danilenko[3], S. Guzhova[4], O. Esipenko[1]

[1] *Institute of Organic Chemistry, Kyiv, Ukraine*
[2] *Institute of Epidemiology and Infectional Diseases, Kyiv, Ukraine*
[3] *Institute of Pharmacology and Toxicology, Kyiv, Ukraine*
[4] *Institute of Bioorganic Chemistry and Petrochemistry, Kyiv, Ukraine*

Last year (ICAR 2010) we have shown that addition antiflogistic to viral inhibitor in vitro potentiated its activity. As next step we treated in vivo flu virus A/H1N1 with simultaneous use rimantadine and amizon (mol: mol). In this case LD_{50} 1500 mg/kg.

Flu virus in dose 10 LD_{50} was applicated mice in a nose. In 1 h rimantadine and amizon in dose 4 mg/ml in 0.2 ml physiology solution was injected intraperitoneally.

Results are below.

Remedy	Mice survival, %	
	After 5 days	After 14 days
Rimantadine	60	60
Amizon 60	40	
Rim. + Am.	100	80
Tamiflu	80	70
Control	0	0

Combine action rimantadine and amizon is more potent on flu occasion in mice.

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An Analogue of the Antibiotic Teicoplanin Inhibits Dengue Virus Entry *In Vitro*

Tine De Burghgraeve[1,*], Suzanne J.F. Kaptein[1], Jan Paeshuyse[1], Vanesa Ayala-Nunez[2], Maria Preobrazhenskaya[3], Andrea Gamarnik[4], Jolanda Smit[2], Johan Neyts[1]

[1] *Rega Institute, KULeuven, Leuven, Belgium*
[2] *University of Groningen, Groningen, Netherlands*
[3] *Gause Institute of New Antibiotics, Moscow, Russia*
[4] *Fundacion Instituto Leloir, Buenos Aires, Argentina*

The dengue virus is a mosquito-borne virus that belongs to the family of the *Flaviviridae*; it is endemic in (sub) tropical regions. Each year over 50–100 million people become infected with the virus of which about 250,000–500,000 may develop severe and potentially life-threatening conditions, i.e. dengue hemorrhagic fever and dengue shock syndrome. There is neither vaccine, nor therapy available. Here, we report on an analogue of the antibiotic teicoplanin LCTA-949 [devoid of antibacterial activity] that inhibits virus induced CPE in a dose dependent manner (EC_{50} of ~5 μM). This finding is corroborated by the quantification of viral RNA levels in culture supernatant by RT-qPCR (EC_{50} = 4.8 μM). A selectivity index (50% effective concentration/50% cytostatic concentration) of approximately 10 was calculated. Antiviral activity is also observed against other flaviviruses, i.e. the yellow fever virus 17D and the Modoc virus, as well as against the hepacivirus HCV. Time of addition experiments indicate that LCTA-949 inhibits the early stages in the viral lifecycle. This is corroborated by the fact that LCTA-949 lacks activity on DENV subgenomic replicon (that does not contain the structural genes) replication. In addition, in single-virus tracking assays LCTA-949 was shown to inhibit the fusion process. Studies are currently ongoing to unravel the precise mechanism by which LCTA-949 inhibits DENV replication. Insight in the mechanism of action may also shed new light on the early stages of the DENV replication cycle.

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Identification of HIV-1 Reverse Transcriptase Dual Inhibitors by a Combined Shape-, 2D-Fingerprint- and Pharmacophore-based Virtual Screening Approach

Simona Distinto[1,*], Francesca Esposito[2], Johannes Kirchmair[3], Cristina M. Cardia[4], Elias Maccioni[4], Stefano Alcaro[1], Luca Zinzula[2], Enzo Tramontano[2]

[1] *Dip. Scienze Farmaco Biologiche, University of Catanzaro, Catanzaro, Italy*
[2] *Dipartimento di Scienze Applicate ai Biosistemi, University of Cagliari, Cagliari, Italy*
[3] *Department of Pharmaceutical Chemistry, University of Innsbruck, Innsbruck, Austria*
[4] *Dipartimento Farmaco Chimico Tecnologico, University of Cagliari, Cagliari, Italy*

The human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is still one of the most attractive targets in the design of new antiviral agents. It is a key enzyme for viral replication which has two associated catalytic functions: a DNA polymerase activity and a ribonuclease H (RNase H) activity. Even though there are several known inhibitors of the RT-associated DNA polymerase function, only few inhibitors of its RNase H function have been identified so far. Here, we report the first application of virtual screening (VS) methods for discovering new inhibitors of this novel and challenging target. The overall VS campaign consisted of two consecutive screening processes, each of it resulting in a hit list of compounds which were tested experimentally. Firstly, the virtual screening platform ROCS (Rapid Overlay of Chemical Structures) was utilized to perform *in silico* shape-based similarity screening in which an hydrazone derivative, previously shown to inhibit the HIV-1 RNase H, was chosen as a query. Consequently, the most active molecules identified in the first VS were selected as queries for a parallel VS which combined three different LB methods: shape-based, 2D-fingerprint, 3D-pharmacophore VS. The effect of the VS selected molecules on the HIV-1 RT-associated activities was evaluated in biochemical assays. Overall, a set of molecules characterized by different scaffolds were identified as new inhibitors of both RT-associated activities in the micromolar range.

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Antiviral Activity of the MEK-inhibitor U0126 against Pandemic H1N1v and Highly Pathogenic Avian Influenza Virus *In Vitro* and *In Vivo*

Karoline Droebner[1,*], Stephan Ludwig[2], Stephan Pleschka[3], Oliver Planz[1]

[1] *Friedrich-Loeffler-Institut, Institute of Immunology, Tübingen, Germany*
[2] *Justus-liebig Universität, Giessen, Germany*
[3] *Institute of Molecular Virology, Münster, Germany*

Zoonotic, seasonal epidemic and pandemic Influenza is still a formidable foe throughout the world. The emergence of the 2009 H1N1 pandemic swine influenza A virus is a good example on how this viral infection can impact health systems around the world. The continuous zoonotic circulation and reassortment of influenza viruses in nature represents an enormous public health threat to humans. Besides vaccination antivirals are required to efficiently control the disease. In the present work we investigated that the MEK inhibitor U0126, targeting the intracellular Raf/MEK/ERK signalling pathway, reduces the 2009 pandemic influenza strain H1N1v and highly pathogenic avian influenza viruses in cell culture and in the mouse lung. U0126 showed antiviral activity in cell culture against all tested influenza virus strains including oseltamivir resistant strains. We were able to demonstrate that treatment of mice with U0126 via the aerosol route led to (i) inhibition of MEK activation in the lung, (ii) reduction of influenza virus titer compared to untreated controls, (iii) protection of influenza virus infected and U0126 treated mice against a 10× lethal challenge. Moreover, no adverse effects of U0126 were found in cell culture or in the mouse. Thus the principal conclusions of our findings are that U0126 inhibiting the cellular target MEK has an antiviral potential in cell culture and in the mouse model. Since MEK inhibitors are tested in various clinical trials against cancer, indicating that MEK inhibitors might be safe and well tolerated.

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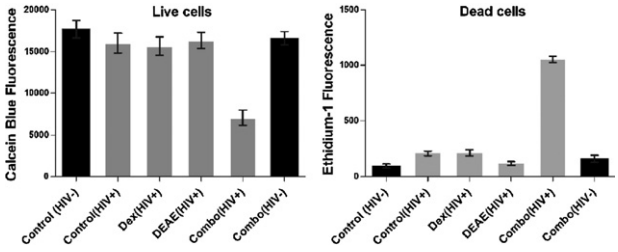
Targeted Elimination of HIV Infected Cells: Synergistic Combination of Dexamethasone and DEAE as a Paradigm

Najoua Elbourkadi*, Lijun Zhao, Ethan Will Taylor

Laboratory for Molecular Medicine, University of North Carolina, Greensboro, USA

Highly Active Antiretroviral Therapy (HAART) has proven to be successful in controlling HIV infection, yet it remains incapable of eradicating the virus. Systemic infection re-emerges upon treatment interruption, requiring a lifetime of costly drug therapy with an ever-present risk of emerging drug resistance. Thus, there is a critical need for the development of new treatment modalities not based solely on virally encoded targets, and with the potential to actually cure HIV infection. Our research introduces the innovative concept of a small molecule drug regimen that has the ability to selectively eliminate HIV infected cells from the body. Using standard colorimetric and fluorometric "Live/Dead" cell labeling techniques, we have developed an easily automated "selective cell death" (SCD) assay, and identified a lead drug combination for the elimination of infected cells, involving a patented combination of two generic FDA approved drugs or their metabolites: the glucocorticoid dexamethasone (Dex) and *N*,*N*-diethylaminoethanol (DEAE), a metabolite of procaine.

As shown in the figure, neither drug alone, but only the combination (Combo), is able to induce cell death, and only in HIV-infected cell cultures ($P < 0.0001$). Prolonged exposure of cells to this drug combination leads to a decline in viral load: two weeks of treatment resulted in a decrease of more than 50% in viral titer relative to untreated control cells. These results provide proof of concept for the utility of our SCD assay, and the ability to identify small molecule drug combinations that can selectively kill HIV-infected cells. Such therapies would complement and enhance the effectiveness of standard antiretroviral drug regimens that inhibit HIV replication.



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Design and Synthesis of New Isatin Derivatives as HIV-1 Reverse Transcriptase Associated Ribonuclease H Inhibitors

Francesca Esposito[1,*], Rita Meleddu[2], Maria Luisa Sanna[2], Simona Distinto[3], Angela Corona[1], Valeria Cannas[1], Enzo Tramontano[1], Maria Cristina Cardia[2]

[1] *Dipartimento di Scienze Applicate ai Biosistemi, University of Cagliari, Cagliari, Italy*
[2] *Dipartimento Farmaco Chimico Tecnologico, University of Cagliari, Cagliari, Italy*
[3] *Dip. Scienze Farmaco Biologiche, University of Catanzaro, Catanzaro, Italy*

The human immunodeficiency virus (HIV) is the etiological agent of the acquired immunodeficiency syndrome (AIDS) in humans. Despite the fact that many therapeutic agents targeted to the viral reverse transcriptase (RT), the multifunctional enzyme which is responsible for the viral genome replication, are already clinically available, none of them is active on the RT-associated Ribonuclease H (RNase H) activity, whose function is essential for viral replication and, hence, is an attractive target for drug development. In the last few years, a few classes of compounds have been identified as HIV-1 RNase H inhibitors, however, none of them was able to reach clinical trial testing. Thus, efforts in the development of new compounds targeting the RNase H activity are relevant to enhance the antiretroviral armamentarium and constitute an attractive challenge for medicinal chemists. In this perspective, within an RNase H drug discovery program, we designed and synthesized a series of differently substituted isatin derivatives and tested them on both HIV-1 RT-associated RNase H and DNA polymerase functions. Within these compounds, isatin derivatives appeared as promising scaffold for the inhibition of the HIV-1 RT-associated RNase H activity. The resulting SAR study may provide significant hints for the determination of the pharmacophoric requirements for the interaction with this viral target.

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Antiviral Activity of the Proteasome Inhibitor VL-01 against Human and Avian Influenza A Viruses

Withdrawn

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In silico Screening of Compounds Targeting Human Cyclin T1 and *In vitro* Evaluation of their Anti-HIV-1 Activity

Takayuki Hamasaki*, Mika Okamoto, Masanori Baba

Division of Antiviral Chemotherapy, Center for Chronic Viral Diseases, Kagoshima University, Sakuragaoka, Japan

The gene expression and transcription step from the integrated proviral DNA is essential for HIV-1 replication. However, drugs blocking this step have not been clinically approved yet. The cellular protein complex p-TEFb (cyclin T1/CDK9) interacts with HIV-1 Tat and the TAR RNA, which plays a crucial role in HIV-1 transcription. Therefore, the interaction among p-TEFb, Tat, and the TAR RNA is considered to be a potential target for inhibition of HIV-1 replication. To identify a lead compound having selective anti-HIV-1 activity, *in silico* screening of compounds targeting cyclin T1 was performed using the molecular docking simulation software MOE (Chemical Computing Group Inc., Quebec, Canada). Since the complex structure of human p-TEFb/Tat/TAR is not available to date, a model structure of human cyclin T1 was constructed by homology modeling based on the complex structure of equine cyclin T1, equine infectious anemia virus (EIAV) Tat, and EIAV TAR RNA (Protein Data Bank ID: 2w2h) and used for the docking study. A putative binding pocket, where small molecule compounds could be bound, was identified within human cyclin T1 model structure. Approximately 3 million compounds were screened according to the primary condition (molecular weight: 350–600 Da, the number of hydrogen bond donor/acceptor < 13, rotatable bonds < 7, and log *P*: 0–6). Then, the selected compounds were further examined for their *in silico* binding to the pocket within cyclin T1 by MOE. Based on the docking score obtained by the screening, 254 molecules were synthesized and examined for their anti-HIV-1 activity in cell cultures. Consequently, 2 compounds with similar chemical structures showed selective inhibition of HIV-1 replication in chronically infected cells (OM-10.1 and U1) stimulated with TNF-α. When their derivatives were synthesized and evaluated for the anti-HIV-1 activity, one compound was found to have higher activity. Studies including their molecular mechanism of action are in progress.

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Novel 2-Styryl-8-hydroxyquinolines (8SQs) Derivatives with Anti-HIV-1 Activity Targeting Viral Integrase and Protease

Anton V. Hinkov[1,*], Kamelia R. Stanoeva[1,2], Sevdalina H. Raleva[3], Vasil G. Atanasov[4], Petya D. Genova-Kalou[3], Radka M. Argirova[3]

[1] *Faculty of Biology, Sofia University "St.Kliment Ohridski", Sofia, Bulgaria*
[2] *Medical Faculty, Medical University-Sofia, Sofia, Bulgaria*
[3] *Dept. of Virology, National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria*
[4] *Faculty of Chemistry, Sofia University "St. Kliment Ohridski", Sofia, Bulgaria*

Earlier it has been reported about anti-HIV-1 integrase activity of styryl-quinolines (Mekouar K. et al., 1998). In Bulgaria six novel 2-styryl-8-hydroxyquinolines (8SQs) derivatives were synthesized and evaluated for anti-HIV-activity in cell culture. The aim of this work was to target anti-HIV-1 activity of novel substances. Supernatants of chronically infected H9/HTLVIIIB were used as a source of HIV-1. Cytotoxicity tests and microtiter infection assays for HIV cytopathic effect in MT4 cells were performed (MTT uptake assay). Reverse transcriptase (RT) activity in supernatants and directly on recombinant RT (Cavidi, Sweden) were checked. Protease as a target was studied by modified screening method using direct spectrophotometric reading of specific substrate utilization by native HIV-1 protease in the absence and presence of the substances studied. To check the integrase as a target, mutants were obtained by serial passaging of virus with continuous exposure to increasing concentrations of active compounds followed by sequencing of *in* region. Mitochondrial toxicity was evaluated by RealTime-PCR as mtDNA:nDNA ratio. Two novel 8SQs: 105B and 241, differing in substitutes at C_2 in the phenol ring, demonstrated inhibition of HIV replication (105B > 95% and 241–70%). 105B showed mitochondrial toxicity accompanied by reducing of both mtDNA and nDNA (mtDNA:nDNA = 0.82 compared to 1.01 and 0.97 in MT4 uninfected and HIV-1 infected cells without inhibitor, resp.). 241 showed no mitochondrial toxicity. Both derivatives exposed no RT inhibition but anti-protease activity (105B–21% and 241–25% resp.). As far as the latter did not explain anti-HIV effect in microtiter assay, we looked for mutations in IN gene in passages 30–32 for 105B and 241. The molecular sequencing found mutations in 105B (N17S, D231I) and in 241 (E10D, D231I), all in *in* region. Evidence demonstrated that 105B and 241 target viral protease and integrase.

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A Novel Method—Amenable for High-throughput Screening Purposes—to Quantify Antiviral Activity Against Viruses that Induce Limited CPE

Dirk Jochmans[1,*], Bernadette G. van den Hoogen[2], Pieter Leyssen[1], Ron A. Fouchier[2], Johan Neyts[1]

[1] *Rega Institute for Medical Research, University of Leuven (KULeuven), Leuven, Belgium*
[2] *Department of Virology, Erasmus MC, Rotterdam, Netherlands*

For antiviral screening purposes, infection of cell cultures with the virus under study, should ideally result in the induction, within just a few days, of (nearly) complete CPE and allow the calculation of acceptable Z′ factors (>0.5). The human Corona virus (NL63) causes only limited CPE on different cell lines (Schildgen et al., J Virol Methods, 2006). Following infection of Vero118 cells, virus-induced CPE was too low to allow readout based on classical

colorimetric methods (such as the MTS assay), even following prolonged incubation times (>7 days). In order to develop an antiviral screenings-assay against NL63, we explored whether a death-cell protease substrate could be used instead. The substrate used is a quenched peptide (bis-AAF-R110), that releases a fluorophore upon proteolytic-cleavage by proteases the latter released from death cells (Niles et al., Anal Biochem., 2007). After different rounds of optimization the following protocol was developed: Vero118 cells in 96-well plate format were infected with NL63 (MOI = 0.01, 200 μL cell culture, 2×10^4 cells/well, IMDM 5% FBS medium). Cultures were subsequently incubated for 5 days at 35 °C after which 20 μL of the peptide solution (16 μM final concentration) was added. Fluorescence was quantified 2 h after incubation at 37 °C. A roughly 3-fold increase in fluorescence intensity in the infected cultures was observed as compared to the uninfected cultures with a low well-to-well variability. Z′ factors calculated from different experiments were in the range of 0.6–0.8, indicating excellent assay quality. An anti-ACE-II polyclonal antiserum (that prevents coronavirus infection in cell cultures) was used as a positive control and allowed to validate the assay for antiviral screening purposes. In conclusion, in conditions where a viability staining is inadequate to quantify virus-induced CPE, a novel simple and convenient method that detects cell-death and that is suitable for high-throughput screening purposes can be employed.

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Effective Prophylactic and Therapeutic Treatment of Yellow Fever Virus with an Adenovirus-vectored Interferon, DEF201, in a Hamster Model

Justin G. Julander[1,*], Jane Ennis[2], John Morrey[1], Jeff Turner[2]

[1] *Institute for Antiviral Research, Utah State University, Logan, USA*
[2] *Defyrus Inc., Toronto, Canada*

Many acute arboviral infections are susceptible to interferon (IFN) therapy in various animal models. Yellow fever virus (YFV) is a prime example, where studies in a hamster model of viscerotropic disease have shown utility of IFN treatment. Human YFV disease cases occur annually despite the availability of an effective vaccine. Unfortunately, due to many factors, including cost and toxicity of IFN therapy, difficulty of conducting clinical trials, and lack of monetary incentive, the clinical development of interferon for YFV and other acute arboviral diseases has not been undertaken. To evaluate the efficacy of DEF201, an adenovirus-vectored interferon, a single intranasal (i.n.) dose was administered 4 h prior to YFV challenge of hamsters. A protective effect was observed with a minimal effective dose below 5×10^5 pfu/animal. Treatment was effective when given therapeutically up to 2 days after virus challenge after a single dose of 3×10^7 pfu/animal. At the same dose, DEF201 also displayed prophylactic protection after single dose administration at 7 days prior to virus challenge. We also investigated a novel delivery system, which distributes DEF201 as a fine mist into the nasal cavity. Further studies were performed to evaluate the effect of DEF201 treatment on late-stage immunization. These studies demonstrate the utility and efficacy of interferon produced by DEF201 in the treatment of YFV in a hamster model.

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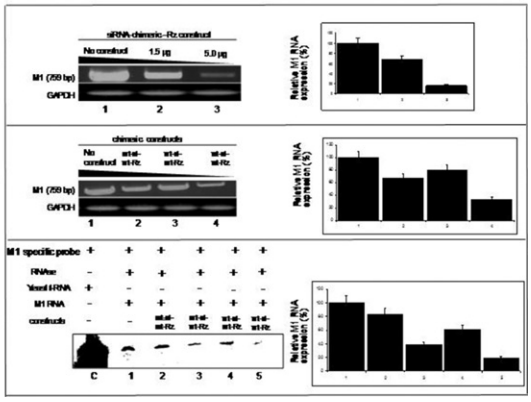
RNA Interference Mediated Gene Silencing of Influenza A Virus: A Tool for Potent Antiviral Therapy

Madhu Khanna[1,*], Prashant Kumar[1], Vikas sood[2], Roopali Rajput[1], Akhil Banerjea[2]

[1] *VP Chest Institute, University of Delhi, Delhi, India*
[2] *National Institute of Immunology, New Delhi, India*

Influenza A virus causes the most prevalent infection of the respiratory tract in humans. Currently available vaccines and antiviral drugs are of limited value because of the rapidly changing antigenic structure of the virus. Clearly, there is a need to develop novel strategies that can potently interfere with the replication of influenza virus .we designed small interfering RNAs against the conserved region of the matrix (M1) and non-structural (NS1) genes of influenza A virus. A decrease in viral gene expression was observed with increasing concentration of siRNAs. We also designed chimeric constructs consisting of siRNA joined by a short intracellular cleavable linker to a known hammerhead ribozyme (Rz) targeted against M1 genome segment of influenza A virus. When this, wt chimeric RNA construct was introduced into a mammalian cell line, along with the M1 substrate, encoding DNA, very significant (67%) intracellular down regulation in the levels of target RNA was, observed. On the contrary, when only the Rz was made, catalytically inactive, keeping the siRNA component unchanged, about 20% reduction in the target M1, specific RNA was observed. This wt chimeric construct showed impressive (>80%) protection against, virus challenge, on the other hand, the selectively disabled mutant constructs were less effective. Therefore, our study demonstrates that the gene silencing technology can provide efficient protection against the influenza virus at desirable levels and may prove as effective antiviral tools.



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Antiviral Effect of the Sulfated Polysaccharide, p-KG03, Against Influenza A Virus

Meehyein Kim[1,*], So-Yeon Kim[1], Hae Soo Kim[1], Joung Han Yim[2], Woo Ghil Lee[1], Chong-Kyo Lee[1]

[1] *Korea Research Institute of Chemical Technology, Daejeon, South Korea*
[2] *Korea Polar Research Institute, Incheon, South Korea*

It has been proposed that the sulfated exopolysaccharide, p-KG03, originating from the marine microalgal *Gyrodinium impudicum* strain KG03, inhibits encephalomyocarditis virus (EMCV) replication *in vitro* and stimulates NO production mediated by a

JNK pathway. In this study we investigated whether p-KG03 could inhibit influenza A virus replication and examined the key steps in the viral replication cycle inducing antiviral activity. Cytopathic effect and plaque assays showed that co- or post-treatment of p-KG03 with influenza A/PR/8/34 (H1N1) into MDCK cells can result in a significant reduction in viral titer (EC_{50}, <1 μg/ml), but not pre-treatment of the polysaccharide. It means that the mode of the antiviral action could be involved in the inhibition of viral entry into cells or the inhibition of early viral RNA replication. Fluorescence microscopy using an NP-specific antibody proved that p-KG03 interferes with nuclear localizaton of viral NP protein at 3 and 5 h postinfection in a dose-dependent manner. However, it was not observed that p-KG03 affects viral RNA polymerase activity in 293T cells where negative GFP RNA flanked by NS 5′ and 3′ UTR is amplified by viral polymerases/NP proteins and expresses GFP protein as a reporter. Taken together, we suggest that p-KG03 has anti-influenza activity by blocking viral entry into cells or by making virus particles non-infectious. Thus it might be worthy of further investigation as a potential anti-influenza compound.

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Excision of AZT and d4T Modulated by Deletions in the β3–β4 Hairpin Loop of HIV-1 Reverse Transcriptase

Mónica Kisic[1,*], Tania Matamoros[1], María Nevot[2], Jesús Mendieta[1], Javier Martinez-Picado[2,3], Miguel A. Martínez[2], Luis Menéndez-Arias[1]

[1] *Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Madrid, Spain*
[2] *Fundació irsiCaixa, Hospital Univ. Germans Trias i Pujol, Badalona, Spain*
[3] *Institució Catalana de Recerca i Estudis Avançats, Barcelona, Spain*

Single-amino-acid deletions affecting residues 67 or 69 in the β3–β4 hairpin loop of HIV-1 reverse transcriptase (RT) have been identified in heavily treated patients. The deletion of Asp67 together with mutations T69G and K70R (Δ67 complex) are usually associated with thymidine analogue resistance mutations (TAMs) (*e.g.* M41L, T215Y, etc.) while the deletion of Thr69 (Δ69) is frequently associated with mutations of the Q151M complex, and rarely found together with TAMs. Biochemical assays showed that in the presence or absence of TAMs, Δ67/T69G/K70R enhances ATP-dependent phosphorolytic activity on primers terminated with 3′-azido-3′-deoxythymidine (AZT, zidovudine) or with 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T, stavudine). However, Δ69 (or the complex S68G/Δ69/K70G) antagonize the effects of TAMs in ATP-mediated excision activity assays. These results were consistent with AZT susceptibility data obtained with recombinant HIV-1 bearing the relevant RTs. Molecular dynamics studies based on models of wild-type HIV-1 RT and mutant RTs Δ69, Δ67/T69G/K70R and D67N/K70R support a relevant role for Lys/Arg70 in the excision reaction. The ε-amino of Lys70 is located >10 Å away from the putative pyrophosphate (PPi) binding site in the Δ69 RT. The loss of interactions between Lys70 and the incoming PPi in Δ69 RT could explain the lower excision activity of this enzyme. These studies also suggested that the substitution K219E could have an effect on thymidine analogue excision/discrimination. Pre-steady-state kinetics revealed minor differences in selectivity between AZT-triphosphate and dTTP, when the deletion-containing RTs (*i.e.* Δ67/T69G/K70R, Δ69 and S68G/Δ69/K70G) were compared with their homologous enzymes having the K219E mutation. However, K219E reduced both ATP- and PPi-mediated excision of primers terminated with AZT or d4T, only when introduced in RTs bearing Δ69 or S68G/Δ69/K70G, providing further biochemical evidence explaining the lack of association of Δ69 and TAMs in HIV-1 isolates.

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Doxycycline in Tick-borne Encephalitis Virus Infection

Liubov Kozlovskaya*, Yulia Rogova, Galina Karganova

Chumakov Institute of Poliomyelitis and Viral Encephalitides, Russian Academy of Medical Sciences, Institut Poliomyelita, Russia

Tick-borne encephalitis (TBE) is endemic flavivirus in many parts of Europe and Asia. It is caused by TBE virus, which is transmitted into humans by tick bites. Ticks are vectors for various human pathogens, bacteria and protozoa. Bacteria includes Borrelia, Anaplasma, and Ehrlichia. One of the most useful drugs for prophylaxis bacterial infections after the tick bite is doxycycline. Mixt-infections of TBE virus and bacteria are very common (10–40%). Previously, structural analysis of Dengue virus E protein showed that doxycycline could have suppressing effect on DV infection in vitro by inhibiting low-pH induced conformational switch into fusogenic state during the entry process. The structure of E proteins is very common for all flaviviruses. Although there are some differences between mosquito- and tick-borne flaviviruses.

In the present study, we evaluated the doxycycline effect on TBE virus infection *in vitro* and *in vivo*.

In vitro experiments on PEK cells revealed that doxycycline addition prior or along with the TBE virus caused increased titers in plaque assay.

In vivo experiments in BALB/c mice showed that doxycycline caused slight positive effect on infectious process post high virus dose inoculation. Possibly, the effect was due to inhibiting of the secondary bacterial infection. There were no differences in mice mortality after small virus dose inoculation in treated and untreated groups. Although the virus titers in CNS of doxycycline treated mice were higher and were fixed more frequently than in infected control group. Analogous picture was observed with Amoksiklav(Amoxicillin + Clavulanic acid), but the effect was less expressed.

Thus, the doxycycline effect on TBE virus infection needs more investigations. Nevertheless, the presented data shows that doxycycline should be used very carefully in prophylaxis after tick bite due to high percent of mixt-infections.

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Antiarboviral Efficacy of Combined Application of Interferon Inducers and Proteolysis Inhibitor

Mykhajloik Kozlovsky[1,*], Vikor Lozitsky[2], Igor Benzel[3], Ihor Lozynsky[1], Sergij Lyakhov[4], Lyudmyla Litvinova[4]

[1] *Lviv Research Institute of Epidemiology and Hygiene, Lviv, Ukraine*
[2] *Ukrainian I.I.Mechnikov Research Anti-Plague Institute, Odesa, Ukraine*
[3] *Danylo Halytskyi Lviv National Medical University, Lviv, Ukraine*
[4] *A.V.Bogatsky Physico-chemical Institute, Odesa, Ukraine*

Earlier, we have shown the possibility to increase production of endogenous interferon owing to the combined application of their inducers (amixin and new phytoremedy SK-19) with proteolysis inhibitor E-aminocaproic acid (E-ACA). This work presents the results of testing the same application method of these

remedies in models of experimental arboviral infections: Tick-borne encephalitis (TBE) and West Nile fever (WNF). Phytoremedy SK-19 was introducing three times with 3 days intervals in doses of 20–50 mg/kg. This treatment and prevention scheme caused resistance of mice to TBE virus within the limits of 24.0–29.3% of protection. Similarly, individual introduction of E-ACA in doses of 1000–2000 mg/kg to infected mice showed low activity (8% of protection). At the same time, the combined application of the above-mentioned preparations using the same schemes provided protection of 35.1–43.8% of infected mice, increasing their survival by 11.1–14.5% compared with the introduction of individual SK-19. A similar pattern was observing when we used amixin and E-ACA in experimental WNF. In this case amixin in an optimal three times injection in dose of 150 mg/kg prevented death of 56.1% of mice, and E-ACA, respectively – 11.2% of mice. Analogous combined use of the above-mentioned preparations was accompanied by appreciable increase of protective action which was characterized by the protection of 67.7–71.5% of animals. It was more by 11.6–15.4% in comparison with an individual introduction of amixin. Also, a significant increase of average duration of life of the animals (by 3.6–4.1 days for TBE and by 5.1–5.6 days for WNF) compared to benchmarks shows the reliable effectiveness of the proposed method of prevention and treatment of TBE and WNF.

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HIV Full-Replication Technology for Identification of Novel HIV Inhibitors from Multiple Sources

Stephan Kremb[1,*], Markus Helfer[1], Horst Wolff[1], Andrea Kleinschmidt[1], Jörg Durner[2], Philippe Schmitt-Kopplin[3], Ruth Brack-Werner[1]

[1] *Helmholtz Zentrum München, VIRO, Neuherberg, Germany*
[2] *Helmholtz Zentrum München, BIOP, Neuherberg, Germany*
[3] *Helmholtz Zentrum München, IÖC, Neuherberg, Germany*

Control of the global HIV/AIDS health threat depends on a continuously flowing pipeline of novel anti-HIV agents for the development of new anti-HIV therapies. HIV with its multiple target-sites and the potential to evade current antiretroviral therapy (HAART) requires a constant and accelerated search for novel therapeutics. Here we report the establishment of a technically streamlined, sensitive and robust cell-based assay system (EASY-HIT) for the unbiased identification and characterization of HIV inhibitors. The assay allows discrimination between inhibitors of early and late phases of HIV replication and yielded high Z' scores (>0.9) and signal stabilities, confirming its robustness. Application of EASY-HIT to screening of various compound libraries identified several novel HIV-inhibitory molecules which are currently under further evaluation as potential lead candidates. Furthermore, we demonstrate the successful application of the assay for the detection of anti-HIV activities in crude biological extracts. Natural products with their largely unexplored diversity present a promising source for the discovery of novel anti-HIV drugs. Extracts derived from terrestrial plants as well as marine macroalgae showed strong activities against various targets of the viral replication cycle such as attachment, viron fusion and provirus integration. Several follow-up assays were applied for the characterization of inhibitory activities with a strong focus on viral entry as well as Tat- and Rev-dependent HIV gene expression.

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An Antiviral Assay to Identify Inhibitors of the Human Metapneumovirus that is Amenable for High-throughput Screening Purposes

Pieter Leyssen[1,*], Bernadette G. van den Hoogen[2], Dirk Jochmans[1], Ron A. Fouchier[2], Johan Neyts[1]

[1] *Rega Institute for Medical Research, University of Leuven (KULeuven), Leuven, Belgium*
[2] *Department of Virology, Erasmus MC, Rotterdam, Netherlands*

Human metapneumovirus (hMPV) is a recently discovered RNA virus in the pneumovirus subfamily of the Paramyxoviridae. Similar to RSV, it is an important cause of respiratory tract infections in infants, young children, and in immunocompromised individuals. Specific antiviral therapy is not available. Here we describe the setup of an antiviral assay against hMPV that is suitable for high-throughput screening purposes. To this end GFP-expressing recombinant hMPV NL/1/00 (De Graaf et al., 2007, J Virol Methods) was employed. Vero118 cells were infected with this recombinant virus (MOI = 0.01, 200 μL cell culture, 2×10^4 cells/mL, IMDM medium) after which cultures were further incubated for 4 days at 37 °C. Fluorescence was quantified using a Saphire^2 system. This method proved to be suitable for high-throughput screening it combined a homogenous assay set-up with good assay quality (Z': 0.5–0.6). Ribavirin was used as a positive control and allowed to validate the assay for antiviral screening purposes. In conclusion an antiviral assay was successfully optimized to a format that allows high-throughput screening for inhibitors of hMPV replication.

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PHYTOCHIK: Biodiversity As A Source of Selective Inhibitors of CHIKV Replication

Pieter Leyssen[1,*], Marc Litaudon[2], Jean-Claude Guillemot[3], Philippe Rasoanaivo[4], Jacqueline Smadja[5], Ameenah Gurib-Fakim[6], Bruno Canard[3], Françoise Guéritte[2]

[1] *Rega Institute for Medical Research, University of Leuven, Leuven, Belgium*
[2] *Centre de recherche de Gif, Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France*
[3] *AFMB, UMR 6098, Campus de Luminy, Marseille, France*
[4] *Institut Malgache de Recherches Appliquées, Antananarivo, Madagascar*
[5] *Laboratoire de Chimie des Substances Naturelles et des Sciences des Aliments, Faculté des Sciences et Technologies-Université de La Réunion, Saint-Denis, Reunion*
[6] *Centre for Phytotherapy Research, Ebene, Mauritius*

Chikungunya virus (CHIKV) is an alphavirus that is transmitted to humans mainly by the Aedes aegypti or albopictus mosquito. Ever since the outbreak on Reunion Islands in 2005–2006, CHIKV is considered to be an emerging virus. In particular arthralgia or arthritis, affecting multiple joints, is very pronounced during the clinical course, and may last for as long as 2 years. Currently, no vaccine nor a selective antiviral drug is available for the prevention or treatment of this debilitating viral infection. Chloroquine is active in cell culture and may alleviate the symptoms of arthritis by acting as an anti-inflammatory agent, although this latter is still under investigation. In 2008, the CRVOI (Centre de Recherche et de Veille sur les maladies émergentes dans l'Océan Indien)

has funded a research program that brings together four areas of expertise: the use of plants as natural source for the treatment of disease (ethnopharmacology), the identification and classification of plant species biodiversity, the purification and identification of unique molecules/secondary metabolites of natural origin, and the identification and characterisation of selective inhibitors of virus replication. Three teams, located on Reunion Islands, Mauritius and Madagascar, together with the ICSN, have build a unique sample library consisting out of more than 1500 crude plant extracts that have been evaluated for selective antiviral activity against CHIKV in Leuven and Marseille. Currently, a bio-assay-guided purification of pure substances is in progress, at present yielding the first results. Concomitantly, enzymatic assays are being developed in Marseille to evaluate and possibly characterize in detail the selective inhibitory effect of these compounds.

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Synthesis, Influence of Polymer Molecular Weight on Drug Release and Anti-HIV Activity of PEGylated AZT Conjugates

Wenjun Li[1,*], Peng Zhan[1], Jingde Wu[1], Yu Chang[1], Christophe Pannecouque[2], Erik De Clercq[2], Xinyong Liu[1]

[1] *Shandong University, Jinan, China*
[2] *Katholieke Universiteit Leuven, Leuven, Belgium*

Despite the first anti-HIV drug zidovudine (AZT) remains an important component in highly active antiretroviral therapy (HAART), the efficacy of its therapy is limited because of short terminal half life (1.2 h) and significant dose-related toxicity, as well as rapid emergence of drug resistance. To overcome its deficiency, one of the strategies was applied in preparation of polymeric AZT prodrug (polymer-AZT conjugate) for improvement AZT pharmacokinetic profiles. In earlier work, we had synthesized a sustained-release prodrug of AZT by conjugating with methoxy poly(ethylene glycol) (mPEG, Mw: 2000 g/mol) and found the conjugate effectively sustained release of AZT, prolonged half life and decreased toxicity in vitro. In order to elucidate the influence of molecular weight of mPEG on biological and pharmacokinetic properties, we synthesized a series of mPEG-succinyl-AZT conjugates with different molecular weight of mPEG (Mw: 750, 2000, 5000, or 10,000 g/mol). Drug release assay indicated that the mPEG-succinyl-AZT conjugates were capable of releasing the parent drug in sustained profiles, but there was no clear molecular weight-correlation to be found. The newly synthesized conjugates were also evaluated for anti-HIV activities and cytotoxicity in MT-4 cells. All of the conjugates displayed good activity against HIV replication. Especially for $mPEG_{750}$-succinyl-AZT (2a), it exhibited good inhibition to both wild and mutant strains including K103N and RES056, which were in the same order as the activity of AZT, but its cytotoxicity was lower than AZT. The selectivity index (SI) showed a clear correlation to Mw of mPEG, i.e. the higher Mw of mPEG-succinyl-AZT, the lower SI of the conjugate. In all, $mPEG_{750}$-succinyl-AZT (2a) exhibits better drugability than other polymeric conjugates, which might provide a feasible sustained-released prodrug alternative of AZT in antiretroviral therapy.

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The Protective Action Arbivirin and Aminocaproic Acid during the Experimental Influenza

V. Lozitsky[1,*], A. Fedchuk[1], T. Grydina[1], S. Rybalko[2], V. Cherednichenko[3]

[1] *I.I. Mechnikov Ukrainian Anti-Plague Research Institute, Odesa, Ukraine*
[2] *L.V. Gromashevsky Institute of Epidemiology and Infectious Diseases, Kyiv, Ukraine*
[3] *Zdorovya Pharmaceutical Company, Kharkiv, Ukraine*

The anti-influenza preparation Arbidol is produced in Ukraine under the name of Arbivirin (Ar). It is officially approved and recommended for the prophylactics and treatment of influenza and acute respiratory viral infections (ARVI) in adults and children aged 2 years and more and aminocaproic acid (ACA) is recommended in adults and children without age limits.

Objectives: Study of the anti-influenza activity preparations on the model of the acute influenza infection in mice.

Methods: We have used: high virulent strain of influenza virus A/PR/8/34 (H1N1), adapted to the mice lungs, 11-days chicken embryos, inbred white mice of 15–18 g weight, ACA and Arbidol (substance Zdorovya pharmaceutical company). The animals from experimental and reference groups were infected intranasally under light aether narcosis with 0.05 ml viral solutions in concentrations from 1×10^{-2} to 1×10^{-7} (4 mice for each solution). The animals from reference group have obtained the day before infection, the infection day and the 3 following days the 0.2 ml of placebo (1% starch solution) twice a day *per os*. The mice of the experimental group have obtained the Ar taken in ratio of 60 mg/kg per day added to the 1% starch solution according to the same scheme as the placebo in the reference group was introduced. The mice of the experimental group obtained ACA in ratio of 2 g/kg per day. The death of animals in each group was accounted during the 14 days after infection.

Results: The results obtained certify that LD_{50} in the reference group was equal to 5.75 lg. In the ACA provided group, the LD_{50} was lower by 1.5 lg, and in group Ar by 1.25 lg lower than in the reference group. The mortality was in the ACA group lower by 27%, and in Ar group by 21% lower than in the reference group. The average longevity was in the ACA group was higher by 1.4 days (9.9 days) and in Ar group by 1.9 days higher (10.4 days) than in the reference group (8.5 days).

Conclusions: The use of ACA and Ar according to the prophylactics/treatment scheme has caused the pronounced protective effect during the modeling of the influenza infection in mice.

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Oseltamivir Influences Hepatic Cytochrome P-450 Dependent Oxidative Metabolism in Influenza Virus Infected Mice

Milka M. Mileva*, Lora S. Simeonova, Galya G. Genova, Angel S. Galabov

Department of Virology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

Osletamivir is known as a neuraminidase inhibitor with a highly specific action against influenza A and B viral infections. Designed as a structural analogue of neuraminic acid, oseltamivir competitively binds the active site of the enzyme neuraminidase on the influenza virus surface. Then, it realized its antiviral effect. The purpose of this study was to examine the impact of oseltamivir

on the activity of liver CYP-dependent enzymes in mice experimentally infected with influenza virus A/Aichi/2/68(H3N2) (1.5 LD_{50}). It was found that influenza virus infection is a powerful prooxidant and causes a significant increase of the lipid peroxidation products as well as a decrease of the natural antioxidants (vitamin E, glutathione) and CYP. Moreover, the cytochrome *c*-reductase and the liver monooxygenases (aniline hydroxylase, ethylmorphin-N-demethylase, analgin-N-demethylase and amidopyrine-N-demethylase) are inhibited as compared to the control (non-infected) animals. We found that oseltamivir treatment led to a decrease of the products of lipid peroxidation on the 5th and on the 7th day after the virus inoculation. Besides, oseltamivir had a stabilizing effect on the content of CYP, activities of cytochrome *c*-reductase and liver monooxygenases. These effects were more pronounced on the 7th day as compared to the 5th day after virus inoculation.

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Antiviral Activity of Hemocyanin Isolated from Marine Snail *Rapana venosa*

Nadiya V. Nesterova[1,*], Svitlana D. Zagorodnya[1], Vesela Moshtanska[2], Pavlina Dolashka[2], Galina V. Baranova[1], Anna V. Golovan[1], Anna O. Kurova[1]

[1] *Institute of Microbiology and Virology of NASU, Kyiv, Ukraine*
[2] *Institute of Organic Chemistry, BAS, Sofia, Bulgaria*

Topicality of Epstein-Barr virus (EBV) in the development of human pathology is enormous. This virus is the etiologic agent of acute form of the disease – infectious mononucleosis. The persistence of the virus in the human organism leads to the development of lymphoproliferative disease, the formation of various carcinomas and to the affection of the peripheral and central nervous system. Therefore, the antiviral activity of hemocyanin from *Rapana venosa* (RvH) against Epstein-Barr virus was studied. Hemocyanin RvH was isolated from the hemolymph of marine snails, leaving in the Black sea. The native molecule of RvH consist from two isoforms RvH1 and RvH2. Each structural subunit contains 8 covalently linked functional units (FUa to FUh) with different carbohydrate content. A comparative study of anti EBV activity of two isoforms RvH, as well as individual FUs was carried out. As a model of EBV-infection *in vitro* have been used the line of lymphoblastoid B-cells Raji. We preliminary shown that all preparations of hemocyanin RvH have low toxicity; CC_{50} was about 700 μg/ml. The antiviral activity was determined by a PCR method, using "Àmply Sens 100 R" system (Russia). Preparations were investigated in concentrations range: 1, 10 and 100 μg/ml. The analysis of obtained data allowed determination of the concentrations, oppressing the replication of the virus on 50%. That was shown by reducing of the number of genomic equivalents of EBV DNA on a cell. ID_{50} for the hemocyanin RvH has amounted to 1 μg/ml. At the same time FU RvH1-6 in concentrations of 10 and 100 μg/ml result in a 100% inhibition of EBV. That is not observed during the test of the total RvH. A similar picture is revealed for the RvH2 and its functional unit FU-5. Thus, the study of antiviral activity of investigated hemocyanins found that they have low toxicity and their effective doses were determined. Proceeding from the index of selectivity which is 700 for hemocyanins isolated from *R. venosa*, it is possible to conclude about their availability for the further researches as of drugs that are active against an Epstein-Barr virus.

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Long-term Inhibition of HIV-1 Replication in $CD4^+$ T Cells Transduced with a Retroviral Vector Conditionally Expressing the *Escherichia coli* Endoribonuclease MazF

Mika Okamoto[1,*], Hideto Chono[2], Hiroshi Tsuda[2], Koichi Inoue[2], Junichi Mineno[2], Masanori Baba[1]

[1] *Center for Chronic Viral Diseases, Kagoshima University, Kagoshima, Japan*
[2] *Center for Cell and Gene Therapy, Takara Bio Inc., Otsu, Japan*

Considering the limitations of current antiretroviral therapy for HIV-1 infection, such as chronic toxicity, emergence of drug-resistant mutants, and inability to eradicate latently infected cells, gene therapy appears to be an alternative and desirable approach. We have recently developed a Tat-dependent expression system of MazF, an ACA-specific endoribonuclease derived from Escherichia coli, in a retroviral vector. Upon expression of Tat, MazF is induced in the cells transduced with this vector and can cleave HIV-1 mRNA, since HIV-1 genome has more than 240 ACA sequences. Strong inhibition of HIV-1 replication, irrespective of X4 or R5 strains, was observed without affecting cellular mRNAs in $CD4^+$ T cells (and PBMCs) isolated from different donors, when the cells had been transduced with the MazF-expressing retroviral vector (MazF/$CD4^+$ T cells). Furthermore, the replication of multi-drug resistant clinical isolates was also strongly inhibited in MazF/$CD4^+$ T cells. The proliferation and viability of the MazF/$CD4^+$ T cells were not affected even under HIV-1 infection. Long-term coculture experiments revealed that HIV-1 replication was always lower in the coculture of HIV-1-infected $CD4^+$ T cells with MazF/$CD4^+$ T cells than in that of HIV-1-infected $CD4^+$ T cells with no vector- or control vector-transduced $CD4^+$ T cells for more than 190 days. The HIV-1 proviral sequences of $CD4^+$ T cells after 3 days post-infection was compared with those after 190 days of the coculture cells and there was no substantial difference in the mutation rates of HIV-1 genes even after 190 days of coculture. Thus, the Tat-dependent MazF expression system has great potential for inhibition of HIV-1 replication in vitro without apparent cytotoxicity and may be able to avoid the emergence of resistant strains for a considerable period of time, indicating a possible candidate for treatment of HIV-1 infection.

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SP600125 Inhibits Orthopoxviruses Replication on a JNK1/2-Independent Manner – Implication as a Potential Anti-Poxviral

Anna C.P. Pereira[2,*], Jamaria A.P. Soares[3], Flavia G.G. Leite[4], André F. da Cruz[1], Alice A. Torres[1], Erna G. Kroon[1], Paulo C.P. Ferreira[1], Cláudio A. Bonjardim[1]

[1] *Grupo de Transdução de Sinal, Laboratório de Vírus, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil*
[2] *Universidade Federal do Piauí, Parnaíba, Brazil*
[3] *Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, WI, USA*
[4] *Institute of Science and Technology, Viena, Austria*

The pharmacological inhibitor SP600125 [Anthra(1,9-cd)pyrazol-6(2H)-one 1,9-Pyrazoloanthrone] has been largely employed as a c-JUN N-terminal kinases (JNK1/2) inhibitor. In this study, we evaluated whether pretreatment with SP600125 was able to prevent Orthopoxviruses *Vaccinia virus* (VACV), *Cowpox virus* (CPXV) and modified *Vaccinia virus Ankara* (MVA) replication. Our findings are consistent with the assumption that pre-incubation with SP600125 at 10, 20, 40 or 50 μM, blocked

virus-stimulated JNK phosphorylation, on a dose-dependent manner. Furthermore, at 40 μM, a concentration where ≥90 of the cells were viable, virus replication was significantly reduced, with the decline in virus yields reaching from ≥90 to 99%, depending on the infected cell line (A31, BSC-40 or BHK-21). The decline in virus titers was followed by an arrest verified in the transition from immature virus (IV) to intracellular mature virus (IMV) stage of the morphogenic cycle. Despite the fact that SP600125 can act as an efficient anti-poxviral compound, we also provide evidence that this antiviral effect is not specifically exerted through JNK1/2 inhibition. This conclusion is supported by the fact that viral titers measured after infections of JNK1/2 Knock-out cells were not altered as compared to those obtained from infected-wild-type cells. In contrast, a decline in viral titers was verified when the infection of KO cells was carried out in the presence of the pharmacological inhibitor. SP600125 has been the focus of recent studies that have evaluated its action on diverse viral infections including DNA viruses of *herpesviridae* family. Our data support the notion that SP600125 can be regarded as a potential anti-poxviral compound.

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Anti-viral Properties and Mode of Action of Standardized *Echinacea purpurea* Extract Against Highly Pathogenic Avian Influenza Virus (H5N1, H7N7) and Swine-origin H1N1 (S-OIV)

Stephan Pleschka[1,*], Roland Schoop[2], James B. Hudson[3]

[1] *Institute of Medical Virology, Jutus-Liebig-University, Giessen, Germany*
[2] *Bioforce AG, Roggwil, Switzerland*
[3] *Department of Pathology & Laboratory Medicine, University of British Columbia, Vancouver, Canada*

Influenza virus (IV) infections are a major threat to human welfare and animal health worldwide. Anti-viral therapy includes vaccines and a few anti-viral drugs. However vaccines are not always available in time, as demonstrated by the emergence of the new 2009 H1N1-type pandemic strain of swine origin (S-OIV) in April 2009, and the acquisition of resistance to neuraminidase inhibitors such as Tamiflu® (oseltamivir) is a potential problem. Therefore the prospects for the control of IV by existing anti-viral drugs are limited. As an alternative approach to the common antivirals we studied in more detail a commercial standardized extract of the widely used herb *Echinacea purpurea* (Echinaforce®, EF) in order to elucidate the nature of its anti-IV activity. Human H1N1-type IV, highly pathogenic avian IV (HPAIV) of the H5- and H7-types, as well as swine origin IV (S-OIV, H1N1), were all inactivated in cell culture assays by the EF preparation at concentrations several orders of magnitude below the recommended dose for oral consumption. Detailed studies with the H5N1 HPAIV strain indicated that direct contact between EF and virus was required, prior to infection, in order to obtain maximum inhibition in virus replication. Hemagglutination assays showed that the extract inhibited the receptor binding activity of the virus, suggesting that the extract interferes with the viral entry into cells. In sequential passage studies under treatment in cell culture with the H5N1 virus no EF-resistant variants emerged, in contrast to Tamiflu®, which produced resistant viruses upon passaging. Furthermore, the Tamiflu®-resistant virus was just as susceptible to EF as the wild type virus.

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Novel Chemical Compounds as Potential Blockers to the Swine-Origin Influenza A H1N1 (2009) Virus Replication

Roopali Rajput[1,*], Prashant Kumar[1], Binod Kumar[1], Madhu Khanna[1], Deepti Sharma[2], Divya Mathur[2], Ashok K. Prasad[2]

[1] *Department of Microbiology, Faculty of Medical Sciences, University of Delhi, Delhi, India*
[2] *Bioorganic Laboratory, Department of Chemistry, University of Delhi, Delhi, India*

Background: The swine-origin influenza A H1N1 (2009) virus, identified in late March 2009 in Mexico, had a brutal impact. Moreover, the lack of effective antiviral therapies against the virus assisted in its progress to cause a pandemic. The increasing capability of the influenza A viruses to develop resistance against antiviral drugs is posing a severe problem in the prophylactic measures for the virus control. Thus, new antiviral drugs are required for control of influenza virus infections.

Methodology: In our study, we analyzed the activity of two novel chemical compounds (kindly provided by Dr. Prasad, Department of Chemistry, University of Delhi) against the seasonal and pandemic influenza A viruses both *in vitro* (MDCK cell line) and *in vivo* (Balb/c mice). The cells and mice were infected with influenza virus strains separately and treated with the compounds, code names: CP1 and CP2. The percentage protection offered by these compounds was determined by MTT assay (*in vitro*) and survival assay (*in vivo*). The degree of virus inhibition by these novel compounds was also analyzed by viral plaque assay, real time RT PCR and Western blotting.

Results and conclusion: Percentage viability of the cells, after treatment with the compounds, increased by 54% and 47% in the presence of CP1 and CP2 respectively. *In vitro* experiments exhibited up to 50% inhibition of the viruses. Approx. 40% inhibition of viral gene expression was also observed in the *in vivo* studies using Balb/c mice. Hence, the two new compounds tested by us may prove as effective antiviral measures for prophylaxis and treatment of infections resulting from the seasonal and/or emerging strains of the influenza virus.

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Photodynamic Effect of Phthalocyanine–Zn (II) Complexes on Some Enveloped Viruses

M. Remichkova[1,*], L. Mukova[1], L. Doumanova[1], V. Mantareva[2], I. Angelov[2], V. Kussovski[1], A.S. Galabov[1]

[1] *The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria*
[2] *Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria*

Objective: Various phthalocyanines have been studied for their capacity for photodynamic inactivation of viruses. Two new water-soluble phthalocianine–Zn (II) complexes with different charges – cationic methylpyridyloxy-phthalocyanine (ZnPcMe) and anionic sulfophenoxy-phthalocyanine (ZnPcS), were used for photoinactivation of two DNA viruses, herpes simplex virus type 1 (HSV-1) and vaccinia virus (VV), and two RNA containing enveloped viruses: bovine diarrhea virus (BVDV) and Newcastle disease virus (NDV).

Experimental design: Aliquots of 0.1 ml stock virus were mixed with 0.1 ml solution containing 0.58 μM ZnPcMe or 0.64 μM ZnPcS. The mixtures were irradiated for 5 and 20 min at room temperature by fluence rate 100 mW cm^{-2} controlled by the photometer

equipment Spectra Physics, USA. Infectious virus titer of irradiated sample and non-irradiated control were determined on MDBK and CT cells monolayers.

Results: The two phthalocyanines (ZnPcMe and ZnPcS) showed a marked virucidal effect against HSV-1 at irradiation for 5 and 20 min (Δlogs = 3.0 and 4.0). This effect was weaker against VV (Δlogs = 2.34 and 2.17). BVDV had a low sensitivity to ZnPcMe (Δlog = 2.0) and a high sensitivity towards irradiation with ZnPcS for 5 and 20 min (Δlogs = 5.8 and 5.3). Both complexes were unable to inactivate NDV.

Conclusion: Inactivation of enveloped viruses by the studied phthalocyanines depends on the structure and the composition of the virus envelope.

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Classical Swine Fever Outbreak Containment – Antivirals as an Epidemiologically and Economically Viable Alternative to Emergency Vaccination and Culling

Stefaan Ribbens[1,*], Nesya Goris[2], Johan Neyts[3], Jeroen Dewulf[1]

[1] *Ghent University, Merelbeke, Belgium*
[2] *Okapi Sciences NV, Heverlee, Belgium*
[3] *KULeuven, Leuven, Belgium*

Classical swine fever (CSF), a pig disease caused by a pestivirus, might result in huge economic losses to countries with densely populated pig areas (DPLAs). The EU minimum control measures require depopulation of infected farms, movement restrictions, zoning and surveillance (base strategy). Emergency vaccination is authorised for DPLAs although the base strategy plus culling in a 1 km ring around infected premises is preferred. The Dutch contingency plan reads vaccination of pigs in a 2 km ring as it was as effective as 1 km ring culling using a stochastic model. Drawbacks to vaccination are inherent as differentiation of vaccinated from infected pigs is impossible for live vaccines and E2 marker vaccines suffer from a 10–14 day 'immunity gap' (time between vaccination and protection). Alternatives using small molecules targeting CSFV replication are being explored. Efficacy was shown in proof-of-principle studies with BPIP, an imidazo[4,5-c]pyridine. Oral administration to pigs 1 day prior to CSFV infection and continued for 15 days decreased viremia compared to untreated pigs and CSFV transmission from BPIP-treated pigs to sentinels was reduced. Hence, this study was set up to simulate between-herd CSFV spread with BPIP supplemented to feed in a 1 km ring around infected farms. The effects were compared to 3 other control scenarios: (i) base strategy, (ii) base strategy with 1 km culling and (iii) base strategy with 2 km E2 marker vaccination. The InterSpread Plus model was adapted to simulate subsequent CSFV spread after incursion in a Belgian DPLA. The median number of infected, culled and supplemented farms (respectively 5, 5 and 29) was lowest for the antiviral strategy followed by the vaccination policy. Moreover, antiviral supplementation was calculated to result in the shortest median outbreak duration. The antiviral scenario is as viable as marker vaccination when both direct (culling and disinfection) and indirect (transport bans, reproduction prohibition, etc.) costs are considered; the other scenarios being more expensive. In conclusion, CSF outbreak containment with antivirals is a valid alternative to more 'conventional' measures.

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Identification and Characterization of OBR-5-340 – A Novel Broad-spectrum Anti-human Rhinovirus (HRV) Inhibitor

Martina Richter[1,*], Vadim A. Makarov[2], Olga B. Riabova[2], Peter Wutzler[1], Michaela Schmidtke[1]

[1] *Institute of Virology and Antiviral Therapy, Jena University Hospital, Jena, Germany*
[2] *Institute of Biochemistry, Russian Academy of Science, Moscow, Russia*

HRVs are the major cause of the common cold, a mild and self-limiting upper respiratory tract infection that causes millions of absences from school and work as well as medical consultations annually. In addition, rhinoviral infections can lead to serious complications, e.g. sinusitis, otitis media, bronchitis, pneumonia as well as exacerbating asthma, COPD, and cystic fibrosis. Until now, there is no approved effective antiviral drug for treatment of HRV infections. Here we describe the discovery of OBR-5-340, a novel broad-spectrum anti-rhinoviral compound. It belongs to a series of 100 new pyrozolo-pyrimidine derivatives that were synthesized and tested for cytotoxicity and CPE inhibitory activity against 30 HRV serotypes in HeLa cells. Like most of these derivatives, OBR-5-340 is composed of 3 ring systems, referred to as ring A [aniline], B [pyrazolo[3,4-*d*]pyrimidine], and C [phenyl] and well tolerated. OBR-5-340 inhibits the CPE of all studied HRV serotypes in the nano- and micromolar dose range. An advantage is its strong activity against pleconaril-resistant HRV serotypes, e.g. HRV 5, 42, and 48. Using HRV 5 as example, the antiviral activity of OBR-5-340 was further confirmed by plaque reduction assays as well as virus-yield reduction assays under single-step growth cycle conditions in HeLa cells. Results from mode of action studies demonstrate the inhibition of virus adsorption by OBR-5-340. In conclusion, OBR-5-340 represents a novel potent capsid-binding compound with broad-spectrum anti-rhinoviral activity that warrants further preclinical and clinical development.

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Relationship Between Homocysteine Serum Level and Other Blood Analyses Parameters in HIV-infected Patients

Bernardino Roca*, José Antonio Ferrero, Maria Cruz del Monte, Elena Resino

Hospital General, University of Valencia, Castellon, Spain

Background: To assess the possible association of increased homocysteine serum level with multiple blood analyses parameters in HIV-infected patients.

Methods: This is the second part of a cross-sectional study, carried out as a supplementary task to the usual control required by HIV-infected patients, in the outpatients' clinic of the Hospital General of Castellon, Spain, along two consecutive visits. The possible association of homocysteine serum level with multiple blood analyses parameters and with variables found to be associated with the aminoacid in the first part of the study was assessed with a multiple linear regression analysis.

Results: A total of 145 patients were included. Creatinine was higher than normal in 7 patients (5%), prothrombin time was higher than normal in 36 patients (25%), and a monoclonal gammopathy was detected in 2 patients (1%). An association was found between high homocysteine serum level and the following variables: high creatinine ($P > 0.001$), low folic acid ($P > 0.001$), HIV risk behavior sexual (vs. parenteral) ($P = 0.033$), hepatitis C virus

co-infection ($P = 0.014$), and high height ($P = 0.009$). An association was found between low homocysteine serum level and the following variables: high prothrombin time ($P = 0.027$), and presence of monoclonal gammopathy ($P = 0.019$).

Conclusion: Associations were found between high homocysteine serum level and high creatinine, and between low homocysteine serum level and high prothrombin time and presence of monoclonal gammopathy.

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Control of HIV-infection with Visits Scheduled Every Four or Every Six Months. A Comparative Randomized Study

Bernardno Roca*, Elena Resino, Maria Cruz del Monte, Manuel Roca

Hospital General, University of Valencia, Castellon, Spain

Background: Guidelines generally recommend scheduling visits for monitoring HIV infection every 3–4 months. But those repetitive controls are many times unrewarding and suppose an inconvenience for patients. Studies are needed to determine the most efficient scheduling of those visits.

Methods: Randomized prospective study, carried out in the HIV clinic of the University General Hospital of Castellon, Spain. Control of HIV-infection, in terms of virologic and immunologic response, and adherence to visits, is compared in patients who are scheduled to visits every four versus every 6 months, throughout one year. Patients are included if (1) they are taking antiretroviral therapies of those recommended by guidelines, (2) they have had undetectable HIV-RNA for at least the last two controls, and (3) they give informed consent.

Results: We include 62 patients. Table shows the most relevant results. An intention-to-treat analysis is carried out. Comparisons are made with nonparametric tests. Values are medians, if not otherwise specified.

Conclusions: Visits scheduled every 6 months, instead of every 4 months, do not seem to jeopardize adequate control of HIV infection.

	Every 4 months	Every 6 months	*P*
Number of patients	32	30	–
Gender male, %	78	67	0.312
Age, years	43	41	0.757
Baseline CD4 cell count, per mm^3	465		0.111
Variation of CD4 cell count after 1 year of follow-up, per mm^3	8	−6	0.479
HIV-RNA undetectable after 1 year of follow-up, %	66	80	0.345
Adherence to scheduled visits, %	88	97	0.070

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European Virus Archive

Jean-Louis Romette

UNIVMED, Marseille, France

The EVA consortium is dedicated to the provision of unique collections of high quality and authenticated virus strains through its virtual bio-resource centre, for fundamental and applied research. The core of the European Viral Archives, is composed by the following participants:

No.	Participant organisation name	Short name	Country
1.	Institut de Recherche pour le Développement	IRD	France
2.	Veterinary Laboratories Agency	VLA	UK
3.	Bernhard-Nocht-Institut für Tropenmedizin	BNI	Germany
4.	Universitaetsklinikum Bonn	UKB	Germany
5.	Health Protection Agency	HPA	UK
6.	Université de Genève	UNIGE	Switzerland
7.	Univerza v Ljubljani	UL	Slovenia
8.	Institute of Virology, Slovak Academy of Sciences	IVSAS	Slovakja
9.	Université de la Méditerranée	UNIVMED	France

The nine member laboratories within the EVA consortium will merge their specialised collections to create a catalogue advertising these viruses through a web portal. EVA provides the catalogue to our customer and the strains are supplied, to EVA specifications, by the respective member laboratory remain the property of the originator. Using standardised procedures for virus production, preservation, qualification, and storage procedures, users will be assured of a consistent level of service, irrespective of the lab supplying the virus. All viruses supplied will meet EVA's standard specification on quality and authentication. The EVA consortium is committed to continuous improvement and development within the field of scientific and medical research. This is evident from our objectives, which include:

- The development of optimized methods for the characterization and conservation of viruses within the EVA collection,
- The development of protocols and facilities for the preservation and long term storage of virus,
- The derivation and development of virus products for diagnosis, identification and antiviral therapy.

The EVA consortium quality policy is based on the following principles, in accordance with the recommendations of the EVA Scientific Advisory Board:

- A unique collection of a wide range of viruses within an international network,
- Comprehensively identified virus strains,
- Standardised quality of virus strain and service level to users,
- Assured bio-security of originating laboratories, users and the general public.

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HLA-C-35C/T Variant: Genetic Association to HIV-1 Disease Progression and Functional Links

Alba Ruiz[1,*], Ester Ballana[1], Beatriz Mothe[1,2], Eulalia Grau[1], Bonaventura Clotet[1], Christian Brander[1,3], José A. Esté[1]

[1] *Irsicaixa AIDS Research Institute-HIVACAT, Badalona, Spain*
[2] *Lluita contra la SIDA Foundation, Badalona, Spain*
[3] *Institució Catalana de Recerca i Estudis Avançats (ICREA), Badalona, Spain*

Genomic studies on HIV-1 have identified host determinants influencing viral replication and infection. A greater understanding of their functional effect may provide alternatives to slow disease progression and improve the response to antiretroviral treatment. A variant 35 kb upstream of the *HLA-C* gene (−35 C/T) has been implicated as a major determinant in early phase and late outcomes of HIV infection, influencing steady-state viral load and time to death. The protective −35CC genotype has been associated with

high *HLA-C* mRNA levels and higher surface expression of HLA-C protein. Such increase could enhance T cell responses and lead to a better antiviral control over the course of the infection. To study the effect of the 35C/T variant, 249 HIV-infected patients (92 progressors and 157 long-term non-progressors) and 180 HIV uninfected individuals were selected and genotyped for the −35 C/T SNP. In a subgroup of patients, *HLA-C* mRNA levels and responses to peptides containing optimally defined HLA-C restricted CTL epitopes were determined. An overrepresentation of the −35CC genotype was found in the LTNP comparing with the progressor group (p-value = 0.0005). Measurement of *HLA-C* mRNA levels in a subset of individuals, revealed a 1.7-fold increase in subjects with −35CC genotype compared to −35TT genotype, correlating the −35CC genotype with higher gene expression. When assessing responses to HLA-C peptides containing HLA-C restricted epitopes, subjects with −35CC mounted broader responses than those with −35TT or −35TC genotype (median 1.5 in −35CC versus median 0 in −35CT and −35TT). −35CC genotype is associated to slow disease progression putatively due to a higher HLA-C mRNA levels, that at its turn may confer higher reactivity to HLA-C presented HIV-1, possibly leading to a stronger activity of the effector cell. The prior knowledge of the presence of a protective genetic variant influencing HIV disease progression and its functional study could be useful to delay the decision of starting antiretroviral treatment.

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Discovery of Novel Natural Neuraminidase Inhibitors (NAI) based on *In Silico* Screening and Antiviral Investigations

Michaela Schmidtke[1,*], Judith M. Rollinger[2], Ulrike Grienke[2], Nora Seidel[1], Andi Krumbholz[1], Peter Wutzler[1], Klaus R. Liedl[3], Johannes Kirchmair[3]

[1] *Jena University Hospital, Institute of Virology and Antiviral Therapy, Jena, Germany*
[2] *University Innsbruck, Institute of Pharmacy/Pharmacognosy, Innsbruck, Austria*
[3] *University Innsbruck, Institute of Theoretical Chemistry, Innsbruck, Austria*

The emergence and worldwide spread of ion channel blocker- and oseltamivir-resistant influenza A viruses ask for the discovery of new highly active antiviral drugs. Recently, we identified and characterized a new potential NAI, katsumadain A, from Alpinia katsumadai by combining computational tools, phytochemical and antiviral approaches (Grienke et al., J. Med. Chem., 2010). Here, the knowledge on compound structure and binding was used for shape-focused virtual screening to identify novel promising compounds with significant enhanced NA inhibitory activity. In the results of virtual screening of the NCI database for biological testing that contains more than 200,000 small organic molecules 5 further natural hit compounds were identified. Three of them are flavonoids – a class of plant metabolites that was repeatedly identified to exhibit interesting antiviral and also NA-inhibiting activity. The NA-inhibiting potential of these compounds was tested with influenza virus A/PR/8/34, 3 clinical H1N1v isolates, and an oseltamivir-resistant H1N1 isolate from the season 2008/09 using a chemiluminescence-based enzyme inhibition assay. All 5 compounds strongly inhibited the NA of the oseltamivir-susceptible H1N1 and H1N1v strains. The best activity exhibited artocarpin, a twofold isoprenylated flavon present in different species of the genus Artocarpus. It was active at nanomolar concentrations. Moreover, its 50% inhibitory concentration was 10-times lower than that of katsumadain A. Artocarpin exhibited also high activity against the NA of the oseltamivir-resistant H1N1 isolate. A potential binding mode of these compounds was determined employing ligand-based techniques and protein-ligand docking using representative protein conformations selected from molecular dynamics (MD) simulations. The insights gained from this modeling study and the SAR data will be exploited for the developing of novel inhibitors of influenza NA with improved resistance profiles.

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Synthesis, Anti-HIV and Cytotoxic Activity of Some Novel Isatine-Sulfisomidine Derivatives

Periyasamy Selvam[1,*], Markandavel Chandramohan[2], Christophe Pannecouque[3], E. De Clercq[3]

[1] *Devaki Amma Memorial college of Pharmacy, Malapuram, India*
[2] *Kamarajar Liver Hospital and Research centre, Madurai, India*
[3] *Rega Institute for Medical Research, Katholieke Universiteit, Leuven, Belgium*

Background: The development of antiviral drugs has provided crucial new means to mitigate or relieve the debilitating effects of many viral pathogens. New classes of inhibitors are essential to combat HIV/AIDS. Isatine is a versatile lead molecule for designing potential antiviral agents and its derivatives were reported to possess wide spectrum antiviral activity.

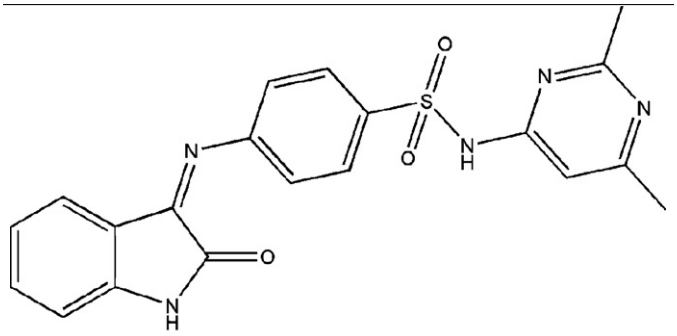
Methods: A series of isatine-sulfisomidine derivatives were screened for antiviral activity against HIV-1 and -2 viruses in MT-4 cell culture. Cytotoxicity of the synthesized compounds was also tested in uninfected MT-4 cells.

Results: All the compounds inhibits the replication of HIV-1 (IIIB) in MT-4 cells (9.22–13.80 μg/ml). The most active compound, SP-A inhibited virus-induced cytopathology by 50% at 9.22 μg/ml and 50% cytotoxicity occurring at a much higher dose more than 125 μg/ml.

Conclusions: SP-A exhibited potency against HIV 1 and are suitable candidate molecules for further investigation.

Anti-HIV activity and cytotoxicity of isatine derivatives.

Compounds	Strain	IC_{50}, μg/ml	CC_{50}, μg/ml
SPIII-A	IIIB	9.22 ± 0.25	>125
	ROD	78.70 ± 5.3	>125
SPIII-B	IIIB	13.5 ± 0	>125
	ROD	>125	>125
SPIII-C	IIIB	12.85 ± 2.33	>125
	ROD	>125	>125
SPIII-D	IIIB	13.80	>89.80
	ROD	>125	>89.80
AZT	IIIB	0.0022	>25
	ROD	0.0011	>25



ISATINE-SULFISOMIDINE

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Studies of HIV Integrase Inhibitory Activity of Novel Isatine Derivatives

Periyasamy Selvam[1,*], Kasthuraiah Maddali[2], Christophe Marchand[2], Yves Pommier[2]

[1] *Devaki Amma Memorial College of Pharmacy, Malapuram 676364, Kerala, India*
[2] *Laboratory of Molecular Pharmacology, NCI, NIH, Bethesda, MD 20892, USA*

Background: The development of antiviral drugs has provided crucial new means to relieve the debilitating effects of many viral pathogens. A rich source for the discovery of new HIV infection inhibitors has been and continues to be, the 'mining' of the large diversity of compounds already available in nature and specifically those from new chemical entities. Isatine (2,3-dioxoindole) is a versatile lead molecule for designing potential antiviral agents and is also reported to possess wide spectrum of antiviral activities. HIV Integrase is a crucial enzyme for HIV replication and an attractive therapeutic target for designing novel anti-HIV agents. To understand the molecular mechanism for the antiviral efficacy of isatine we investigated its inhibitory activity against HIV-1 integrase.

Methods: Novel isatine-sulphadimidine derivatives have been studied against HIV-1 integrase enzymatic activity. All compounds were investigated for both 3′-processing (3′-P) and strand transfer (ST) activity of HIV-1 integrase enzyme.

Results: All compounds exhibited significant inhibitory activity against HIV-1 integrase (3′-P: 5.7–32.5 μM and ST: 3.55–28 μM). The 5-chloro-N-acetyl derivatives (SPIII-5Cl-AC) displayed inhibitory activity against both 3′-P and ST In enzymatic activity (3′-P IC_{50}: 6.8 ± 0.6 μM and ST IC_{50}: 3.55 ± 0.02 μM).

Conclusions: From these studies Isatine derivatives are inhibitors of HIV integrase enzymatic activity. This is the first report showing the anti-HIV-1 IN activity of isatine derivatives. HIV integrase inhibitory activity of isatine derivatives.

Compounds	IC_{50} 3′-P, μM	IC_{50} ST, μM
SPIII-5H	7.4 ± 1.6	6.18 ± 1.3
SPIII-5Cl	5.7 ± 1.0	4.5 ± 1.3
SPIII-5Br	32.5 ± 2.4	28.0 ± 1.2
SPIII-5Me	16.5 ± 2.5	10.6 ± 2.0
SPIII-NA	16.3 ± 1.6	13.6 ± 1.0
SPIII-5Cl-AC	6.8 ± 0.6	3.55 ± 0.02

The results are IC_{50} ± S.D., $n = 4$ for HIV-1 IN inhibitory activity.

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Combined Anti-influenza Virus Effect of Natural and Synthetic Viral Inhibitors

Julia Serkedjieva[1,*], Iskra Ivanova[2]

[1] *Institute of Microbiology, Bulgarian Academy of Sciences, Bulgaria*
[2] *Department of Microbiology, Sofia University, Bulgaria*

The data from the combined application of viral inhibitors suggest that this could be a promising approach in the control of viral infections. Our research group has a substantial experience in this field of research. The aim of the present work was to investigate the combined anti-influenza virus effect of a protease inhibitor (PI), produced by *Streptomyces chromofuscus* 34-1 (SS 34-1) with a number of alternative antiviral agents. Earlier research proved that SS 34-1 inhibited significantly the replication of influenza viruses *in vitro* and *in vivo* (Angelova et al., 2006). The PI was a hydrophobic and a termostable protein, had a molecular mass of 11.2 kDa, isoelectric point of 7.5 and a high content of hydrophobic amino acids and proline. The N-terminal sequence demonstrated its homology to the *Streptomyces* subtilisin inhibitors family. The combined virus-inhibitory effects of SS 34-1 with either Rim – rimantadine hydrochloride, PC – a plant polyphenol-rich extract or a number of protease inhibitors (ACA – ε-amino caproic acid, SS 225 – a microbial PI, STI – soya trypsin inhibitor, Apr – aprotonin and Leu – leupeptin) was tested on the reproduction of influenza virus A/Germany/34, strain Rostock (H7N1) in MDCK cell cultures. The reduction of the virus-induced CPE and the decrease of HA and infectious virus production were used as measures of viral inhibition. The simultaneous use of SS 34-1 + Rim, a selective anti-influenza drug, in doses, which by themselves do not suppress significantly viral replication, resulted in an additive increase of inhibition. The combination SS 34-1 + PC, a plant preparation with established anti-influenza-virus activity, lead to a synergistic limitation of viral replication. The combined effect of SS 34-1 and the protease inhibitors Apr, Leu and STI was indifferent. Only the combinations of SS 34-1 + ACA were of the additive to synergistic type. Our results show that in order to achieve a synergistic virus-inhibitory effect of the combinations it is essential to carefully choose the individual components and the precisely select their doses.

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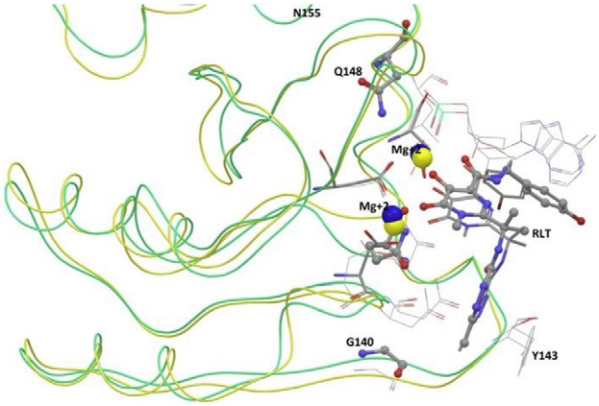
A Catalytic 3D Model Development of HIV-Integrase and Drug Resistance Understanding by Molecular Dynamics Simulation

Ashoke Sharon*, Tuniki Balaraju, Chandralata Bal

Department of Applied Chemistry, Birla Insititute of Technology, Mesra, Ranchi, India

Even though 25 years of an anti-HIV drug discovery, resistance and cross resistance possess major threat. The approved drug Raltegravir is currently available to target virally encoded enzyme "HIV Integrase". The slow drug development against integrase may attribute due to poor 3D structural and catalytic information of this enzyme. The recent reports explain the molecular basis of catalysis by integrase and their inhibitors. However, a complete model giving a detailed account of various biochemical reactions controlled by integrase, its inhibition and drug resistance is still to be understood. Therefore, we report a creation of integrase model using homology followed by global minimization, validated by experimental facts. This 3D model explains the possible mode of action during strand transfer. The studies were further extended to understand the molecular basis of drug resistance by molecular dynamics simulation. The dual mutant G140S-Q148H, having negligible impact on virus replication, however dynamics result demonstrate geometrical constrain for the binding of either Raltegravir or Elvitegravir and causing high resistance. Furthermore, the flexible catalytic loop has been studied for the compensatory role of mutant S140, which favors stabilization by initiating a polar interaction within the loop by its associative residue N117.

The present computational studies demonstrate the two metal-ion mechanism of potential molecules as INSTIs and present a valid 3D catalytic model. This model further provides insight for new drug discovery through the graphical and empirical drug response in comparison to biochemical evidence against WT and mutant strain (Q148H, N155H, G140S, G140S/Q148H) of HIV Integrase.



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Susceptibility to Neuraminidase Inhibitors and M2 Blockers of Some Seasonal Influenza Strains Isolated in Bulgaria 2004–2007

Lora Simeonova[1,*], Galina Gegova[1], Lucia Mukova[1], Angel S. Galabov[1], Rositza Koceva[2], Sylvie van der Werf[3]

[1] *Department of Virology, The Stephan Angeloff Institute of Microbiology, BAS, Sofia, Bulgaria*
[2] *National Reference Laboratory of Influenza and Acute Respiratory Infections, NCIPD, Sofia, Bulgaria*
[3] *Unité de Génétique Moléculaire des Virus à ARN, Institut Pasteur, Paris, France*

M2 blockers and neuraminidase inhibitors are two classes of drugs currently approved for prophylaxis and treatment of seasonal influenza A virus infections. The frequency of antiviral drug resistance has increased dramatically over the last 10 years and therefore monitoring of susceptibility to licensed inhibitors is an essential component of influenza surveillance and therapy in Bulgaria and worldwide. Phenotypic and molecular techniques were applied for detection of resistance of influenza viruses (H1N1) and (H3N2) strains isolated in Bulgaria 2004–2007 to neuraminidase inhibitors and M2 blockers. IC_{50} values of rimantadine were determined by CPE inhibition in cell cultures. IC_{50} values of oseltamivir were evaluated fluorimetrically by neuraminidase susceptibility assay with MUNANA substrate. RT-PCR and sequencing were carried out for evaluation of gene segments coding HA, NA and M2 proteins with subsequent phylogenetic analysis. From overall 26 influenza strains (H1N1) and (H3N2) 22 were sensitive and 4 (two H1N1 and two H3N2) were resistant to rimantadine hydrochloride in CPE inhibition assay. 17 isolates were subjected to fluorescent assay and their susceptibility to oseltamivir and zanamivir were determined. IC_{50} of zanamivir varied from 1.05 nM to 5.28 nM and osletamivir IC_{50} were from 0.28 nM to 1.31 nM. Sequencing revealed S31N and V27T mutations in transmembrane region of M2 protein conferring resistance to adamantanes in A/Sofia/1250 (H3N2) strain. The virus remained susceptible to neuraminidase inhibitors. In all other viruses no mutations associated with either to M2 blockers or neuraminidase inhibitors were found. The HA, NA, and M sequence data of the H1N1 and H3N2 viruses were assembled and clustered and phylogenetic trees were constructed using the neighbor-joining method and bootstrap analysis.

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Prophylactic Activity of mDEF201 Against Vaccinia Virus Respiratory Infections in Mice

Donald F. Smee[1,*], Min-Hui Wong[1], Eric Sefing[1], Ramtin Rahbar[2], Jane Ennis[2], Jeffrey D. Turner[2]

[1] *Utah State University, Logan, USA*
[2] *Defyrus Inc., Toronto, Canada*

An attenuated adenovirus type 5 vector containing an interferon alpha gene has been developed for the treatment of viral infections. We investigated mDEF201 (the vector containing mouse interferon) for the treatment of lethal respiratory infections in mice caused by vaccinia virus (WR strain). In the first experiment, mDEF201 was administered intranasally at 100 thousand, 1 million, or 10 million PFU/mouse one time only at 24 h prior to virus exposure. This afforded 90–100% protection from death and reduced day 5 lung virus titers approximately 8-, 80-, and 800-fold, respectively. Lung weights and lung hemorrhage scores, which increase during infection, were also significantly reduced by the treatments. Cidofovir (100 mg/kg/day i.p., once-daily for 2 days starting at +24 h) reduced viral titers 40-fold. A dose-responsive protection from body weight loss was afforded by mDEF-201 treatments, with minimal weight loss seen in groups receiving the two highest doses. This protection was superior to the effect of cidofovir. An empty adenovirus vector was similar to placebo in its activity. High doses (10 or 100 million PFU/mouse) of mDEF201 were required to prevent death when the vector was given 6 h after virus exposure. Extended prophylaxis was performed with a single mDEF201 administration one time only at 28, 21, 14, 7, or 3 days pre-virus challenge. All of these treatments protected mice from the lethal infection. None of the mice treated at these times exhibited weight loss during the infection but showed steady weight gain. Surviving animals from this infection were subjected to re-challenge with vaccinia virus. They all lost minimal body weight and survived, whereas naïve infected animals died. These results demonstrate the potent prophylactic activity of mDEF201 against this orthopoxvirus infection.

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Novel Derivatives of Abacavir – Synthesis and Activity Against Human Immunodeficiency Virus-Type 1 in Cell Culture

Kamelia R. Stanoeva[1,2,*], Ivanka G. Stankova[3], Anton V. Hinkov[2], Ivailo I. Alexiev[4], Petya D. Genova-Kalou[5], Radoslav I. Chayrov[3], Radka M. Argirova[6]

[1] *Medical Faculty, Medical University – Sofia, Sofia, Bulgaria*
[2] *Faculty of Biology, Sofia University "St.Kliment Ohridski", Sofia, Bulgaria*
[3] *Department of Chemistry, South-West University "Neofit Rilski", Blagoevgrad, Bulgaria*
[4] *National HIV Confirmation Laboratory, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria*
[5] *Laboratory for Cell Cultures, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria*
[6] *Laboratory of Retroviruses, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria*

Abacavir (ABC) is clinically associated with hypersensitivity reactions, risk for cardiovascular disease, etc. A possible way to minimize side effects is by modifying chemical structure. Three abacavir (ABC) esters and dipeptides (Gly-ABC, Z-Gly-ABC and

Z-Gly-Gly-ABC) were synthesized using thiazole-containing amino acid glycine – Gly and evaluated for anti-HIV activity on MT-4 cells. Cytotoxicity experiments showed Z-Gly-Gly-ABC and Z-Gly-ABC were more cytotoxic (CC50 = 70 μM and 100 μM respectively) than Gly-ABC (CC50 = 650 μM) which in turn proved less cytotoxic than ABC (CC50 = 160 μM). Gly-ABC inhibited HIV-1 III B replication measured by infectivity and reverse transcriptase (RT) activity, IC50 was detected to be 6.5 μM. Mitochondrial toxicity was established, although decline in both mitochondrial and nuclear DNA were found. Further, 32 passages of HIV-1 in MT-4 cells with increasing concentrations of Gly-ABC were carried out and RT region of the resulting virus was sequenced using Opengene, TRUGENE kit. IC50 increased up to 5 times compared to initial IC50. Both mutations found – K122E and T165I are not related to ABC as well as to other nucleoside reverse transcriptase inhibitors. Z-Gly-ABC and Z-Gly-Gly-ABC were not further evaluated due to their cytotoxicity. In conclusion, a new less cytotoxic derivative of ABC – Gly-ABC – was synthesized with high anti-HIV activity due to RT inhibition, low mitochondrial toxicity and most probably characterized by a high genetic barrier to resistance.

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Efficacy of Zanamivir Administered by Different Routes and at Different Times for Treatment of an Influenza A/CA/04/2009 (H1N1) Virus Infection in Mice

Bart Tarbet*, Deanna Larson, Min-Hui Wong, Donald Smee

Institute for Antiviral Research, Utah State University, Logan, USA

Zanamivir is recommended for administration by inhalation. However, the intranasal route is problematic in mice because that route exacerbates the infection. This study determined the dose-responsive effects of zanamivir administered by three routes (i.p., i.m., or p.o.) against challenge infection of mice with influenza A/CA/04/2009 (pandemic H1N1) virus. Zanamivir doses of 3 and 10 mg/kg were effective at increasing survival regardless of the route of administration. The dose of 1 mg/kg also produced significant survival results when administered by the i.m. or p.o. routes. We also evaluated the antiviral effects of delayed zanamivir treatment, when initiated at 4, 24, and 48 h post-challenge infection. Twice daily treatments with zanamivir at 3 and 10 mg/kg for 5 days were compared to placebo controls matched for the route of administration. All mice survived challenge infection in the groups treated with 10 mg/kg zanamivir beginning 4 and 24 h post-infection. In addition, all treatment groups, at all three starting times showed significant improvement in survival compared to placebo controls. However, mortality was observed at the 3 mg/kg dose for the group receiving zanamivir by the i.p. route at all three staring times, and for the i.m. treatment group starting at 24 h post-infection. None of the treatments significantly prevented weight loss during the initial 8–10 days following infection. However, an improvement in mean body weight, observed as more rapid weight gain after day 12 post-infection, was almost exclusively observed for the 10 mg/kg dose at all three staring times. These results demonstrate that zanamivir administered by different routes can be used effectively to treat an influenza virus infection in mice, and that treatment can be delayed for 24 or 48 h post-infection and remain efficacious.

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Inactivated Vaccine Against Tick-Borne Encephalitis Virus as Surrogate Vaccine Against Omsk Hemorrhagic Fever Virus

Liubov Terekhina[1,*], Nataliya Pripuzova[2], Mikhail Vorovitch[2], Yulia Rogova[2], Lidiya Romanova[2], Nataliya Tereshkina[2], Andrey Timofeev[2], Galina Karganova[2]

[1] *Federal State Unitary Enterprise on Manufacture of Bacterial & Viral Preparation of Chumakov Institute of Poliomyelitis and Viral Encephalitides of Russian Academy of Medical Sciences, Institut poliomyelita, Russia*

[2] *Institute of Poliomyelitis and Viral Encephalitides of Russian Academy of Medical Sciences, Institut poliomyelita, Russia*

Tick-borne encephalitis virus (TBEV) and Omsk hemorrhagic fever virus (OHFV) are mammalian tick-borne flaviviruses. Viruses are closely related, and the *p*-distance is about 0.25 counted for the whole ORF. These viruses show cross-reactivity in neutralization test and other serological reactions. OHFV cause an acute disease with hemorrhagic syndrome and neurological symptoms. The virus is spread in Siberia (Russia) with sporadic cases, and there are shared foci of TBEV and OHFV in the area. Nowadays there is no any vaccine against OHFV.

In the present work we tried to evaluate the protective immunity against OHFV that is caused by immunization with inactivated vaccine against TBEV in mice and monkeys. First of all we modeled the Omsk hemorrhagic fever in BALB/c mice with hemorrhagic syndrome and neurological symptoms like paralysis. Virological and pathomorphological studies showed that two-time intramuscular vaccination of mice with TBEV vaccine protected 70% of animals from infection with $100LD_{50}$ OHFV.

The acute clinical symptoms did not registered in green monkeys (Cercopithecus aethiops) after OHFV inoculation. The OHFV infection in the monkeys was observed by clinical blood analysis and viremia, and by virus spread in CNS and viscerals after the autopsy. The two-time intramuscular vaccination with TBEV vaccine did not prevent the OHFV infection but strikingly decreased the level of virus spread and abnormal changes in blood samples. The TBEV vaccine does not prevent the OHFV infection but weakens the symptoms.

Summarizing all the facts we can conclude that TBEV vaccine could be considered only as a surrogate vaccine in urgent cases OHFV infection.

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Efficacy of Aminocaproic Acid use for Prevention of Some Infectious Diseases in Organized Collectives

V. Trihleb[1,*], V. Lozitsky[2], V. Smorgunova[1], S. Pozdnyakov[2], A. Voronkov[3]

[1] *Main Military Medical Clinical Center, Kyiv, Ukraine*

[2] *Ukrainian I.I. Mechnikov Anti-Plague Research Institute, Odesa, Ukraine*

[3] *Research and Design Institute of Chemical Technology "Chemtechnology", Severodonetsk, Ukraine*

Previously, our studies have established the participation of proteolysis in the interaction of virus with the host organism. Aminocaproic acid (ACA), an inhibitor of proteolysis slows proteolysis raising and penetration of viruses into cells. Inhibition of replication of influenza virus types A and B, parainfluenza, adenoviruses, with the ACA in different cell systems was revealed. ACA leads to the stimulation of defense reactions of organism. Application of ACA on

the model of experimental influenza is effective for intranasal, oral and parenteral methods of administration. Prophylactic efficacy of ACA was also established. Use of ACA for the prevention and treatment of influenza and acute respiratory diseases (ARDs) in children and adults was allowed in the Ukraine in 2009. With the scope to study the ACA prophylactic activity studies in organized collectives, we have prescribed it per-orally in 2.0 g dose four times a day during a week. The monitoring was performed in two independent collectives (923 young adults males aged 18–19 years old) during acute respiratory diseases appraisal. As the reference, 4 groups were taken (2 from each collective) but without E-ACA application. The obtained results shows: compare to the high morbidity rates in the reference group of the first tested collective in the basic group preventively treated with ACA, number ARDs has decreased in two times. At the same time compare to the morbidity rate growth of ARDs (by 15–27%), quinsy (by 14–21%) and pneumonia (by 6–7%) in the reference groups of the second tested collective, of the main examined group, treated with ACA, pneumonia morbidity rate has decreased up to five times, while the ARDs and quinsy levels were stabilized. The smallest number of cases of these infections in both teams surveyed recorded in the period of ACA application. The results obtained allow recommend the use of ACA for the efficient prophylactics of ARDs, quinsy and pneumonia in the organized collectives in the period of increased incidences of these infections.

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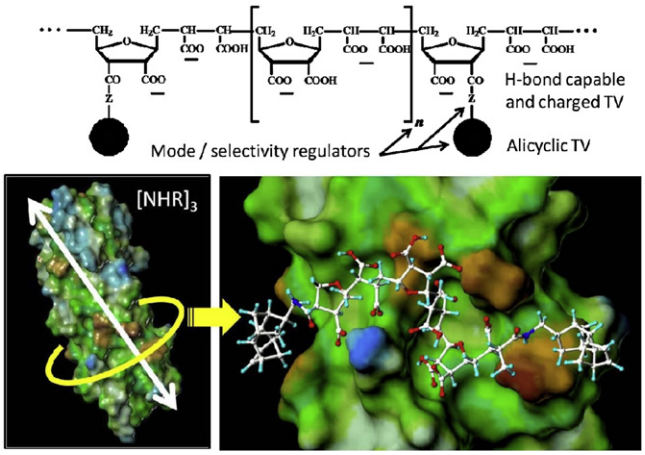
Polymer-coupled Systems for Blocking the Viral Fusion 1. Modeling *in silico* the *in vitro* HIV-1 Entry Inhibitors

V. Tsvetkov[1,2,*], A. Veselovski[2], A. Serbin[1,3]

[1] *Biomodulators RC, Health RDF, Moscow, Russia*
[2] *Inst Biomedical Chemistry, RAMS, Moscow, Russia*
[3] *Inst Petrochemical Synthesis, RAS, Moscow, Russia*

A trimer hairpin complex of viral class I fusion proteins heptade repeat regions (**NHR** – **CHR**) is a crucial trigger for fusion in entry mechanism of the human immunodeficiency virus type 1 (**HIV-1**) and of many other (*retro-*, *orthomyxo-*, *paramyxo-*, *corona-*) enveloped viruses. So an inhibition of the fusion mediators should be considered as a key drug design strategy against these viruses. In focus of the HIV/AIDS at least three traditional routes, via small-molecules (**1**), peptides (**2**), and antibodies (**3**), are developed. But all ones in principle cannot provide a drug-resistance preventing efficiency because of mono-point/site mode of action (1/2) or of inadequate adaptability (2/3) to the target mutations. Here we exploring a complementary approach based on water-soluble biocompatible synthetic polymers with flexible chain backbone, modified via targeting vectors (**TV**) designed for recognition-blocking the NHR tri-helices core of HIV-1 gp41. The polymeric substances with controlled hetero-functional TV (H-bond capable, electrostatic selective to positive Lys/Arg sites, and suitable to dock in hydrophobic pockets) were synthesized, evaluated *in vitro* for HIV-1 entry inhibition, and explored by computational docking and molecular dynamics. The TVs satisfy to point/site docking (likely the small molecules/antibodies), and H-bond-capable and charge driven polymeric chain blocks the target predominantly along the a-helices (similarly the C34/related peptides). But in contrast with the known NHR blockers the newly studied polymeric systems possess the unique adaptability to combined axial-co-belting (from pocket to pocket) multi-blockage both along and around the tri-helices complex, for example:



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Discovery and Development of Orally Active Antivirals for the Treatment of RSV: Identification of BTA9881 and a 2nd Generation Candidate

Simon Tucker[1,*], Alistair Draffan[1], Jennifer Fenner[1], Jega Iswaran[1], Angela Luttick[1], Mike McCarthy[2], Gary Pitt[1], Jane Ryan[1]

[1] *Biota Holdings Limited, Melbourne, Australia*
[2] *MedImmune Inc, Gaithersburg, USA*

Respiratory syncytial virus (RSV) is the predominant cause of acute lower respiratory tract infection (LRTI) in children. It has been estimated that in the United States 4–5 million children up to 4 years of age will develop an acute RSV LRTI annually. More than 125,000 children are admitted to hospital for RSV related illness in the United States each year. RSV infection is also a major cause of morbidity and mortality in high risk adult populations where infection rates can range from 50 to 80% depending on the underlying medical condition. Small molecule inhibitors of RSV fusion have been described by several groups. Compounds with this mechanism are thought to interfere with F-mediated fusion *via* one of two domains on the glycoprotein. The Biota RSV fusion inhibitor program encompasses several classes of inhibitors with examples advanced from the discovery stage to Phase I clinical trials. The most developed class to date is the imidazoisoindolones and examples from this series are orally bioavailable with excellent pharmacokinetic profiles in multiple species. Data will be presented to describe the characterization and development of the series from early research "hits" through preclinical development and nomination of BTA9881 as a clinical candidate. BTA9881 demonstrated excellent pharmacokinetic properties and a good safety profile at doses up to 400 mg per day in humans in a Phase I single ascending dose trial. Research has continued to identify distinct structural classes of potent and selective RSV-F inhibitors. A second generation candidate has been identified with a superior screening profile to BTA9881 and results from *in vitro* and *in vivo* studies will be discussed.

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Mechanistic Studies on a Novel Hydrophobic Derivative of Aglycoristocetin with Potent and Broad Activity Against Influenza Viruses

Evelien Vanderlinden[1,*], Els Vanstreels[1], Kurt Vermeire[1], Dirk Daelemans[1], Pal Herczegh[2], Ferenc Sztaricskai[2], Lieve Naesens[1]

[1] *Rega Institute for Medical Research, Leuven, Belgium*
[2] *Department of Pharmaceutical Chemistry, University of Debrecen, Debrecen, Hungary*

We previously reported on the in vitro anti-influenza virus activity and structure-activity relationship of hydrophobic derivatives of the glycopeptide compound aglycoristocetin [Naesens et al., Antiviral Res., 2009]. In Madin-Darby canine kidney (MDCK) cells infected with different strains of influenza A/H1N1, A/H3N2 and B viruses, the lead compound 8e displayed an antivirally effective concentration of 0.4 μM. The concentration producing 50% inhibition of cell proliferation was 67 μM, yielding an antiviral selectivity index of 167. Virus yield at 72 h p.i. was reduced by 3 logs at 5 μM 8e. Inhibition of virus replication by 8e was confirmed in A549 and Vero cells. Influenza virus fully retained its sensitivity to 8e after 11 sequential virus passages in MDCK cells in the presence of 8e (at concentrations up to 25 μM). In time-of-addition studies, 8e lost activity when added 1 h or later p.i., showing that 8e inhibits an early step in virus replication. 8e produced no inhibitory effect on binding of virus to MDCK cells at 4 °C. Inhibition of hemagglutinin (HA)-mediated membrane fusion was excluded, since 8e had no effect on virus-induced red blood cell hemolysis at low pH, nor on polykaryon formation of HA-expressing cells exposed to low pH. Besides, 8e did not inhibit the conformational change of the HA at low pH, as observed in a tryptic digestion assay. Confocal microscopy on influenza virus-infected MDCK cells stained with anti-nucleoprotein antibody at 1 h p.i., revealed that 8e causes a marked inhibition of the nuclear entry of the virus. Studies with reference compounds are ongoing to unravel precisely how 8e interferes with the influenza virus entry process. The aglycoristocetin derivative 8e represents a new class of potent and broad-acting influenza virus inhibitors with potential therapeutic relevance.

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Avoidance of Coxsackievirus Drug Resistance by Using a Novel Scheme of Combining Anti-Enteroviral Inhibitors *In Vivo*

Ralitsa Vassileva-Pencheva*, Angel S. Galabov

Department of Virology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

We present a novel scheme for combined application of anti-enteroviral substances in coxsackievirus infections in mice, which consists of a consecutive alternating, not simultaneous, administration of the substances in combination. This study summarizes the results from the experiments with two coxsackieviruses – CVB1 (neurotropic) and CVB3, as the latter is represented by two strains – cardiotropic strain Woodruff and neurotropic strain Nancy. A triple combination of enteroviral replication inhibitors showing good efficacy was selected – its effectiveness is expressed in lengthening of the mean survival time and about 50–60% reduction of mortality rate in infected mice as compared both to the placebo group, partner compounds used alone every day, and to the same combination applied simultaneously every day. Studies of the drug sensitivity of viral brain isolates from mice, treated with this combination and the combination partners indicate that virus isolates from the group treated with the alternating combination not only preserve, but even increase their sensitivity to the drugs.

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Inhibition of Enveloped Virus Infection of Cultured Cells by Valproic Acid

Angela Vázquez-Calvo[1,*], Juan-Carlos Sáiz[2], Francisco Sobrino[1], Miguel A. Martín-Acebes[2]

[1] *CBMSO, (UAM-CSIC), Spain*
[2] *Dpto, Biotecnología, INIA, Spain*

Valproic acid (VPA) is a short chain fatty acid commonly used for treatment of neurological disorders. As VPA can interfere with cellular lipid metabolism, its effect on the infection of cultured cells by viruses of seven viral families relevant to human and animal health, including eight enveloped and four non-enveloped viruses, was analyzed. VPA drastically inhibited multiplication of all the enveloped viruses tested, including the zoonotic lymphocytic choriomeningitis virus and West Nile virus (WNV), while it did not affect infection by the non-enveloped viruses assayed. VPA reduced vesicular stomatitis virus infection yield without exerting a major blockage of either viral RNA or protein synthesis. In contrast, VPA drastically abolished WNV RNA and protein synthesis, indicating that this drug can interfere at different steps of enveloped virus infection. Thus, VPA can contribute to understand crucial steps on viral maturation and to develop future strategies against infections associated with enveloped viruses.

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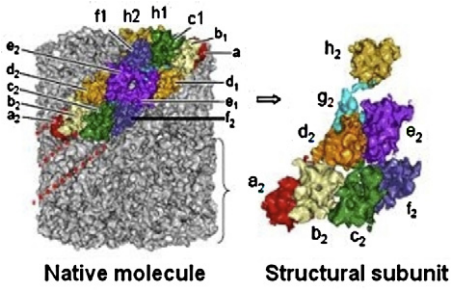
Antiviral Effect of Molluscan Haemocyanines

Lyudmila Velkova[1,*], Lubomira Nikolaeva-Glomb[2], Lucia Mukova[2], Aleksander Dolashki[1], Pavlina Dolashka[1], Angel S. Galabov[2]

[1] *Institute of Organic Chemistry with Centre of Phytochemistry 9, Sofia, Bulgaria*
[2] *The Stephan Angeloff Institute of Microbiology, Sofia, Bulgaria*

Hemocyanins (Hcs) are oxygen-binding glycoproteins, freely dissolved in the hemolymph, of many arthropods and mollusks. The structure and oligosaccharide moieties of the molluscan Hcs *Rapana venosa* and *Helix lucorum* have been determined and recently received particular interest due to their immunostimulatory properties. Hemocyanins also have been found to show antiviral activity. In the present study the antiviral effect is tested against the *in vitro* replication of human respiratory syncytial virus (hRSV) and influenza virus A/Aichi/2/68/H3N2 by the CPE-inhibition assay. The complete molecules of Hcs do not show antiviral effect. But a marked antiviral activity of the structural subunits and the functional units is found against the replication of hRSV. Their effect

against the replication of influenza A virus is weaker. The antiviral activity seems to be due to the glycosylation of the structural subunits RvH1 and RvH2 and of the functional units as well, where the carbohydrate chains are exposed on the surface of the molecule and some of the moieties can bind to viral proteins. It is assumed that the complete molecules of the hemocyanins do not possess any antiviral activity because of the fact that in this case the carbohydrate chains are buried in between the whole molecule and therefore, are unable to interact with viral proteins. The antiviral activity is present only in the case when the carbohydrate chains are exposed externally.



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Novel Inhibitors of Nuclear Translocation of HIV-1 Integrase

Kylie Wagstaff*, Stephen Rawlinson, Anna Hearps, David Jans

Monash Univ, Monash, Australia

Infectious diseases, such as those caused by HIV remain highly significant health burdens world-wide, due to a lack of effective treatments and the ability of many viruses to develop resistance to anti-viral agents. During the infectious cycle, specific viral proteins, including those from cytoplasmically replicating viruses, enter the host cell nucleus in order to perform functions essential to the viral lifecycle. A particularly intriguing example is HIV-1 integrase (IN), which plays an essential role in infection in integrating the HIV genome into that of the infected host cell. Most IN-based anti-viral compounds target IN/DNA interaction, but since IN must first enter the nucleus before it can perform these critical functions, nuclear transport of IN is also an attractive target for therapeutic intervention. Here we describe a novel screening assay (Wagstaff et al., 2005, 2010) for identifying inhibitors of protein nuclear import, based on amplified luminescent proximity homogeneous assay (AlphaScreen) technology, which is high-throughput, efficient and cost-effective. We use the assay to screen for and identify specific inhibitors of the interaction between IN and its nuclear transport receptor Importin (IMP) α/β. Importantly, we demonstrate that one of the identified compounds, Mifepristone (Mif), is effective in preventing active nuclear transport of IN in transfected cells, and hence may represent a useful anti-HIV therapeutic. The screen also identified broader-spectrum inhibitors of IMPα/β such as Ivermectin (Ive), which will be useful tools for nuclear transport research in the future. That the activity of Mif and Ive can be validated in living/infected cells underpins the utility of this novel screening approach.

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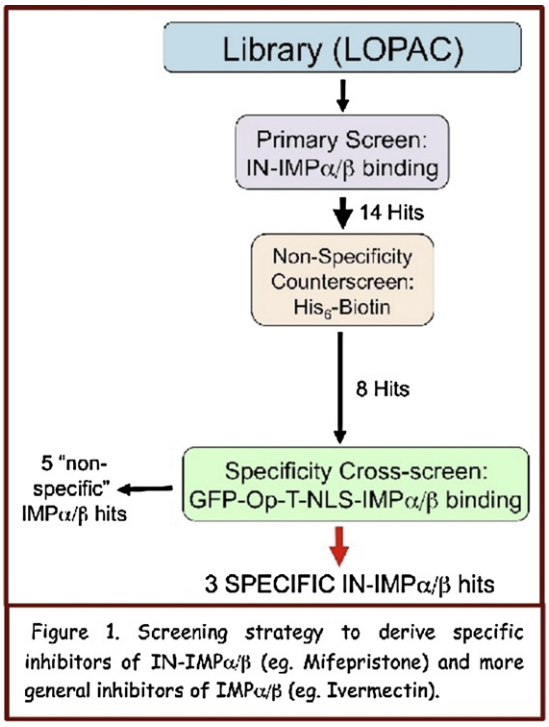

Figure 1. Screening strategy to derive specific inhibitors of IN-IMPα/β (eg. Mifepristone) and more general inhibitors of IMPα/β (eg. Ivermectin).

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Development of Antimicrobial Peptides as Topical Microbicides for the Prevention of HIV

Karen M. Watson[1,*], Ashlee D. Boczar[1], Guangshun Wang[2], Robert W. Buckheit Jr.[1]

[1] *ImQuest BioSciences Inc., Frederick, USA*
[2] *University of Nebraska, Omaha, USA*

In the absence of an effective HIV vaccine, topical microbicides represent an important strategy for preventing the sexual transmission of HIV, the predominant mode of HIV transmission worldwide. Women now account for 46% of all adults living with HIV worldwide. The dynamics of the epidemic demand the development of safe, effective and acceptable female-controlled chemical and physical barrier methods, including topical microbicides, to reduce HIV transmission. An approved vaginal microbicide does not yet exist despite extensive development efforts. Although the field of microbicides had its first success with an approved NrTI (Tenofovir) and continuing development seems to focus heavily on other approved therapeutics (Dapivirine and Maraviroc), other strategies should continue to be explored. We have identified HIV-1 and HSV-2 inhibitory mammalian antimicrobial peptides (AMPs) through extensive evaluation of diverse peptides included in a unique AMP database developed at The University of Nebraska Medical Center. Several lead peptides from the database, as well as derivative peptides rationally engineered to enhance efficacy and reduce toxicity, have been identified with significant anti-HIV-1 and anti-HSV-2 inhibitory potential. These lead AMPs have been further evaluated in microbicide-specific mechanism and range of action evaluations, including virus transmission inhibition assays as well as activity in the presence of seminal and vaginal fluids. These data would suggest that the peptides act at an early stage of replication (inactivation, entry or RT) and against all virus subtypes. We believe that antimicrobial peptides have the potential to

be developed as a component of a combination microbicide product to prevent the sexual transmission of viral, bacterial and fungal organisms.

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dsRNA binding characterization of full length recombinant wild type and mutants *Zaire ebolavirus* VP35

Luca Zinzula*, Francesca Esposito, Daniela Pala, Enzo Tramontano

Dipartimento di Scienze Applicate ai Biosistemi, University of Cagliari, Cagliari, Italy

The multifunctional VP35 protein is a major virulence factor by which the highly pathogenic Ebolaviruses (EBOVs) evade host innate immune response. VP35 is essential for EBOVs replication as a component of the viral RNA polymerase complex, it is a key participant to the nucleocapsid assembly, and it also binds to dsRNA antagonizing RIG-I like receptors antiviral signaling and, ultimately, inhibiting the host interferon (IFN) production. Insights in the VP35 dsRNA binding, ascribed to its C-terminal IFN inhibitory domain (IID), have been recently revealed through structural and functional analysis of the C-terminal truncated version of the protein. Here, we report the first biochemical characterization of the dsRNA binding of the full length, His-tagged recombinant VP35 (rVP35) of the *Zaire ebolavirus* species. For this purpose we established a new *in vitro* magnetic pull down assay, validating it for compound screening also by assessing the inhibitory ability of the auryntricarboxylic acid which showed an IC_{50} value of 50 μg/mL. Optimal biochemical parameters for dsRNA binding and K_d values for dsRNA with different length were obtained through competition binding studies. Furthermore, the dsRNA binding properties of the R305A, K309A and R312A rVP35 mutants, known for their defective dsRNA binding-mediated inhibition of the host IFN response in cell culture were assessed. Interestingly, results showed that, as compared to wild type rVP35, all three rVP35 mutants displayed a modified migration pattern in gel mobility shift assays and, when tested in the magnetic pull down assay, they displayed a significantly increase of the K_d values for dsRNA binding.

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Role of Cathepsin A and Lysosomes in the Intracellular Activation of Novel Anti-papillomavirus Agent GS-9191

Gabriel Birkus[1,*], Nilima Kutty[1], Christian Frey[1], Riri Shribata[1], Tsuifen Chou[2], Carston Wagner[2], Martin McDermott[1], Tomas Cihlar[1]

[1] *Biology Department, Gilead Sciences, Foster City, USA*
[2] *Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, USA*

GS-9191, a bis-amidate prodrug of nucleotide analogue 9-(2-phosphonylmethoxyethyl)-N^6-cyclopropyl-2,6-diaminopurine (cPrPMEDAP), was designed as a topical agent for the treatment of papillomavirus-associated proliferative disorders such as genital warts. It has been previously shown that cPrPMEDAP is deaminated to guanine nucleotide that is further metabolized to active nucleoside triphosphate analog. In this study, we investigated the mechanism of conversion of GS-9191 to cPrPMEDAP. We observed that GS-9191 is hydrolyzed in the presence of lysosomal carboxypeptidase cathepsin A (CatA) in vitro and is less efficiently metabolized in CatA-deficient fibroblasts compared to control cells. In addition, knock-down of CatA by siRNA reduced the intracellular accumulation of GS-9191 metabolites. However, intracellular CatA levels did not correlate with the susceptibility of tested cell lines to GS-9191, indicating that the CatA step is unlikely to be rate-limiting for the activation of GS-9191. Further analysis showed that upon the hydrolysis of the carboxylester bond in one of the GS-9191 amidate moieties, the unmasked carboxyl group displaces L-phenylalanine 2-methylpropyl ester from the other amidate moiety. The formed cPrPMEDAP-L-phenylalanine conjugate (cPrPMEDAP-Phe) is not metabolized by Hint1 (histidine triad nucleotide binding protein 1) phosphoramidase, but undergoes a spontaneous degradation to cPrPMEDAP in acidic pH that can be significantly enhanced by the addition of SiHa cell extract. Pre-treatment of SiHa cells with bafilomycin A or chloroquine resulted in 9-fold increase in the intracellular concentration of cPrPMEDAP-Phe metabolite and the accumulation of GS-9191 metabolites in lysosomal/endosomal fraction. Together, these observations indicate that the conversion of GS-9191 to cPrPMEDAP occurs in lysosomes via CatA-mediated ester cleavage followed by the release of cPrPMEDAP, most likely through the combination of enzyme-driven and spontaneous pH-driven hydrolysis of cPrPMEDAP-Phe intermediate.

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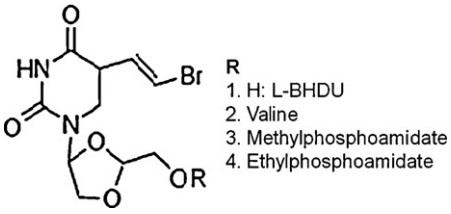
Dioxolane L-Nucleoside Analogs Prevent Varicella-Zoster Virus Replication in Fibroblasts and Skin Organ Culture

Chandrav De[1,*], Satish Chavre[2], Chung. K. Chu[2], Jennifer Moffat[1]

[1] *SUNY Upstate Medical University, Syracuse, USA*
[2] *University of Georgia, Athens, USA*

The α-herpesvirus varicella-zoster virus (VZV) causes chickenpox (varicella) and shingles (zoster). Current treatments are acyclovir and its derivatives, phosphonoformate, and brivudin (Europe only). Live, attenuated vaccines (Varivax, Zostavax) lower the incidence of primary and recurrent infections. Additional antiviral drugs with increased potency are needed, especially for resistant VZV strains and to treat post-herpetic neuralgia. We found that the bromovinyl uracil derivative (L-BHDU) was effective against VZV in culture and in a mouse model, so 3 related prodrugs were evaluated for their effects on VZV-BAC-Luc replication in HFFs and skin organ culture (SOC). Virus spread was measured by bioluminescence imaging. The ethyl- and methylphosphoamidate derivatives were similar to L-BHDU, with EC_{50} 0.1–0.3 μM in HFF at 48 hpi. In SOC, the EC_{50} of L-BHDU and the ethyl derivatives were similar (methyl not tested). The valyl derivative was most potent, with an EC_{50} of 0.038 μM in HFFs and 0.05 μM in SOC at 6 dpi. At 2 μM, these compounds did not affect HFF proliferation, and they were nontoxic up to 200 μM over 3 days. HFF cells treated with these compounds (2 μM) appeared normal and VZV plaque size was reduced. Additional tests will be conducted to evaluate these compounds against VZV strains resistant to acyclovir, their effects on viral DNA synthesis, and their effectiveness in the SCID-Hu skin implant mouse model. Overall, the results indicate that L-BHDU prodrugs, especially the valyl derivative, show promise as novel antiviral agents for treating VZV infections.



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Evaluation of Approved Antivirals for Inhibition of Xenotropic Murine Leukemia-Related Virus (XMRV) in Cell-Based Assays

Tracy L. Hartman*, Robert W. Buckheit Jr.

ImQuest Biosciences, Frederick, USA

Xenotropic murine leukemia-related virus (XMRV), a retrovirus discovered in 2006, has been controversially associated with human prostate cancer and chronic fatigue syndrome (CFS). XMRV nucleic acid or proteins are found in 27% of prostate cancers and in 68% of chronic fatigue syndrome patients, and in less than 4–6% of normal controls, suggesting an association between the virus and human disease. To date there is no effective treatment for CFS. Twenty-eight drugs approved for use in humans were evaluated against XMRV replication in vitro. Drugs used to treat HIV-1 infection, as well as compounds used to treat other virus infections, were evaluated. Published literature indicates little similarity between HIV-1 and XMRV proteins: 28% homology at the amino acid level of protease, 17% homology with RT, and 14% homology with integrase; making it difficult to predict which anti-HIV agents may be effective against XMRV. Several drugs from each major class of antiretroviral agents: nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTI, NtRTI and NNRTI), integrase inhibitors (II), and protease inhibitors (PI). An in vitro assay utilizing PG-4 cells infected with XMRV collected from 22Rv1 human prostate cancer cells was developed to measure inhibition of virus replication. Efficacy and toxicity data for the approved antiviral agents will be reported, as well as evaluation of combined antiviral agents in the anti-XMRV assay.

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The Design and Synthesis of Pyrolle-Carbaldehydes as HIV-1 Integrase Strand-Transfer Inhibitors

Raymond Hewer[1,*], Telisha Traut[1,2], Bradley Williams[2], Judy Coates[1]

[1] *Mintek, Johannesburg, South Africa*
[2] *University of Johannesburg, Johannesburg, South Africa*

In silico screening of commercial databases through an optimised integrase structure identified a pyrolle-carbaldehyde with high docking scores. Virtual derivatisation of the core structure was undertaken to optimise favourable interactions and ~100 analogues were synthesised through a facile one-pot reaction. Biological evaluation of the water-soluble HCl-salt derivatives identified a number of compounds that actively inhibited the strand-transfer step as determined through direct enzymatic assays. In particular, 10 orally bioavailable compounds proved active against HIV-1 integrase in the low micromolar range (IC_{50}'s <10 μM). Here, we investigate the binding mode of these compounds within the active site of the integrase model, discuss the cytotoxicity, solubility and inhibitory activity of select compounds and establish a preliminary structure–activity-relationship.

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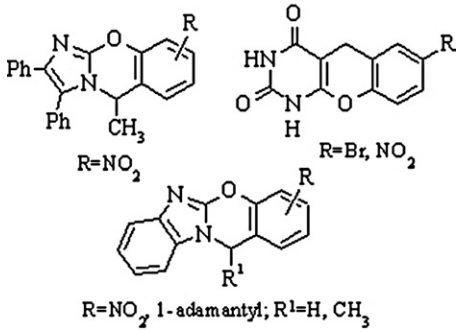
Identification and Characterization of Azolo-1,3-benzoxazines and Condensed Benzopyrans as Potent Non-nucleoside Inhibitors of Orthopoxviruses

Yuri Klimochkin[1,*], Vitalij Osyanin[1], Natalia Sidorina[1], Marina Leonova[1], Evgeny Belanov[2], Olga Serova[2], Sergey Balakhin[2], Nikolay Bormotov[2]

[1] *State Technical University, Samara, Russia*
[2] *FSRI SRC VB "Vector", Koltsovo, Russia*

The potential use of variola virus as a biological weapon has renewed efforts in the development of antiviral agents against orthopoxviruses. Additionally, there is a growing threat from zoonotic poxvirus outbreaks, particularly monkeypox, cowpox, and vaccinia, all close relatives of variola. There are still no proven effective drugs for the treatment of severe orthopoxvirus infection. Thus, further development of new antiviral agents for the control of orthopoxviruse infections is urgently needed. During our investigation we have synthesized series of azolo-annelated 1,3-benzoxazines and condensed benzopyrans. Among compounds having activity against vaccinia poxvirus (Variola vaccinia) it is necessary to note small molecular weight fused heterocycles such as 12H-benzimidazo[2,1-b][1,3]benzoxazines, 2,3-diphenyl-5H-imidazo[2,1-b][1,3]benzoxazines and 1,5-dihydro-2H-chromeno[2,3-d]pyrimidin-2,4(3H)-diones. While a majority of these compounds moderately inhibited vaccinia virus, the 12-methyl-2-nitro- and 2-(1-adamanthyl)-12H-benzimidazo[2,1-b][1,3]benzoxazines exhibited the most potent anti-orthopoxvirus activity with an IC_{50} of 0.008 mM and 0.012 mM, respectively. A novel cascade Michael addition–intramolecular nucleophilic cyclization approach based on o-quinone methide generation as intermediates has been used for construction of these compounds.

Acknowledgment: The work is supported by the FCP «Scientific and pedagogical staff of innovative Russia in 2009–2013».



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Differential Expression of Host Cellular Factors upon HIV-1 Reactivation

Paula Ordonez Suarez*, Takayuki Hamasaki, Masanori Baba, Mika Okamoto

Division of Antiviral Chemotherapy, Center for Chronic Viral Diseases, Kagoshima University, Kagoshima, Japan

The development of antiretroviral therapy (ART) has achieved almost complete suppression of HIV-1 replication in infected individuals (plasma viral load <50 copies/mL). However, integrated HIV-1 remains hidden in long-lived reservoir cells, such as latently infected $CD4^+$ T lymphocytes and monocyte/macrophages. As a consequence, infected individuals receiving ART often experience the rebound of plasma viral load at the time of discontinuation of ART. Since latent HIV-1 can be reactivated from the reservoir cells leading to viral dissemination and disease progression, a clear understanding of the mechanisms of viral latency and reactivation is a key to designing novel therapeutic agents and achieving eradication of these viral reservoirs. Recent genomic studies have identified several cellular factors associated with viral latency, suggesting that a subset of host-virus interactions is triggered during HIV-1 reactivation. In order to identify cellular factors differentially expressed upon HIV-1 reactivation, a comprehensive and wide genome expression analysis was conducted using a model of promyelocytic cells latently infected with HIV-1. Microarray expression profiles were analyzed at different time points after stimulation for inducing active viral replication in the latently infected cells. In this study, a subset of genes was differentially expressed in the stimulated cells when compared to the unstimulated cells and their uninfected parental cells (332 genes upregulated vs. 102 genes downregulated). Significant upregulation was observed for genes related to signal transduction, immune response, G-protein coupled receptor protein signaling pathway, cell–cell signaling, ion transport and cell adhesion. Our results highlight the role of a subset of host factors involved in viral latency, indicating their potential use as novel cellular targets for inhibition of HIV-1 replication and inhibitor design. Undergoing studies, including knockdown of the genes and evaluation in other cell lines and primary T lymphocytes, are aimed to elucidate the role of selected host factors in viral replication.

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Recycling of HIV Particles is Required for Infection by Endocytosed Virus after Cell to Cell Transfer

Marc Permanyer*, Ester Ballana, José A. Esté

IrsiCaixa, AIDS Research Institut, Badalona, Spain

Although it has long been assumed that HIV entry occurs by direct fusion between virus and cellular membranes at the cell surface, recent alternative evidences suggests that HIV particles may fuse with endosomal membranes, being this route the only productive entry pathway. Thus, the role of endocytosis in HIV replication and whether or not endocytic virus transfer represents an escape mechanism from the immune system or therapeutic agents remains highly controversial. Parallel cocultures, in the presence of different entry inhibitors, between HIV-1-infected MOLT NL4-3 effector cells and previously stimulated or non-stimulated primary CD4+ lymphocytes, induced the transmission of high amounts of HIV-antigen (around 20% of p24 positive T cells in all conditions, except for Leu3a which inhibited 75% of transfer in both cases). After coculture, purified trypsin-treated target cells were left in culture in different medium conditions, designed specifically to evaluate the infectious pathway taken by the captured HIV particles. Purified, trypsin-treated target cells became infected if left in culture medium. Conversely, cells did not become infected if cultured in the presence of mAb IgGb12, an agent that blocks virus attachment to CD4 (6 and 1.3-fold increase in supernatant p24 production in non- and stimulated CD4+ T cells, respectively). Importantly, similar results were obtained if the transfer of HIV particles occurred in the presence of the HIV entry inhibitors BMS155 or AMD3100. Our results suggest that HIV could not infect cells through direct fusion from within endosomal compartments and required recycling to the cell surface to initiate a productive infection. Consequently, the HIV-transmission process is presented here as an itinerant virus reservoir, capable to generate trans-infection after the release of the HIV particles to the extracellular environment. Since cell-to-cell HIV transfer is considered one of the most efficient mechanisms of HIV spread, new insights in the mechanism of HIV transfer allows the identification and characterization of novel potential targets for HIV anti-retroviral therapy.

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Inhibition of Influenza Virus-induced NF-kB and ERK Activation can Simultaneously Reduce Both, Virus Titres and Cytokine Expression *In Vitro* and *In Vivo*

Ruth Pinto[1,*], Susanne Herold[2], Lidija Cakarova[2], Katrin Hoegner[2], Jürgen Lohmeyer[2], Oliver Planz[3], Stephan Pleschka[1]

[1] *Institute of Medical Virology, Justus-Liebig-University, Giessen, Germany*
[2] *Department of Internal Medicine, University of Giessen Lung Centre, Giessen, Germany*
[3] *Friedrich-Loeffler-Institute (FLI), Tübingen, Germany*

Influenza virus (IV) infection can cause severe pneumonia and lead to acute respiratory distress syndrome and death. So far therapeutic actions are limited to vaccines and a few anti-viral drugs. These drugs target functions of the virus itself thereby selecting drug-resistant variants. During their replication IV activate ERK and the transcription factor NF-kB. Both result in pro-viral as well as anti-viral effects by promoting nuclear export of the viral genome to be packaged into progeny virions and by inducing expression of pro-inflammatory host defence factors. Apart from tissue damage caused by the lytic replication of the virus, an imbalanced overproduction of anti-viral cytokines ("cytokine burst") can lead to severe lung damage as observed in human infections with highly pathogenic avian IV (HPAIV) of the H5N1-type. Recently we have shown that inhibition of NF-kB activity reduces virus titre *in vitro* and *in vivo*. As a proof of principle we now analyzed whether it is possible to target both aforementioned pathways with specific inhibitors in order to reduce virus titres as well as virus-induced cytokines, simultaneously. We show that reduced activity of both pathways by specific inhibitors indeed leads to decreased virus titres and cytokine expression. This was not only true *in vitro* for permanent A549 cells or primary mouse alveolar epithelial cells infected with human IV or HPAIV, but also *in vivo* in IV-infected mice. Our results hereby demonstrate for the first time *in vitro* and *in vivo* that inhibition of ERK and NF-kB activity can be used to reduce virus titres and modulate pro-inflammatory cytokine expression, concurrently. This could provide new rationales of future therapeutic strategies to treat influenza virus pneumonia.

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A viable Human Influenza A Virus Lacking Neuraminidase (NA) Activity-isolation and Characterization

Martina Richter[1,*], Sandor Nietzsche[2], Elke Bogner[3], Peter Wutzler[1], Michaela Schmidtke[1]

[1] *Institute of Virology and Antiviral Therapy, Jena University Hospital, Jena, Germany*
[2] *Centre of Electron Microscopy, Jena University Hospital, Jena, Germany*
[3] *Institute of Virology, Charité University Hospital, Berlin, Germany*

NA inhibitor (NAI) genotyping of 25 human influenza A viruses (H3N2) that circulated between 2002 and 2006 in Germany revealed a mixture of full-length and defective NA genes in one of these clinical isolates. In the present study, plaque isolation and purification were applied to get clones of NA deletion mutants and wild-type virus for further characterization. Twenty plaques were randomly picked. Twelve of them were produced by viruses that contained deleted NA genes and 8 by wild-type virus. Three defective as well as three full-length clones were three times plaque purified. Sequence analysis of plaque-purified NA deletion mutants showed that the incomplete gene segment contained 3 deletions and encoded a 25 amino acid protein lacking the coding capacity for the active center of the enzyme. As expected, the defective viruses possessed no neuraminidase activity in a chemiluminescence-based NA assay in contrast to the wild-type virus. Interestingly, the NA-lacking mutant was able to undergo multiple cycles of replication in MDCK cell culture. This was proved by determination of viral titers in the supernatant and by detection of nucleoprotein in infected cells 10, 24, and 48 h p.i. In contrast to the virus expressing full-length NA, the plaque-purified isolates lacking enzymatic activity achieved ~5000-times lower viral yields and virus spread was highly restricted. Differences in virus release were further studied by electron microscopy without and with immunogold labelling. The results from plaque purification as well as RT-PCRs of the NA gene proved that a high amount of NA-deficient viruses was present in the original sample. The full-length NA segment was very faint or not detected by gel electrophoresis whereas a smaller band (~560 bp) was abundantly present. Probably, the shorter defective NA gene segment has a replicative advantage. Wild-type viruses might support the release of mutant viruses from infected cells and act as "helper" virus for spread. Further studies are needed to understand the clinical and therapeutical relevance of these findings.

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Favipiravir (T-705) Treatment of Experimental Arenaviral Infection Initiated after the Onset of Clinical Disease

Brian B. Gowen[1,*], Michelle Mendenhall[1], Andrew Russell[1], Donald F. Smee[1], Yousuke Furuta[2]

[1] *Utah State University, Logan, USA*
[2] *Toyama Chemical Company, Ltd., Toyama, Japan*

Lassa and Junín viruses are the most notable of the *Arenaviridae* family of viruses that cause viral hemorrhagic fevers. Ribavirin is the only antiviral drug indicated for the treatment of these severe arenaviral diseases, but has limited efficacy in advanced cases of disease and is associated with toxicity. To further advance preclinical development of T-705 (Favipiravir), a promising inhibitor of arenavirus infection, we have refined and characterized a model of acute arenaviral infection in outbred guinea pigs based on challenge with an adapted strain of Pichindé virus (PICV). Intraperitoneal challenge with 500 plaque-forming units of our guinea pig-adapted passage 19 strain of PICV caused a diffuse infection that was uniformly lethal, associated with fever, weight loss, thrombocytopenia, coagulopathy, and increases in serum aspartate aminotransferase (AST) concentrations. Oral favipiravir treatment (300 mg/kg/day, twice daily for 14 days) reversed disease in sick animals presenting with marked fevers and thrombocytopenia, with all animals fully recovering from PICV-induced disease even when therapy was initiated one week after the infection. Favipiravir effectively reduced viremia and serum AST levels measured during the course of infection. In addition, fever was almost immediately reduced after the initiation of treatment. Limited efficacy was observed when animals were dosed with 150 mg/kg/day or less of favipiravir. The higher dose requirement for favipiravir in the guinea pig PICV infection model compared to the hamster system is not likely due to reduced sensitivity of the p19 guinea-pig adapted strain as it was found to be equally sensitive to the inhibitory effects of favipiravir in cell culture. Further, plasma concentrations achieved following oral favipiravir administration in guinea pigs and hamsters were comparable. We hypothesize that the disparity in effective dosage may be due to less efficient conversion of T-705 to the active triphosphate form or a more rapid systemic elimination in guinea pigs.

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Poster Session 2: Hepatitis Viruses, Herpes Viruses, Pox Viruses, Other Antiviral Agents and Medicinal Chemistry *Chairs*: 4:00–6:00 pm Sofia 3 and Kyota

Identification of Alphavirus Inhibitors by Using Virus-Based Assays and a Chikungunya Replicon Cell Line

Tero Ahola[1,*], Leena Pohjala[1,2], Pasi Kaukinen[1], Age Utt[3], Margus Varjak[3], Andres Merits[3], Päivi Tammela[2]

[1] *Institute of Biotechnology, University of Helsinki, Finland*
[2] *Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, Finland*
[3] *Institute of Technology, University of Tartu, Estonia*

We have previously developed luciferase-based methods to screen for inhibitors of Semliki Forest virus (SFV) replication, and discovered novel antiviral nucleosides and betulin-derived compounds, which inhibited both SFV and Sindbis virus (Antiviral Res. 78, 215–222; J. Nat. Prod. 72, 1917–1926). Here, we extend our studies to chikungunya virus (CHKV) by developing a persistent replicon cell line. A screen of 124 natural compounds and 234 clinically approved drugs and other pharmaceutical compounds revealed inhibitors of alphavirus replication in both categories. The hit compounds included 5,7-dihydroxyflavones apigenin and naringenin, coumarins bergapten and coumarin 30 and six drug molecules with 10*H*-phenothiazinyl structure, all showing IC_{50} values against SFV in the low micromolar range. Coumarin 30 and naringenin represented the most potent compounds, with IC_{50} values 0.4 μM and 2.2 μM, respectively, when assayed against a luciferase containing SFV marker strain. The hit compounds also reduced SFV and Sindbis virus induced cytopathic effect and inhibited SFV production in virus yield experiments. A CHKV replicon was constructed containing the virus replicase proteins together with puromycin acetyltransferase, EGFP and *Renilla* luciferase marker genes, and the replicon was transfected into BHK21 cells to yield a stable cell line. A noncytopathic replication phenotype was achieved by combining nsP2 Pro718 to Gly substitution and a five

amino acid insertion within CHKV nsP2. The replicon cell line was characterized and adopted for antiviral screening in 96-well plate format. The hit compounds identified against SFV were assayed in the replicon system, and the flavonoids apigenin, chrysin, naringenin and silybin were found to suppress CHKV replicon expression levels. The stable replicon cell line developed in the course of this work is a highly useful tool in studies of CHKV replication inhibitors. Recently, we have validated the CHKV replicon cell line for screening in a fully automated 384-well format, and initiated screening of larger chemical libraries.

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New Antiviral Substances of Indoloquinoxaline and Diphenyl Nature

Georgiy V. Antonovych[1,*], Olena Bogorad-Kobelska[1], Nadia M. Zholobak[1], Sergiy A. Lyakhov[2], Maria O. Shibinska[2], Sergiy A. Andronati[2], Mykola Ya. Spivak[1]

[1] *D.K. Zabolotny Institute of Microbiology and Virology NAS of Ukraine, Kyiv, Ukraine*
[2] *O.V. Bogatsky Physicochemical Institute NAS of Ukraine, Odesa, Ukraine*

A range of newly synthesized indoloquinoxaline and diphenyl derivatives was tested for toxicological, antiviral (AV) and interferon-inducing properties in attempt to develop novel AV substances with lower toxicity, higher effectiveness and different pattern of biological properties than that of current medications. Tilorone dihydrochloride was chosen as reference substance. 6-[3-(4-Morpholine)ethyl]-6H-indolo[2,3-b]quinoxaline dihydrochloride and 4,4′-bis-[2-(diethylamino)ethoxy]diphenyl dihydrochloride, further referred to as I1 and D1, demonstrated the best properties. In vitro experiments were carried out on L929, EPT cell cultures, splenic and peritoneal ex vivo murine cells. Test substances turned to be significantly less toxic than official preparation. Maximum tolerable dose for D1 and I1, determined on L929 cells, was 5× and >37× higher than that of tilorone. This tendency repeated in acute toxicity tests on mice; both substances can be classified as low toxic. IFN-inducing activity in case of all tested cell cultures and 3 preparations was most efficient at 6 mkg/ml. Achieved IFN titers were also similar, which indicates certain common mechanisms of action. In vivo D1 stimulated much higher IFN titers in serum than tilorone, while I1 possessed similar effectiveness. Both test substances activated cellular link of immunity more intensively and differed from each other and reference drug by the cytokine profile. AV effect was observed in vitro and in vivo for both test agents. It was not exclusively mediated through IFN system, but was at least partly based upon direct AV properties against RNA and DNA containing viruses. I1 was more efficient in prophylactic application, while D1 and tilorone were active in case of therapy as well. Expressed antiviral properties, pronounced IFN-stimulating and immunomodulating potential allow us to consider both tested substances as novel antiviral drugs. Low toxicity and wide range of effective concentrations grant certain advantages over official AV drug–tilorone.

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Pegasys As A Second Line of Effective Treatment Plan for G3 Non Responders of Conventional Therapy

Binish G. Arshad[1,*], Abida Raza[2], Samina Shakeel[1], Muhammad A. Anwar[3]

[1] *Quaid-i-Azam University, Islamabad, Pakistan*
[2] *Nuclear Medicines Oncology and Radiotherapy Institute, Islamabad, Pakistan*
[3] *PAEC General Hospital, Islamabad, Pakistan*

In Pakistan most common Genotype is 3a and above 70 percent response to 24 weeks treatment plan has been observed in our previous study (Raza et al., 2010). Available treatment plans (TPs) in Pakistan are: combinational treatment of conventional interferon 3MIU plus Ribavirin 1200 mg/day for 24–48 weeks (TP-1), Pegasys 180 μg/week plus Ribavirin 1200 mg/day for 24 weeks as a first line of treatment (TP-2), Pegintron 180 μg/week plus Ribavirin 1200 mg/day for 24 weeks as a 1st line of treatment (TP-3), Pegasys 180 μg/week plus Ribavirin 1200 mg/day for 24 weeks as a 2nd line of treatment (TP-4) for non responders of TP-1. Four available treatment plans (TPs) are compared to have the comparative effectiveness against HCV genotype 3a. In this cohort study, patients were categorized into four groups as stated above. Included study subjects were 25, 25, 20 and 22 for groups 1–4 and were given treatment plans 1–4, respectively. Viral load before and after the treatment were performed on Rotorgene 3000™ Real Time PCR system using AJ Roboscreen extraction and quantification modules. Response rate was found 76, 80 and 80 and 81.8% in four treatment plans. Study data suggests that for non responders of conventional therapy, pegasys therapy will be effective as a 2nd line of treatment plan. This will also help in counciling the HCV on treatment patients to adhere to therapy.

Reference

Raza, A., et al., 2010. Therapeutic response guided Interferon (IFN) therapy among patients chronically infected with hepatitis C virus. Antiviral Research 86, A1–A78.

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Effective Treatment Plan for G3 Patients

Withdrawn

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Cost Effective Rapid Virological Response Guided Peginterferon Therapy Plan in HCV Genotype 3 Pakistani Population

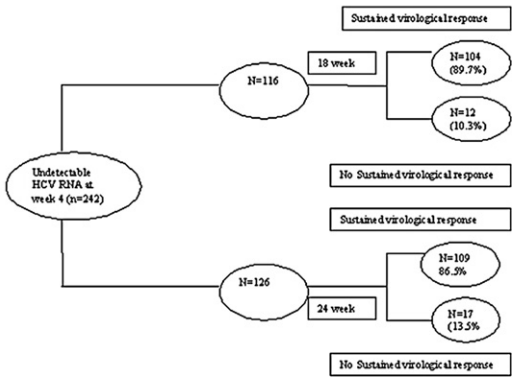
Hafsa Aziz[1,*], Abida Raza[1], Uzma Adeeb[2], Amin Athar[3], Muzaffar L. Gill[2]

[1] *Nuclear Medicines Oncology and Radiotherapy Institute, Islamabad, Pakistan*
[2] *Islamabad Specialist Clinic, Islamabad, Pakistan*
[3] *IBB Punjab University, Lahore, Pakistan*

Being poor country standard care of treatment in Pakistan against Hepatitis C is conventional interferon combination therapy. But response rate against genotype 3 has been found ~65% in our previous studies. Peginterferon therapy is highly expensive. It is important to tailor the treatment plans on individual

treatment response. In current study main objective is to assess Sustained virological response (SVR) rate with respect to 18 vs. 24 week treatment plan in RVR cases. In this retrospective study 316 seropositive genotype 3 HCV patients were selected who were receiving pegintron alfa 2a (180 μg/week) plus ribavirin (weight based) therapy. Treatment duration of these patients was individualized on the basis of week 4 virological response, defined as rapid virological response (RVR). Patients with RVR (Fig. 1) were randomized to 18 week (group A, $n = 116$) and 24 week (group B, $n = 126$). Patients with no RVR ($n = 74$) were allocated as group C and treated for 24 weeks. High SVR was observed in group A (89.7%) and group B (86.5%) as compared to group C (59.5%). High SVR was observed in both 18 vs. 24 weeks treatment (group A vs. group B) but the difference is non significant ($p = 0.31$). The difference in SVR rate in group B vs. C with 24 weeks treatment was found highly significant ($p < 0.001$). Data suggests that 18 weeks treatment of pegintron with weight based ribavirin is equally good in RVR achieving patients.

Figure 1: Predictability of sustained virologic response in 18 and 24 week treatment groups with rapid virological response after peginteron combination therapy



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Antiviral Activity of *Cymbopogon nardus* (L.) Rendle Fractions Against HSV-1

Adibah A. Bahtiar[1,*], Nazlina Ibrahim[1], Ismail Ahmad[2]

[1] *Universiti Kebangsaan Malaysia, Bangi, Malaysia*
[2] *Universiti Malaysia Sarawak, Sarawak, Malaysia*

An *in vitro* study was carried out to investigate the antiviral activity in four hexane fractions of *Cymbopogon nardus* (L.) Rendle towards HSV-1. Cytotoxicity assay was conducted towards Vero cell line using MTT assay to determine the fraction concentration that cause 50% cell death (CC_{50}). Plaque reduction and virus reduction assays were used to evaluate antiviral properties of test fractions. Two treatments were used to determine the fraction mode of action. Pre-treatment [(C + V) + A] was done by inoculating the virus (V) to the cells (C) before being treated with fraction (A). Post-treatment [(C + A) + V], involved inoculation of the cells with virus after treatment with fraction. Five different concentrations of fractions were used. HSV-1 dose was fixed at ~50 pfu/well. Cytotoxicity test showed 1.0 CC_{50} values for the four fractions ranged between 0.078 mg/ml and 0.240 mg/ml. Moderate antiviral activity was observed in both treatments, with 32.44% reduction showed by F117 in post-treatment while in pre-treatment assay, F147 showed a reduction of 42.93% (Fig. 1). Similar reduction was also observed in virus yield assay where F117 reduced virus yield by 39.43% and F147 reduced virus yield by 39.91% (Fig. 2). In conclusion, test fractions F117, F123, F147 and F166 of *C. nardus* were not cytotoxic and have moderate antiviral activity against HSV-1 as shown by plaque reduction assay. These fractions will be combined to test whether there will be an increase in antiviral activity.

Keywords: *Cymbopogon nardus*; HSV-1; Plaque reduction assay; Virus yield reduction assay

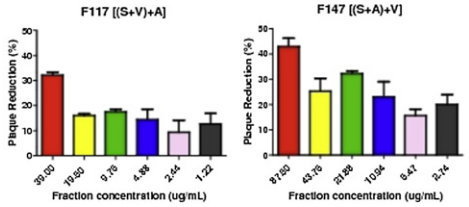

Figure 1: Percentage of plaque reduction in post-treatment [(S+V)+A] and pre-treatment assay [(S+A)+V].

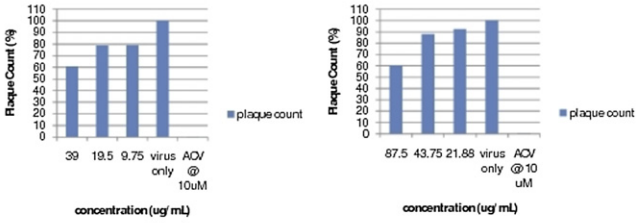

Figure 2: Effect of test fraction on virion production in virus yield reduction assay. Total virion was harvested after 48 hours incubation with test fraction and titrated to quantify total virion produced compared to control (total virion from infected cells without treatment). ACV plaque count was 0.25%.

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Amino Acid Substitutions At Residue 207 of Viral Capsid Protein 1 (VP1) Confer Pleconaril Resistance in Coxsackievirus B3 (CVB3)

Heike Braun[1,*], Vadim A. Makarov[2], Olga B. Riabova[2], Peter Wutzler[1], Michaela Schmidtke[1]

[1] *Institute of Virology and Antiviral Therapy, Jena University Hospital, Jena, Germany*
[2] *Laboratory of Biochemistry of Stresses of Microorganisms, RAS Institute of Biochemistry, Moscow, Russia*

Pleconaril binds into a hydrophobic pocket within VP1 of entero- and rhinoviruses, stabilizes the viral capsid and prevents viral adsorption and/or uncoating. Previous studies showed that pleconaril susceptibility of CVB3 correlates with the amino acid in position 1092 of the hydrophobic pocket. In the present study, nine independently derived resistant variants of the clinical isolate CVB3 97927 were isolated under pleconaril treatment in HeLa cells. Pleconaril did not inhibit the plaque production of these plaque-picked and three times plaque-purified resistant isolates at the maximum noncytotoxic drug concentration. Sequence analysis of the genome region coding for the capsid proteins VP1, VP2, VP3 und VP4 revealed substitution of isoleucine by methionine at residue 92 of VP1 in three of these resistant isolates. Six others possess a single amino acid substitution at residue 207 of VP1 ($3 \times$ Ile1207 → Arg, $3 \times$ Ile1207 → Lys) that was not described until now. To localize this newly identified resistant mutation, structure models of VP1 were generated with the Swiss–PdbViewer. The results indicate that amino acid in position 1207 belongs to the GH-loop of VP1 and is located near to the heel of the foot-shaped pocket. This loop was previously shown to undergo conformational changes during drug binding. Furthermore, the virulence of the pleconaril-sensitive CVB3 97927 and 3 of its pleconaril-

resistant variants were compared in one-step-growth experiments. The results did not reveal differences between pleconaril-sensitive and -resistant variants. Taken together, the results indicate that in addition to amino acid substitutions in position 1092 of the hydrophobic pocket, Ile1207 → Arg and Ile1207 → Lys correlate with a pleconaril-resistant phenotype. Possibly, substitution of amino acid 1207 of the GH-loop hinders conformational changes of this loop observed during drug binding and by this manner drug entry into the binding pocket or supports receptor binding and leads so to a reduced anti-CVB3 activity of pleconaril.

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Engineering Genetic Suppressor Elements against Hepatits C Virus

Zhilei Chen*, Rudo Simeno

Texas A&M University, College Station, USA

Hepatitis C virus (HCV) infection is a major public health problem, putting infected individuals (~180 million worldwide) at risk of developing cirrhosis, hepatocellular carcinoma and liver failure. The current standard interferon-based therapy for HCV cures only ~50% of patients infected with the most common genotype. In this study, we describe the selection of a new class of antivirals against HCV – genetic suppressor elements (GSEs). GSEs are protein/nucleotide sequences derived from a gene or genome of interest that act as transdominant inhibitors of a particular biological function. Using an engineered hepatoma cell line – n4mBid – that undergoes significant HCV-induced cytopathic effect and cell culture-derived HCV (HCVcc), we developed an efficient selection system for isolating anti-HCV GSEs from a library comprising a fragmented HCV genome. Target cells expressing the 4th round enrichment library showed significant resistance to HCV cytopathic effect and reduced levels of permissivity to HCV infection. These studies represent the first report of successful selection of genetic anti-HCV elements. We are currently isolating individual GSEs from the selected cell population and characterizing their specific effects on the HCV life cycle.

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3′,5′di-*O*-Trityluridine Inhibits Flavivirus (Dengue and Yellow Fever Virus) Replication and Targets the Viral RNA Dependent RNA Polymerase

Tine De Burghgraeve[1,*], Suzanne J.F. Kaptein[1], Kai Dallmeier[1], Barbara Selisko[2], Michael Jacobs[3], Bruno Canard[2], Arthur Van Aerschot[1], Johan Neyts[1]

[1] *Rega Institute, KULeuven, Leuven, Belgium*
[2] *Université de la Méditerranée, Marseille, France*
[3] *Royal Free & University College Medical School, London, United Kingdom*

The dengue fever virus (DENV) and the yellow fever virus (YFV) are members of the genus flavivirus in the family *Flaviviridae*. An estimated 50–100 million cases of DENV infections occur each year and approximately half a million patients require hospitalization. There is no vaccine or effective antiviral treatment available. There is an urgent need for potent and safe inhibitors of DENV replication; ideally such compounds should have broad-spectrum activity against flaviviruses.We report on the activity of 3′,5′di-*O*-trityluridine (DiTU) on flavivirus replication. The compound inhibits induction of DENV- and YFV-induced cytopathic effect (CPE) with the EC_{50} values in the low micromolar range. This was confirmed in virus yield reduction experiments, where dose-dependent inhibition of viral RNA synthesis was observed (DENV-2 EC_{50} = 1.2 μM; YFV-17D EC_{50} = 0.8 μM; Selectivity index > 125). Moreover, DiTU also efficiently inhibited viral protein synthesis in the same concentration range. Activity was demonstrated in DENV subgenomic replicons (which encodes only non-structural viral proteins) (EC_{50} = 3 μM) indicating that the compound inhibits intracellular events of the viral replication cycle. This observation was corroborated by the time-of-drug-addition studies, where DiTU was shown to inhibit flavivirus replication at a time point that coincides with the onset of intracellular viral RNA synthesis. DiTU efficiently inhibited highly purified DENV-2 polymerase (EC_{50} = 1.8 μM). Drug-resistant variants are currently being selected, but even following 10 passages, no such variants have so far been selected. In conclusion, our data indicate that the anti-flavivirus activity of DiTU is the result of inhibition of the viral RNA dependent RNA polymerase, that this molecule has a high barrier to resistance and that it does not act as a nucleoside analogue.

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Identification of a Novel Antiviral Drug Targeting at Host Apoptotic Responses

Linlin Gu*, Qianjun Li

University of Alabama at Birmingham, Birmingham, USA

Bluetongue virus (BTV) is a multi-layered, double-stranded RNA and the prototype virus in the genus *Orbivirus* within the *Reoviridae* family. BTV is one of the most important diseases of domestic livestock, including sheep, goat, cattle, horse and other domestic animals, with $3 billion/year loss worldwide. Recently, the re-emerging of BTV has caused a major outbreak of disease in cattle and sheep in several countries across northern and western Europe. We present the identification and characterization of a novel antiviral against BTV by targeting at the host apoptotic response. This novel small molecule antiviral compound belongs to one of the six clusters of antivirals against BTV (Li et al., 2009), identified via a high throughput screening of a 200,000 compound library. This compound showed an IC_{50} at 0.69 ± 0.15 μM, with very low cytotoxicity (CC50 > 100 μM), demonstrated that it is high selective against BTV with a Selective Index (SI_{50}) over 100. This compound also reduced the BTV plaque formation by 2–3 logs in standard plaque assay. The Time-of-Addition assay showed that this compound inhibits the late event of the BTV viral life-cycle. Mechanism of action studies indicate that this compound might interact with the host apoptotic/autophagic mechinary. The identification and characterization of a novel antiviral against BTV could be further developed a new control and prevention measure against BTV.

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New Caledonian Plants As A Source of Dengue Virus Inhibitors

Jean-Claude Guillemot[2,*], Pierre-Marie Allard[3], Paul Coulerie[1], Cecilia Eydoux[2], Françoise Gueritte[3], Edouard Hnawia[1], Canard Bruno[2], Litaudon Marc[3]

[1] *Laboratoire de chimie des substances naturelles, LIVE-UNC EA 4243, Université de la Nouvelle-Calédonie, Nouméa, New Caledonia*
[2] *AFMB-ESIL, UMR 6098, Universités d'Aix-Marseille I et II, Marseille, France*
[3] *Centre de Recherche de Gif ICSN, CNRS, Gif-sur-Yvette, France*

The dengue virus reached to epidemic proportion in all tropical and subtropical areas and even spread to some temperate regions. It is nowadays the most widespread and prevalent arthropod-borne viral disease of humans, affecting more than 50 million people each year. The virus group consists of 4 serotypes causing similar symptoms ranging from mild febrile illness to a life-threatening dengue hemorrhagic fever. Research for vaccine or antiviral treatments is now a priority for public health. Extracts of barks and leaves from different endemic plants of New Caledonia described in traditional Melanesian pharmacopeia as fever treatment, or with specific phytochemical interests were examined for their activity against dengue virus. The screening assay was made on the RNA polymerase part of NS5 enzyme, which is essential for the virus replication. This enzyme is specific of the virus and common to the four serotypes. Bioguided fractionation of the inhibitory extracts against dengue virus enzyme NS5, led us to the isolation of several pure compounds. Some of these compounds inhibit specifically the dengue polymerase, and not other viral polymerase. Furthermore, two of them are not cytotoxic and do inhibit the dengue replicon. This integrated strategy permit us to isolate new compounds with an antiviral potential.

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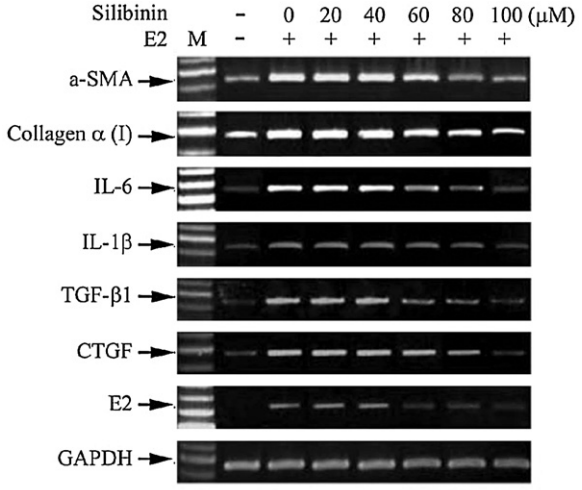
Silibinin Abolish the Enhanced Expression of Fibrosis-Related Molecules Caused by Hepatitis C Virus E2 Protein

Ming-Ju Hsieh[1,*], Yen-Chen Liu[3], Tzy-Yen Chen[2], Hui-Ling Chiou[3,4]

[1] *Institute of Biochemistry and Biotechnology, Chung Shan Medical University, Taichung, Taiwan*
[2] *Department of Internal Medicine, Chung Shan Medical University Hospital, Taichung, Taiwan*
[3] *School of Medical Laboratory and Biotechnology, Chung Shan Medical University, Taichung, Taiwan*
[4] *Department of Clinical Laboratory, Chung Shan Medical University Hospital, Taichung, Taiwan*

Chronic Hepatitis C virus (HCV) infection may lead to the liver fibrosis, cirrhosis and eventually hepatocellular carcinoma. The current standard-of-care for HCV infection is a long-term administration of pegIFNα and ribavirin with only approximately 50% response rate for genotype 1 infected patients while side effects of various severities and viral resistance may lead to treatment failure. Therefore, development of new or auxiliary remedies to improve response rate or alleviate side effects will be of great value. Our previous study indicated that E2 protein may involve in the process of hepatic fibrogenesis through an oxidative damage-related pathway. In this study, treatment with silibinin, a compounds extracted from herbs, was conducted on E2-expressing cells and RT-PCR analysis was performed to show that E2-enhanced expression of fibrosis-related molecules, including α-SMA, collagen α(I), TGF-beta1, connective tissue growth factor (CTGF), IL-6 and IL-1beta, MMP-2, were all abolished by a treatment with silibinin. Further studies demonstrated that silibinin inhibited α-SMA and collagen α(I) overexpression by regulating pathways involved Smad, p38-MAPK and AKT. Results from this study suggested that silibinin may possess inhibitory capability for the progression of liver fibrogenesis



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Antiherpes Activities of Some Medical Plants from the Lamiaceae

Kalina A. Kostova[1,*], Anton V. Hinkov[1], Stoyan A. Shishkov[1], Daniel G. Todorov[1], Milena A. Dimitrova[2], Zhenya P. Yordanowa[2], Veneta M. Kapchina-Toteva[2]

[1] *"St. Kliment Ohridski" University, Faculty of Biology, Laboratory of Virology, Sofia, Bulgaria*
[2] *"St. Kliment Ohridski" University, Faculty of Biology, Department of Plant Physiology, Sofia, Bulgaria*

Chloroform, ethanol, methanol and water extracts, derived from wild and *in vitro* propagated *Lamium album* L. and *Leonorus cardiaca* L. significantly blocked herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) replication in MDBK cells without apparent cytotoxicity. The chloroform extracts had most potent antiviral activity. The 50% effective doses (IC_{50}) of the chloroform extracts from native *L. cardiaca* were identically – 80 μg/ml. The inhibitory effects of the other extracts were similar or slight. The IC_{50} value on the in vitro extracts from *L. album* was 550 μg/ml and 467 μg/ml, respectively and on the in vivo extracts were 668 μg/ml and 780 μg/ml. The viral replications ware suppressed with 90% after addition of the chloroform extracts in maximal nontoxic concentrations (MNC). The methanol *in vitro* extract and chloroform *in vivo* extract suppressed extracellular HSV-1 above 90% – Δlog 4 and Δlog 1.5, respectively.

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Antiviral Activity of a Thioglycoside Derivative Mimicking Tunicamycin Structure

Ewelina Krol[1,*], Dawid Nidzworski[1], Grazyna Pastuch-Gawolek[2], Grzegorz Grynkiewicz[3], Wieslaw Szeja[2], Boguslaw Szewczyk[1]

[1] *University of Gdansk, Gdansk, Poland*
[2] *Silesian Technical University, Gliwice, Poland*
[3] *Pharmaceutical Institute, Warsaw, Poland*

Tunicamycin has an antiviral effect against a broad spectrum of viruses affecting the first step of N-glycosylation process. However, the therapeutic use of tunicamycin has been limited by its toxicity in animals. Recently, using classical swine fever virus (CSFV), which can cause an acute, highly infectious and economically damaging disease in swine and wild boars, we reported that tunicamycin analogues – uridine derivatives of 2-deoxy sugars, possess significant antiviral properties affecting late steps of glycosylation process. Another group of compounds mimicking tunicamycin structure was synthesized in order to investigate whether these compounds also exhibit antiviral properties against CSFV. A lead compound – a thioglycoside derivative – designed GP6 was identified as the most selective inhibitor. The antiviral activity analysis of GP6 included the study of the inhibitor on penetration and propagation of CSF virus (using plaque reduction and virus yield assay), and on maturation of viral envelope glycoproteins by immunoblotting. GP6 effectively arrested viral growth in swine kidney cells (SK6) at a 50% inhibitory concentration (IC_{50}) of 5 ± 0.12 mg/ml without significant toxicity for mammalian cells. Moreover, it reduced the formation of viral glycoproteins E2 and E^{rns} in a dose-dependent manner. We have excluded the possibility that the inhibitor acts at the replication step of virus life cycle using real time RT-PCR method. Time of drug addition studies demonstrated that virus adsorption is the main target of GP6 inhibitor. Further experiments are needed for investigating whether this compound can be used as a safe antiviral agent against other members of Flaviviridae family and other RNA viruses.

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Hepatitis E Virus (HEV) Proteome and RNA Silencing Suppressors (RSS): A Search

Amit Kumar*, Subrat K. Panda, Hemlata Durgapal

Department of Pathology, All India Institute of Medical Sciences, New Delhi, India

Background/Aims: HEV is an emerging pathogen responsible for sporadic and epidemic hepatitis. Etiological agent is a single-stranded positive sense RNA encapsidated in capsid. Viral genome replicates via negative sense intermediate. The dsRNA intermediates can trigger RNAi response against HEV. Thus, presence of RSS in the HEV genome cannot be ruled out. Here we report the absence of RSS activity in the HEV proteome.

Methods: We have designed a RNAi-RSS system. siRNAs against Firefly luciferase (Fluc) gene were designed, synthesized, converted into shRNA cassette and cloned. HEV genes encoding Methyltransferase, Helicase, Replicase, ORF2 and ORF3 proteins were cloned individually in eukaryotic expression vector. Fluc shRNAs were validated by co-transfection with target (pcDNA3-Fluc) in Huh7 cells. Inhibition was determined by dual luciferase assay at 48 h post-transfection. Suppressor assay was performed by co-transfecting Fluc shRNA along with target and individual viral protein expressing constructs into Huh7 cells. shRNA against Enterovirus 70 was used as unrelated control. Empty vector transfected along with target was used as reference control. Flock house Virus (FHV) protein B2 was used as a positive control for silencing suppression. HEV protein which shows any RSS activity was further evaluated as enhancer of HEV replication in the presence of pre-validated anti-HEV shRNA. HEV RNA was co-transfected with individual HEV protein expressing vector and shRNA designed against 3′ NCR of the virus. Using Real Time PCR, post 48 h transfection, RSS activity of viral protein was determined.

Results: Methyltransferase, Helicase, Replicase and pORF2 proteins did not show any RSS activity in dual luciferase RSS assay. pORF3 gave a strong RSS activity comparable with that of FHV B2 protein. But when HEV genome was targeted with pre-tested shRNA against HEV along with pSG1-ORF3, no enhancement effect was observed as demonstrated by Real Time PCR.

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Investigation of the Effects on Early Secretory Pathway in Cultured Cells and Potential Application of Antiviral Substances

V. Levakova*, A.S. Galabov

Department of Virology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

Early secretory pathway is a dynamic place where microtubule motor proteins play key role in the transport of cargo. Previous study characterised the activity of two main microtubule motors dynein and kinesin within the distance between endoplasmic reticulum (ER) and Golgi apparatus using fluorescence microscopy. Our present study is focused on the anterograde transport from ER towards Golgi membranes. We have described dynein association to membraned along microtubule filaments and we are now interested to explorer further its associated traffic events along microtubules. Using fluorescent antibodies we labelled membranes from the early secretory pathway to characterise p115 ERGIC membrane activator in Hela cells which were treated or nontreated with *E. coli* O157:H-. At the first step we detected alterations in p115 labelling using CLSM. We want to introduce as a next step some antiviral substances routinely used in our laboratory to elucidate possible effects on membrane movement in the anterograde transport and characterize Golgi/ERGIC membrane morphology in tissue culture cells. This research will provide prospectives to apply our approach in investigation of viral infections and other pathogen causative agents.

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Virucidal Activity of Calluses' Extracts from Tobacco-Plants

V. Lozitsky*, A. Fedchuk, T. Grydina, L. Mudryk, L. Shitikova, L. Socheslo

Ukrainian I.I. Mechnikov Anti-Plague Research Institute, Odesa, Ukraine

Background: Plants are a rich source of substances with antiviral and/or virucidal activity. We have previously founded antiviral effect of protease inhibitors from soy beans, haricot, potatoes and of powder from wheat germ.

Objectives: We have studied virucidal activity of calluses' extracts from tobacco-plants in this research.

Methods: Freeze-dried powder were obtained from calluses. Extracts of them were prepared at the rate of 100 mg to 9.9 ml

of special medium. The extracts were clarified by centrifugation, and determined their virucidal activity. Influenza virus A/Hong Kong/1/68 (H3N2) was diluted with a special medium containing (experiment) and not containing the calluses' extract (control) to a concentration of 10,000 LD_{50}. Control and experimental samples were incubated at 4 °C for 20 h and then at a temperature of 37 °C for 4 h. Number of infectious virus in the samples was determined by titration on fragments of chorio-allantoic membranes of 12–14 days old chick embryos.

Results: Held on 3 experiments with each of the extracts. LD_{50} in control was 4 lg. The highest levels of the virucidal activity had calluses' extracts from *Nicotiana suaveolens* and *Nicotiana alata* (distinctions from control were 3.2 and 2.3 lg LD50 accordingly). Calluses' extracts from *Nicotiana pauciflora* and *Nicotiana goodspedii* did not demonstrated virucidal activity. Virucidal activities of calluses' extracts from *Nicotiana exelsior*, *Nicotiana rustica* and *Nicotiana trigonophylla* were highly expressed (distinctions from control were from 1.5 to 1.9 lg LD_{50}).

Conclusion: Calluses' extracts from different tobacco-plants have wide spectrum of virucidal activities levels.

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Impact of HIV Coinfection on State of Immunology of Patients with Chronic HCV-Infection

Natallia V. Matsieuskaya*, Vladimir M. Tsyrkunov

Medical University, Grodno, Belarus

Aim of study: to evaluate features of T-cellular immune response and associated cytokine profile in HIV/HCV-infected patients according to clinical and immunological stage of HIV-infection.

Materials and methods: Serum levels of the cytokines (interleukin-2 (IL-2), IL-4, IL-8, IL-6, tumor necrosis factor α (TNF-α), γ-interferon (IFN-γ), IL-10) and CD4+, CD8+ Ò-lymphocytes of blood have been investigated by ELISA method (DRG International, Inc., USA) and flow cytometry (Becton Dickinson facs caliber, USA) in 3 groups of patients. 1 group – 33 patients with HIV/HCV coinfection on 1 and 2 clinical stages of HIV-infection (WHO clinical classification, 2006); 2 – 10 patients with HIV/HCV on the 3 and 4 stages of HIV-infection 3 group – 25 patients with HCV-infection. Control group included 20 health people.

Results: We have detected that in the first group of patients hyperstimulation of immunity system took place: these patients had significantly higher level of IL-8 (medians, Mann–Whitney *U*-Test) (170 pg/ml vs. 110 pg/ml, $p < 0.02$), IL-2 (2.4 and 1.15 U/ml, $p < 0.02$), INF-γ (1.5 IU/ml vs. 1.15 IU/ml, $p < 0.05$), IL-4 (125.4 pg/ml vs. 85 pg/ml, $p < 0.05$) and CD8+ lymphocytes number (926 ± 579.8 vs. 450.3 ± 264.7, $p < 0.01$) in comparison with HCV-infected patients. Progression of immunosuppression and development of 3rd and 4th stages of HIV-infection has been associated with fast decreasing of cellular immunity response, shift to Th2-type immunity response, activation of systemic inflammatory response because of manifestation of opportunistic infections.

Conclusion: On early stages of HIV-infection immunity control under HCV in HIV/HCV coinfected patients is more strong in comparison with mono HCV-infection. Study of immunopathogenesis of HCV/HIV coinfection may be a key for new approaches in elaboration of antiviral treatment of HCV-infection.

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Styrylpyrone Derivative of *Goniothalamus umbrosus* Inhibit HSV-1 Infection During Viral Early Replication Cycle

Norefrina S. Md Nor*, Nazlina Ibrahim

Universiti Kebangsaan Malaysia, Bangi, Malaysia

We have observed a potent HSV-1 antiviral activity by Styrylpyrone derivative (SPD) extracted from the root of *Goniothalamus umbrosus.* In the virus yield reduction assay, we observed reduction of virus yield after 48 h of treatment with SPD, with effective concentration (EC_{50}) were determined at 2.290μM in post-treatment study. Significant 100-fold reduction of virus plaque forming unit were observed when infected cell treated with SPD at the concentration of 12.5 μM. We investigated the possibility of antiviral activity being retained with lower concentrations of SPD. We found 16-fold and 4-fold reduction when infected Vero cell were treated with 7.5 μM and 5 μM respectively. In plaque reduction assay, more than 95 percent of HSV-1 plaque successfully reduced with treatment of 12.5 μM of SPD, confirming the antiviral activity exhibited by SPD. Furthermore, in the time dependent study, more than 75 percent reduction observed when SPD were administered at 2 h post-infection and the reduction percentage then dropped with the delay of the treatment time. The time-dependent activity may have suggested inhibition of viral early replication cycle. However, time removal experiment showed that 75 percent of reduction could only be observed after 10 hours post-treatment with SPD (results not shown). Thus, this might indicate longer time is needed for the adsorption of SPD into the cell before it can react. In this regards, the earlier treatment being administered to infected cells, the higher the chances for SPD to react at the intended HSV-1 cycle during infection. On the other hand, SPD mode of action might actually target the later time-point in HSV-1 virus cycle.

Keywords: HSV-1; Styrylpyrone derivative; Viral inhibitory activity

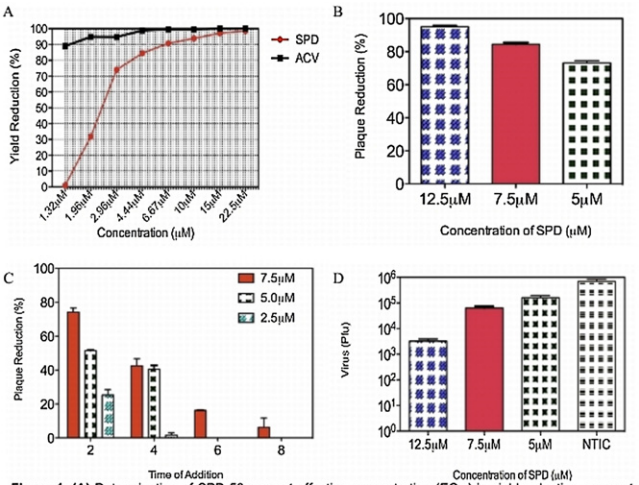

Figure 1. (A) Determination of SPD 50 percent effective concentration (EC_{50}) in yield reduction assay at 2.290μM.**(B)** Determination of SPD antiviral activity in virus plaque reduction assay.**(C)** Determination on effect of different time of treatment additions on HSV-1 plaque reduction at different SPD concentrations.**(D)**Titration of virus yield expressed as plaque forming unit (pfu) when infected cells were treated at different doses compared to non-treated infected cells (NTIC).

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Hepatitis C Virus Vaccine Candidates from Chimeric Hepatitis B Core Virus-like Particles Carrying Different Fragments of HCV Non-structural Protein 3

Marija Mihailova[1,*], Anastasija Dovbenko[1], Janis Bogans[1], Andreas Walker[2], Velta Ose[1], Irina Sominskaya[1], Sergei Viazov[2], Pauls Pumpens[1]

[1] *Latvian Biomedical Research and Study Centre, Riga, Latvia*
[2] *Institute of Virology, Essen University Hospital, Essen, Germany*

Hepatitis C virus (HCV) persists in up to 85% of infected individuals as a chronic infection characterized by liver infiltration of inflammatory cells that can lead to fibrosis, cirrhosis and hepatocellular carcinoma. Chronic HCV infection results from weak or absent T cell responses. Pegulated-interferon-alpha (INF-α) and ribavirin, the standard of care for chronic HCV, have numerous immune effects but are not potent T cell activators. A potent immune activator such as TLR9 agonist CpG oligonucleotide (CpG) may help current treatment approaches. It was shown that vigorous T helper and cytotoxic T cell response to nonstructural protein 3 (NS3) of HCV plays significant role in the clearance of the virus. Therefore the aim of this study was to create unique type of HCV immunogen capable of induction of specific HCV cellular immune response. Chimeric virus-like particles bearing different NS3 regions, containing several CD4+ and CD8+ epitopes, were created on the basis of hepatitits B virus core protein (HBc). To enhance the immunogenicity of these chimeric capsids, immunostimulatory CpG oligonucleotides were packaged into the particles. Such type of HCV immunogen could serve as effective vaccine candidate.

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The Crimean Congo Hemorrhagic Fever European Consortium: Modern Approaches to Diagnostics, Epidemiology, Prevention, Therapy and Preparedness

Ali Mirazimi[1,*], Jerome Weinbach[2], Manfred Weidmann[3], Anna Papa-Konidari[4], Peter Leyssen[5], Gülay Korukluoğlu[6], Friedemann Weber[7]

[1] *Swedish Institute for Infectious Disease Control, Stockholm, Sweden*
[2] *Inserm-Transfert, Paris, France*
[3] *Universitaetsmedizin Goettingen, Goettingen, Germany*
[4] *Aristotelio Panepistimio Thessalonikis, Thessalonikis, Greece*
[5] *Katholieke Universiteit Leuven, Leuven, Belgium*
[6] *Refik Saydam National Public Health Agency, Ankara, Turkey*
[7] *Philipps Universität Marburg, Marburg, Germany*

Large outbreaks of Crimean Congo Hemorrhagic fever virus (CCHFV) in several European countries and neighbouring areas are on the rise. To date, there is no vaccine available and no selective antiviral drug for the management of the disease. The general knowledge of migration, epidemiology, re-assortment and recombination of the virus is very limited. To fill these gaps, the CCH Fever project proposes to create a multidisciplinary collaborative research environment by bringing together selected competitive advantages such as: operative capacity with appropriate high security research facilities, reference centers and clinical samples from endemic areas and an international network of e xperienced researchers. This multidisciplinary research consortium will facilitate the progress in several key research areas of the field.

This program will mainly focus on (i) developing sensitive and biosafe state-of-art diagnostic tools for CCHFV, (ii) gathering the forces and resources in Europe to build a Biobank of clinical samples, (iii) building a comprehensive database consisting in clinical, laboratory and surveillance data, (iv) taking advantage of unique and state-of art tools to progress towards vaccine candidates and specific antivirals against this bio-treat and (v) disseminating the appropriate knowledge to the health care workers in endemic regions.

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Nitazoxanide is an Indirect Inhibitor of HCV Replication Through Modulation of Cellular Kinase CKI Alpha to Enhance HCV NS5A Hyperphosphorylation

Abigail Montero*, Prasanth Viswanathan, Chansuek Yon, Brent Korba

Georgetown University Medical Center, Washington, USA

Nitazoxanide (NTZ, Alinia®, Romark Laboratories, LC) is a licensed thiazolide anti-infective that is currently in advanced stages of clinical development for the treatment of chronic hepatitis C. Previous studies demonstrated that NTZ and its active metabolite, tizoxanide (TIZ), exhibit potent antiviral activity against multiple genotypes of HCV in cell culture, but the antiviral mechanism has remained undetermined. The current investigation sought to provide support for an antiviral mechanism of action. HCV replicon cell lines were treated with NTZ, and the levels and status of viral and specific cellular proteins were followed by Western blot, immunoprecipitation, and *in vitro* assays for enzymatic activities. TIZ was inactive against HCV polymerase, protease, and helicase in enzymatic assays. The overall rate of reduction of HCV proteins in NTZ-treated HCV replicon cell lines was consistent with loss of viral RNA template. Intracellular membrane preparations (where replication of HCV is localized) revealed that 48–72 h of NTZ-treatment induced a 4–6-fold enhancement of hyperphosphorylated HCV NS5A (p58), and a similar reduction in the levels of basally phosphorylated NS5A (p56). The phosphorylation state of NS5A is established as a regulator of the switch from active viral genome replication to packaging and assembly; overproduction of p58 is known to inhibit HCV replication. Casein kinase I-alpha (CKIa), is the cellular kinase responsible for conversion of HCV NS5A p56 to p58. CKI activity in intracellular membrane preparations from NTZ-treated HCV replicon cells was 3–4-fold higher than those from untreated cells, as measured in enzymatic assays. However, TIZ had no direct effect on purified CKIa activity in enzymatic assays, including autophosphorylation. These data provide a primary antiviral mode of action for NTZ against HCV: overproduction of the hyper-phosphorylated form of HCV NS5A, associated with enhancement of the cellular enzyme activity responsible for NS5A hyperphosphorylation. Since TIZ appears to have no direct effect on CK1a in enzymatic assays, we hypothesize the primary cellular target for TIZ is a protein involved in the upstream regulation of CK1a.

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Adenosine Deaminase-like Protein 1 (ADAL1) Catalyzes Removal of Different Alkyl Groups from N^6- or O^6-substituted Purine or 2-Aminopurine Nucleoside Monophosphates

Eisuke Murakami*, Haiying Bao, Ralph Mosely, Jinfa Du, Michael J. Sofia, Phillip A. Furman

Pharmasset, Inc, Princeton, USA

N^6-Methyl-AMP/dAMP aminohydrolase has been shown to be involved in the metabolism of pharmacologically important N^6-substituted purine nucleosides and 5′-monophosphate prodrugs thereof. Such compounds include abacavir, an approved anti-HIV agent, and GS-9219, a cytotoxic agent that targets lymphoid cells that is currently in early phase clinical trials. Currently, there are several O^6-substituted guanosine-5′-monophosphate prodrugs in clinical or preclinical development as anti-HCV agents including PSI-352938, a 3′,5′-cyclic phosphate prodrug with an O^6-ethyl substitution on the guanine and PSI-353661 and INX-189 phosphoramidate prodrugs containing O^6-methyl guanine. These compounds require removal of the O^6-alkyl group from the guanine base prior to metabolism to the active 5′-triphosphate. Therefore we assessed the ability of purified recombinant human N^6-methyl-AMP aminohydrolase to use O^6-substituted purine 5′-monophosphates as substrates. Human N^6-methyl-AMP/dAMP aminohydrolase was cloned, using primers described by Schinkmanova et al. (Collect. Czech. Chem. Commun., 2008), and overexpressed in *E. coli*. Mass spectroscopic analysis followed by amino acid sequence analyses indicated that the protein was adenosine deaminase like protein isoform 1 (ADAL1). Furthermore, activity and molecular weight profiles indicated that N^6-methyl-AMP/dAMP aminohydrolase and ADAL1 were indeed the same enzyme. An extensive structure-activity relationship study showed that ADAL1 was able to catalyze removal of different alkyl groups from not only N^6-substituted purine or 2-aminopurine nucleoside monophosphates but also from O^6-substituted purine nucleotides. The ADAL1 activity was susceptible to modifications in the phosphate moiety but not to changes in the sugar moiety. Overall, our data indicated that ADAL1 specifically acts at the 6-position of purine and 2-aminopurine nucleoside monophosphates.

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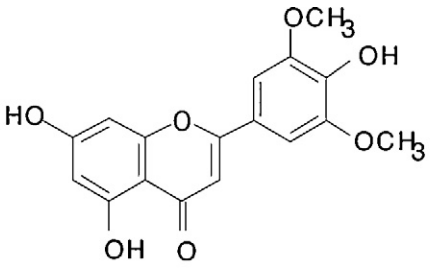
Inhibition of Human Cytomegalovirus Replication by Tricin (4′,5,7-Trihydroxy-3′,5′-dimethoxyflavone)

Tsugiya Murayama[1,*], Ying Li[1,2], Rie Yamada[1], Hidetaka Sadanari[1], Keiko Matsubara[1], Yuuzo Tuchida[2], Mamoru Koketsu[3], Kunitomo Watanabe[3]

[1] *Hokuriku University, Kanazawa, Japan*
[2] *Hououdou Co. Ltd., Shinagawa, Japan*
[3] *Gifu University, Gifu, Japan*

Human cytomegalovirus (HCMV) persists as a lifelong latent infection. However, HCMV is frequently activated in immunocompromised individuals, such as patients with AIDS or organ transplants, thereby causing severe morbidity and eventual mortality. Symptomatic HCMV infection has been treated with ganciclovir (GCV), but the appearance of GCV-resistant viruses is a recurrent problem in the treatment of immunocompromised patients with HCMV infection. Although PFA and CDV have been used in combination with GCV for the treatment of GCV-resistant HCMV, these treatments are not always successful. Therefore, effective new anti-HCMV agents and regimens need to be developed.

In this study, we show that the tricin (4′,5,7-trihydroxy-3′,5′-dimethoxyflavone), a derivative from Sasa albo-marginata, have anti-HCMV properties in the human embryonic fibroblast cell line MRC-5. On plaque assay, tricin showed dose-dependent inhibitory properties from 0.05 to 1.2 μM, but tricin had no virucidal effects on cell-free HCMV. Treatment with tricin 1 h before, or 1 h or 3 h after viral infection significantly suppressed HCMV replication. Moreover, tricin inhibited the expression of immediate early (IE) mRNA and DNA polymerase (UL54) mRNA in HCMV-infected cells. Western blot analysis also demonstrated that tricin decreased the expression of IE antigen (especially IE2) and cyclooxygenase 2 (COX-2) expression in HCMV-infected cells. In the presence of tricin, prostaglandin E2 (PGE_2) protein accumulation by HCMV infection was completely inhibited. These results suggest that tricin is a novel compound with potential COX inhibitor-dependent anti-HCMV activity.



Structure of tricin (4', 5, 7-trihydroxy-3', 5'-dimethoxyflavone)

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Identification of Bicyclic Sulfone Inhibitors of HHV-6 Targeting the HHV-6 U77 Helicase

Lieve Naesens[1,*], Graciela Andrei[1], Robert Snoeck[1], Daniel Gerry[2], Joseph E. Banning[2], Phillip D. Wilkerson[2], Zoe M. Renew[2], Chad E. Stephens[2]

[1] *Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium*
[2] *Department of Chemistry and Physics, Augusta State University, Augusta, USA*

We previously reported on the promising anti-HHV-6 activity of bicyclic sulfone derivatives (Naesens et al., 2006. Antiviral Res. 72, 60). We now examined the structure-activity relationship of a series of newly synthesized structural analogues. Their anti-HHV-6-activity was determined in HHV-6A (GS)-infected HSB-2 cells and HHV-6B (Z29)-infected MOLT-3 human T-lymphoblast cells, using a microscopic CPE reduction assay and real-time PCR quantitation of viral DNA. Some of the novel compounds were superior to the original compound, both in antiviral activity and selectivity. This strong inhibitory effect on HHV-6 replication was confirmed in HHV-6-infected fresh human cord blood lymphocytes. In order to identify the antiviral target, a resistance study was performed in which HHV-6A (GS) was serially passed in the presence of increasing concentrations of one bicyclic sulfone compound. After eight virus passages, a mutant virus was obtained with strong resistance to the bicyclic sulfones (antiviral EC_{50} values >300 μM), while its sensitivity to foscarnet and cidofovir was the same as that of control virus. DNA sequencing on the resistant virus revealed an isoleucine to methionine substitution at position 318 of the HHV-6 U77 helicase. Protein alignment showed that the Ile-318 residue in HHV-6 U77 is identical in both HHV-6 A and B variants and lies adjacent to motif IV, which is highly conserved among the herpesviruses and is required for DNA helicase activity. The I318M substitution selected by the bicyclic sulfones lies in an HHV-6 U77 region that aligns

with a sequence of HSV-1 UL5 helicase that contains most resistance mutations to herpes simplex virus (HSV) helicase-primase inhibitors, several of which are in (pre)clinical testing. The finding that these bicyclic sulfones act as HHV-6 helicase inhibitors opens new perspectives for the development of HHV-6 specific antiviral compounds.

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Antiviral Effect of Oxoglaucine in Combination with Some Enterovirus Replication Inhibitors

Lubomira Nikolaeva-Glomb*, Adelina Stoyanova, Anna Metodieva, Angel S. Galabov

The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

Despite the fact that enteroviruses are ubiquitous human pathogens, up to date there is no specific antienteroviral drug available. A clear need exists for the development of effective inhibitors of enterovirus replication, as well as for different approaches to enhance the effect of the existing ones. Recently in our laboratory the antienteroviral effect of oxoglaucine *in vitro* was described. In the present study an attempt is made to enhance the activity of oxoglaucine by combining it in dual concomitant combinations with other inhibitors of enterovirus replication, i.e. disoxaril, 2-(alpha-hydroxybenzyl)benzimidazole (HBB), guanidine-hydrochloride, 2-(3,4-dichlorophenoxy)-5-nitrobenzonitrile (DNB) and ribiavirin. The effect of the dual combinations was studied as regards the *in vitro* replication of poliovirus 1, strain LSc-2ab) and coxsackievirus B1. The combined effect character was analyzed by the three-dimensional model for evaluating drug interactions proposed by Prichard and Shipman. The combinations of oxoglaucine were additive or synergistic ones with the exception of those with ribaviin. The latter were mildly antagonistic. The highest volume of synergy was observed when oxoglaucine was combined with DNB. The same antiviral effect was achieved with doses much lesser than the necessary ones if drugs were applied alone. The synergy contributed to the higher selectivity of the combination. Greater synergy was usually observed against the replication of poliovirus 1 in comparison to coxsackievirus B1. In conclusion, combining oxoglaucine with other enterovirus inhibitors expectedly lead to enhanced reduction of virus replication and decrease of toxicity. The most promising synergistic combination in this respect was the dual combination of oxogaucine and DNB.

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Antiadenoviral Assay, Based on the Quantitative Detection of Infected Cells Containing Virus-induced Intranuclear Inclusion Bodies

Lidiya N. Nosach*

Institute of Microbiology and Virology NASU, Kyiv, Ukraine

Activity of compounds against adenoviruses (AdV) usually is determined using indirect tests of AdV replication: the cytopathic effect (CPE), plaque formation or the viability of infected cells. All these assays are based on the development of degenerative changes in infected cells in the multiple cycle of AdV reproduction. CPE is induce penton and it may take place at incomplete AdV reproduction when structural AdV proteins are synthesis abundant but the formation of infectious virus to is absent. Development of CPE at complete reproduction cycle is accompanied by the accumulation of viral DNA, proteins and virions in the nucleus and the formation DNA-containing intranuclear inclusion bodies. In order to the accurate estimation of antiadenoviral activity we recommend to use method based on the quantitative detection of number infective cells containing AdV-induced intranuclear inclusion bodies. The inclusions were revealed by luminescent microscopy using the acridine orange. Previously optimal conditions of infection must been determined. The virus control must have nearly 80% cells with inclusions at 48 h p.i. We determined the efficacy of some antiviral compounds against AdV type 1 by with method in a comparison with imunofluorescence assay to reveal the number of cells producing the hexon antigen. Both test showed dose-dependent antiviral effect of ribavirin, 6-azacytidine and 6-azauridine and the correlation of received values. However, the reveal of infected cells with intranuclear virus-induced inclusion bodies is easy to perform, rapid and eliminate the need labeled reagents. EC_{50} was calculated as a concentration decreasing the percentage of inclusion-positive or hexon-positive cells on 50% in comparison to control. Ribavirin had EC_{50} nearly 32 μM. EC_{50} of 6-azacytidine and 6-azauridine ranged between 4 and 8 μM. This method may by used to determine more precisely antiadenoviral activity of compounds witch were showed effect in indirect test of AdV replication.

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Triterpenoids from *Platycodon grandiflorum* Inhibit Hepatitis C Virus Replication

Sang Jin Park[1,*], Jong Hwan Lim[1], Jae Won Yang[1], Jung Cheul Shin[1], Eun-Joo Kim[4], Joo Won Suh[2], Soon B. Hwang[3], Jong Woo Kim[1,2]

[1] *Laboratory of Infectious Disease, B&C Biopharm Co. Ltd., Suwon, Republic of Korea*
[2] *Division of Bioscience and Bioinformatics, Myongji University, Yongin, Republic of Korea*
[3] *Ilsong Institute of Life Science, Hallym University, Anyang, Republic of Korea*
[4] *Department of Pharmacology, Korea Institute of Toxicology, Korea Research Institute of Chemical Technology, Daejeon, Republic of Korea*

Hepatitis C virus (HCV) afflicts approximately 200 million people worldwide. Currently there are no directly acting antiviral agents available to cure HCV patients. In this study, while searching for new anti-HCV agents from natural products, we found a potent inhibitor from extracts of *Platycodon grandiflorum* (PG) that inhibited HCV RNA replication. PG has been known to possess anti-allergic, neuro-protective, anti-obesity, immune regulative and anti-inflammatory activities. However, whether PG has an anti-HCV therapeutic activity has not yet been investigated. We found that PG-extracts efficiently inhibited RNA replication in Huh7 cells harboring HCV replicon. Furthermore, six triterpenoids (PD, PD_2, PD_3, DPD, DPD_2, and PA) were identified as active components involved in anti-HCV activity. Each of these components directly inhibited RNA-dependent RNA polymerase activity of HCV NS5B protein. Our computational molecular modeling data showed that these triterpenoids bound near the active site of HCV polymerase. Importantly, these triterpenoids exerted synergistic antiviral activity in combination either with IFN-α or NS5A inhibitor (BMS 790052), or with HCV protease inhibitor (Danoprevir: ITMN-191). Moreover, we also found that triterpenoids of PG provided good

hepatoprotective effect. In conclusion, triterpenoids extracted from PG efficiently suppressed HCV replication in HCV replicon system and could be a novel natural anti-HCV therapeutic agent.

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Combinatorial Anti-arenaviral Therapy with the Small Molecule SKI-1/S1P Inhibitor PF-429242 and Ribavirin

Antonella Pasquato [1,*], Cylia Rochat [1], Juan Carlos de la Torre [2], Stefan Kunz [1]

[1] *University Hospital Center and University of Lausanne, Lausanne, Switzerland*
[2] *The Scripps Research Institute, La Jolla, USA*

Arenaviruses are enveloped negative strand viruses that cause acute and chronic infections. Several Arenaviruses can cause severe hemorrhagic fever in humans. In West Africa Lassa virus causes several hundred thousand infections per year, while Junin, Machupo, Guanarito, and Sabia virus have emerged in South America. So far, only one drug is licensed against arenaviruses, the nucleoside analogue Ribavirin (Rib), which is effective when given early in disease, but shows only minor therapeutic effects in late stages of the infection. Previous works demonstrated that processing of the arenavirus glycoprotein precursor (GPC) by the cellular proprotein convertase site 1 protease (S1P), also known as subtilisin-kexin-isozyme 1 (SKI-1), is crucial for cell-to-cell propagation of infection and production of infectious virus. Recently, the SKI-1/S1P inhibitor PF-429242 was shown to inhibit Old World arenavirus GPC processing, cell-to-cell propagation, and infectious virus production. In the present study, we assessed the activity of PF-429242 against processing of the GPCs of the genetically and structurally more distant New World arenaviruses and found potent inhibition of processing of the GPCs of Junin, Machupo, and Guanarito virus. Using the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV), we studied the potency of PF-429242 in the context of acute and chronic infection. In line with published data, PF-429242 potently inhibited acute LCMV infection. PF-429242 was also highly active against chronic infection and drug treatment resulted in rapid extinction of the virus without emergence of drug-resistant variants. In a combinatorial drug approach, we found that PF-429242 potentiated the anti-viral effect of Rib in treatment of acute and chronic infection. Taken together, we showed that the SKI-1/S1P inhibitor PF-429242 is broadly active against GPC processing of all major human pathogenic arenaviruses. Apart from being potent in acute infection, the drug is remarkably active in clearing chronic infection and potentiated the anti-arenaviral activity of Rib.

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Generation of dsRNAs Targeting VP1 and VP3 Gene Regions of Coxsackievirus B1 Utilizing Bacteriophage ϕ6 Polymerase Complex

Nikolay M. Petrov [1,*], Dennis H. Bamford [2], Ralitsa Vassileva-Pencheva [1], Angel S. Galabov [1]

[1] *Department of Virology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria*
[2] *Institute of Biotechnology, University of Helsinki, Helsinki, Finland*

Coxsackievirus infections can result in a wide variety of disease syndromes including upper respiratory tract symptoms, aseptic meningitis, pericarditis, pleurodynia, myocarditis, and encephalitis. No anti-Coxsackievirus drugs are currently licensed and treatment is directed toward ameliorating symptoms. This places the need of new tools to control virus infections. RNA interference is a mechanism for silencing the transcriptional product of an activated gene. Its high conservancy and sequence-specificity are the basis for its huge potential for therapy against various infectious diseases, genetic disorders, and cancer. A way for high-quality and cost effective production of large quantities of double-stranded RNA is the use of virus-based systems targeting specific regions of the virus genome. T7 RNA polymerase and RNA-dependent RNA polymerase of bacteriophage ϕ6 were used to generate dsRNA molecules from 3′ part of VP3 and 5′ part of VP1 gene regions of Coxsackievirus B1. For large amounts of dsRNA production we utilized *Pseudomonas syringae* cells that constitutively express the bacteriophage ϕ6 complex, and plasmids with the target sequences placed under T7 promoter.

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Inhibition of Herpes- and Adenovirus Replication by Extract of *Artemia salina* Cysts from Crimean Hypersaline Lakes

Olga Yu. Povnitsa [1,*], Lidiya N. Nosach [1], Irina I. Rudneva [2], Valentin G. Shayda [2], Nadiya V. Nesterova [1]

[1] *Institute of Microbiology and Virology NASU, Kyiv, Ukraine*
[2] *Institute of Biology of the Southern Seas NASU, Sevastopol, Ukraine*

Adenoviral infection is a widely distributed human pathology and takes the second place for its spread among acute respiratory infections after influenza. So far, no specific and effective medicines have yet been developed for the treatment of local and systemic forms of adenoviral infection. The diseases caused by Herpes simplex virus (HSV) are widely distributed. Treatment of these infections is the most significant medical problem. At present for the treatment of these diseases nucleoside (acyclovir, ganciclovir, penciclovir, cidofovir) analogues are used. But the appearance of resistant virus is current problem in the treatment of patients and deficiency in the antiviral preparations caused their toxicity. Therefore, it is very important to develop new antiviral drugs against this virus. Very promising approach is the antiviral screening of products derived from natural sources including marine fauna and flora, bacteria, fungi, and green plants. We have studied the antiviral activity against Herpes simplex virus (HSV-1, strain US) and Human adenovirus type 2 (Adh2) of the protein extracts (PE) which were produced from *Artemia* cysts collected in Crimean hypersaline lakes. Anti-viral experiments were performed in vitro on *Hela* cells. PE was tested for antiviral activity against HSV-1 by a plaque reduction assay. The rate of Adh2 like viral reproduction inhibition was evaluated by the decrease percentage of the cells with specific viral intra-nuclear inclusions. The highest inhibitory effect was observed when PE was added to cells immediately after absorption of viruses at 1 h.p.i. It was determined that PE in concentration of 16 μg/ml inhibited the reproduction of HSV-1, EC_{50} of PE was 2.8 μg/ml, and selectivity index (SI) was 7. PE in concentration 2 μg/ml decreased the number of infected Adh2 cells with intranuclear inclusions on 99%, EC_{50} of PE was 0.3 μg/ml, SI was 60. The compounds of these extract probably act on the inhibition of late stages of the viral reproduction. These results suggest that PE have a potential value as a source of new effective compounds against human adenovirus.

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European Consortium on Antiviral Drug Development: SILVER

Jean-Louis Romette*, Ernest Gould

UNIVMED, Marseille, France

The project aims to identify Small molecule Inhibitor Leads Versus Emerging and neglected RNA viruses (SILVER). It will focus its activities on selected medically important RNA viruses for which the development of drugs is considered essential (Dengue-, entero- and paramyxoviruses), whereas other relatively neglected and/or emerging RNA viruses will be explored to identify the most promising viral protein targets and antiviral compounds. A pipeline strategy will be employed to progress this work plan, whilst multidisciplinary workgroups (WAVEs) will ensure the proper flow of information, data, exploitation and dissemination. This organization allows any virus and viral target to enter the pipeline at its current state of knowledge and art. Targets for potential drugs include infectious virus, structurally characterised viral enzymes and other proteins. Leads for currently available antiviral drugs have been identified by screening compound libraries in virus-infected cell culture systems and *in vitro* assays using purified viral enzymes. Selective inhibitors of viral replication have also been (and are being) derived using detailed structural knowledge of viral proteins and structure-based drug design. Hits will be assayed using individual viral protein targets and replicative proteins in complex with viral RNA. The potential protective activity of the most potent inhibitors, that have a favourable (*in vitro*) ADME-tox profile, will be assessed in relevant infection models in animals. The consortium will also be prepared to re-align its specific research objectives immediately in the case of emergence of new RNA viruses threatening human health during the lifetime of the project. To this end, one workpackage will develop an "outbreak pipeline" in which SILVER partners will collaborate with worldwide network. The combined virus group-specific expertise of these specialists will ensure a broad coverage of human RNA viruses, which in the case of a novel emerging agent should maximize its processing speed and incorporation into the drug development pipeline. Licenses on promising compounds or compound classes will be presented to the interested pharmaceutical industry and will provide a multidisciplinary framework for rapid knowledge-based response to emerging infections.

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***In-vitro* Screening for Compounds Active Against Polyomavirus BK: A Seven Year Experience**

S. Parmjeet

University of Pittsburgh, Pittsburgh, USA

Background: Polyomavirus BK causes nephropathy in solid organ transplant patients and hemorrhagic cystitis after bone marrow and stem cell transplantation. Antiviral drugs with proven efficacy are not available.

Methods: 1061 compounds were selected for screening based on known biology of the virus and published literature on other small DNA viruses. This effort was supplemented by a homology modeling based virtual screening of 5.1 million compounds directed at the viral capsid protein VP-1 and the large T antigen (LTA). Crystal structures of SV40 VP-1-ganglioside and LTA-ATP ligand complexes were used as templates for construction of the homology models. Anti-BKV activity of compounds was confirmed by direct measurement of BKV Gardner strain replication in WI-38 cells using real time PCR. Toxicity to host cells was evaluated by the neutral red assay and by quantifying the housekeeping gene aspartoacylase as an index of host cell replication.

Results: Based on EC50, selectivity index, or FDA approval for other indications, compounds worthy of further pursuit found in the following categories: (i) Nucleotide analogs: HDP-cidofovir, ODE-cidofovir, HDP-cidofovir-(S)-HPMPA, ODE-(S)-HPMPA, PMEA derivatives. (ii) Malonitrilamide inhibitors of dihydro-orotate dehydrogenase: leflunomide, FK778. (iii) Topoisomerase inhibitors: camptothecin, ciprofloxacin, NSC 270718. (iv) Receptor analogs: BTB11968. (v) Anti-microbial peptides: hecate, tachyplesin. (vi) Anti-VP-1 monoclonal antibodies with >90% virus neutralizing ability. (vii) Commerically available immunoglobulin preparations. (viii) Compounds interfering with intra-cellular transport: chloroquine, nystatin. (ix) Kinase inhibitors: Tyrphostin RG13022, STO-18584, STO-18812, STO-18816, Erlotininb, Sorafenib.

Conclusions: Several compounds with in-vitro anti-BKV activity have been identified. Further testing is desirable in additional cell lines, multiple viral strains, and in animal models. Drugs already approved by FDA for other indications can proceed directly to human trials. Computerized homology modeling has shown the conceptual feasibility of discovering additional drugs that may disrupt viral capsid assembly and LTA mediated ATP-dependent unwinding of BKV DNA prior to its replication.

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Studies on Anti-HSV Activity and Cytotoxicity of *Morinda citrifolia* L. Noni Leaf

Periyasamy Selvam[1,*], Julie M. Breitenbach[2], Katherine Z. Borysko[2], John C. Drach[2]

[1] *Devaki Amma Memorial College of Pharmacy, Chelembra, Malapuram, India*

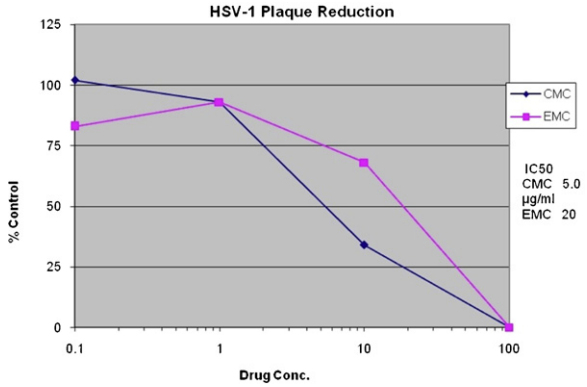
[2] *School of Dentistry and College of Pharmacy, University of Michigan, Michigan, Ann Arbor, USA*

Backround: The development of antiviral drugs has provided crucial new means to mitigate or relieve the debilitating effects of many viral pathogens. A rich source for the discovery of new HSV infection inhibitors has been and continues to be, the 'mining' of the large diversity of compounds already available in nature and specifically those from botanical extracts. *Morinda citrifolia* is used in the Indian system of medicine for the treatment of variety of diseases and enriched with flavinoids, anthroquinone and glycoside, but antiviral activity against Herpes Simplex Virus (HSV) not yet been studied, based on this fact present work is to study HSV inhibitory activity of different extracts of *Morinda citrifolia*

Method: The chloroform (CMC) and ethanol (ETMC) leaf extracts of *M. citrifolia* have been evaluated for antiviral activity against HSV in HFF cells by plaque reduction assay. The both extracts of the leaf of *M. citrifolia* were also investigated for cytotoxicity in KB (oral cancer) cells.

Results: Chloroform and ethanolic extracts of *M. citrifolia* exhibited inhibitory activity against of HSV activity at the concentration of 5 and 20 μg/ml, respectively. Chloroform and ethanolic extracts of *M. citrifolia* also exhibited cytotoxic activity against of KB cells at the concentration of 45 and 100 μg/ml respectively.

Conclusion: Anthroquine, flavinoid and alkaloids are the principle active constituents of *M. citrifolia*, which may responsible for HSV inhibitory activity. This is the first report showing the anti-HSV activity of *M. citrifolia*.



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Antiviral Activity of Carbohydrate-containing Biopolymers of *Pseudomonas chlororaphis* subsp. *aureofaciens*

Victoria V. Shepelevitch*, Volodymyr V. Shubchynskyy, Ludmila D. Varbanets, Elena A. Kiprianova

Zabolotny Institute of Microbiology and Virology, Kiev, Ukraine

Saprophytic soil bacteria *Pseudomonas chlororaphis* subsp. *aureofaciens* produce a wide set of antibacterial and antifungal substances. Strains of this species *UCM** B-*111* and *UCM* B-*306* are the components of biopreparation gaupsin which is used in Ukraine as a tool of plants protection from fungal and bacterial diseases. We have shown that gaupsin inhibited *in vivo* for 97-80% the development of tobacco mosaic virus (TMV) strain U1 in *Datura stramonium*, *Nicotiana tabacum* and *Nicotiana sanderae* plants during three vegetation seasons. Lipopolysaccharides (LPS) obtained from cells of both *Pseudomonas chlororaphis* subsp. *aureofaciens* strains using Westfal-Yann water-phenol method proved to be highly active antiviral agents. Their antiviral activity was 98–100% at concentration 10–1 mg/ml, 57–69% at 0.1, 43–44% at 0.01 and 14–11% at 0.001 mg/ml. It is interesting that LPS isolated from other bacterial species (*Rahnella aquatilis*, *Ralstonia solanacearum*) were not active against TMV or even stimulated the the necrosis formation. The cultural fluids of strains B-*111* and B-*306* grown in industrial or semisynthetic medium just as the thermostable water-soluble preparations isolated from fermentation broth by evaporation, dialysis and liophylization were also active against TMV. They inhibited TMV infectivity for 99–97% in concentration 10 mg/ml; the antiviral effect reduced to 20–24% at concentration 0.1 mg/ml. Only traces of proteins and nucleic acids and small amount of neutral monosaccharides (4–25%) have been found in LPS and extracellular polysaccharides which allows to suppose the presence of significant quantity of uronic acids in their composition. This originality of monosaccharide composition of studied preparations may be probably responsible for their unique antiviral effect.
*Ukrainian Collection of Microorganisms

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European Training Network on (+)RNA Virus Replication and Antiviral Drug Development

Frank van Kuppeveld[1,*], Eric Snijder[2], Alexander Gorbalenya[2], Bruno Canard[3], Ralf Bartenschlager[4], Johan Neyts[5], Andrea Brancale[6], Chris McGuigan[6]

[1] *Radboud University Nijmegen, Nijmegen, The Netherlands*
[2] *Leiden University Medical Center, Leiden, The Netherlands*
[3] *Université de la Méditerranée, Marseille, France*
[4] *University Hospital of Heidelberg, Heildelberg, Germany*
[5] *Rega Institute KU Leuven, Leuven, Belgium*
[6] *Cardiff University, Cardiff, Wales, United Kingdom*

Antiviral drug development requires a detailed understanding of virus replication and effective translation of this knowledge into drug discovery. Europe needs well-trained experts with multidisciplinary skills to advance this field. However, few, if any, European training institutes have the broad know-how required to provide such a comprehensive training programme. The EUVIRNA partnership aims to fill this gap with the proposed EUVIRNA training programme, which is a Marie Curie Initial Training Network (ITN) funded by EC FP7 (FP7-people-2010-ITN). The EUVIRNA partnership consists of six outstanding European academic partners and four industrial partners (Tibotec-Virco, Pike Pharma GmbH, Riboxx GmbH, and Okapi Sciences NV), and an associated partner specialized in education (Virology Education). All EUVIRNA partners are recognized leaders in their field, ensuring state-of-the-art training possibilities, and their skills are highly complementary. EUVIRNA aims to introduce 17 Early Stage Researchers (PhD students) and 3 Experienced Researchers (postdoctoral researchers) to state-of-the-art knowledge and technology applied in molecular virology and antiviral therapy, with both local and network-wide training activities. Individual research projects, research training workshops and intersectoral secondments will be supplemented with complementary skills courses to improve career development and perspectives. The industrial partners are actively involved in the entire programme, and will furthermore organize a 1-week industry-oriented conference aimed at further bridging the gap between academia and industry. Thus, EUVIRNA offers talented researchers a multidisciplinary and intersectoral training programme and prepares them for a future leading role in European molecular virology research and antiviral drug development.

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Ellagitannins as New Highly Efficient Inhibitors of Herpes Simplex Virus Replication and Synergists of Acyclovir

Nelly Vilhelmova[1,*], Adelina Stoyanova[1], R. Jacquet[2], S. Quideau[2], Angel S. Galabov[1]

[1] *Department of Virology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria*
[2] *Université de Bordeaux, Institut des Sciences Moléculaires (CNRS-UMR 5255), Institut Européen de Chimie et Biologie, Pessac Cedex, France*

Nonahydroxyterphenoyl-bearing *C*-glucosidic ellagitannins castalagin, vescalagin and grandinin possess a pronounce inhibitory effect on the replication of acyclovir (ACV)-sensitive strains of HSV types 1 and 2 in MDBK cells. An especially high activity against HSV-1 manifested castalagin, its SI attaining values higher than 1000, comparable to or exceeding SI values of ACV. Moreover, the three ellagitannins inhibited markedly the replication of ACV-resistant strains of HSV-1 and HSV-2 (casta-

lagin SI values of 1048 and 97.6, respectively, being registered). The combination effect of ellagitannins with ACV was studied through the three-dimensional analytical approach of Prichard and Shipman for evaluation of the impact of drug–drug interactions. A markedly synergistic character of the ellagitannins–ACV combinations effects was registered on the replication of both HSV-1 and HSV-2 ACV-sensitive strain. Testing of combinations ellagitannins plus ACV against HSV-1 and HSV-2 strains resistant to ACV demonstrated also marked synergistic effects. The synergism was more pronouncedly expressed towards HSV-1 as compared to HSV-2 strain. Data obtained showed that ACV as a partner in the combinations with ellagitannins against ACV-resistant HSV strains could be applied in comparatively lower concentrations. The data we collected demonstrate the high potential of *C*-glucosidic ellagitannins as antiherpetic agents. Obviously, one of these substances, castalagin, applied alone or in combination with ACV, could be considered as a perspective for further anti-herpesvirus chemotherapeutic studies.

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In Vitro Combination Therapy with Tegobuvir (GS-9190) is Highly Efficient in Curing Cells from HCV Replicon and in Delaying/Preventing the Development of Antiviral Resistance

Inge Vliegen[1,*], Jan Paeshuyse[1], I-hung Shih[2], Gerhard Pürstinger[3], Steven Bondy[2], William A. Lee[2], Weidong Zhong[2], Johan Neyts[1]

[1] *Rega Institute, Leuven, Belgium*
[2] *Gilead Sciences, Inc., Foster City, USA*
[3] *University of Innsbruck, Innsbruck, Austria*

Tegobuvir (GS-9190) is a novel non-nucleoside inhibitor of HCV RNA replication with proven activity in HCV infected patients. When combined with either the protease inhibitor VX-950, the nucleoside polymerase inhibitor 2′-*C*-methylcytidine or various non-nucleoside polymerase inhibitors in short-term assays, an overall additive antiviral activity was calculated. A slight synergistic effect was observed when low concentrations of GS-9190 were combined with low concentrations of either a benzimidazole or benzofuran non-nucleoside polymerase inhibitors. It was next studied whether prolonged culturing of replicon-containing cells in the presence of combinations of GS-9190 with other DAA delayed or prevented resistance development against either compound. When GS-9190 (at concentrations of 6, 30 or 150 nM) was added to replicon-containing cells that were cultured in the presence of suboptimal concentrations of VX-950 or the various polymerase inhibitors, resistance development against these compounds was either markedly delayed or completely prevented. Next, the potential of various combinations to clear cells of HCV replicons was evaluated. GS-9190, at the concentration of 150 nM, was able to cure replicon-containing cells after a single passage when combined with VX-950. The triple combination of GS-9190 (11 nM), VX-950 and 2′-*C*-methylcytidine resulted in clearance of replicon RNA after two passages. In contrast, the inhibitors when used alone at 3-fold higher concentrations were not able to cure the cells from the replicon after 6 passages. In conclusion GS-9190 resulted in an additive to slightly synergistic antiviral effect when combined in short term antiviral assays with other DAA in vitro. Antiviral combinations containing low concentrations of GS-9190 are highly effective in curing cells from their replicon and in delaying or preventing the development of resistance against other DAA.

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Human Papillomavirus Genotype Distribution in Women in Montenegro

Danijela Vujošević[1,*], Vineta Vuksanovic[1,2], Mario Poljak[3], Nebojša Joksimovic[4]

[1] *Institute of Public Health, Podgorica, Yugoslavia*
[2] *University of Montenegro, Podgorica, Yugoslavia*
[3] *Institute of Microbiology and Immunology, Ljubljana, Slovenia*
[4] *Clinical Centre of Monetengro, Podgorica, Yugoslavia*

Infection with high-risk genotypes of human papillomaviruses (HPV) is the main etiological agent of cervical cancer, the second most common form of cancer in women worldwide. Despite the existence of screening programs, the disease is still responsible for more than 10,000 deaths registered annually in the European Union. Information on distribution of HPV genotypes in women in a certain country is important for the selection of the appropriate screening test for detection of HPV infection in a given country. Therefore, the purpose of this study was to determine the range and frequency of HPV genotypes in women in Montenegro. HPV genotypes were determined using the method of enzyme restriction of PCR products amplified with group-specific primers MY09/MY11 and restricted with seven different restriction endonucleases. In all 189 women cervical smears were taken during a routine gynecological examination at the Clinical Center in Podgorica.

Out of the total number of women HPV infection was found in 1/5 of participants (20%, 38/189). Genotyping performed in 38 HPV DNA positive women shows that the HPV genotype 16 is dominant in Montenegro (36.8%, 14/38). The second most frequent HPV infection is with HPV genotype 58 and it is found in 10.5% of participants. HPV 31 and HPV 6 infections are present in 7.9% of women, while infections with other genotypes were demonstrated individually by 2.6%. Mixed infection was demonstrated in 18.4%.

Also, in our group of participants it was found that mixed HPV infection, with more than one HPV genotype is dominant in younger women (aged 25–30 years) and with at least one high-risk or probably high-risk HPV genotype. According to the results of our research we believe that in active search of women, who are more likely to develop cervical cancer, it necessary to do the tests that are able to detect broader spectrum of high-risk HPV genotypes. Sensitive detection of multiple HPV genotypes in patients is, also, especially important for the determination of persistence of infection and timing of usage type-specific vaccines.

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Enhanced Cellular Penetration of ODE-(S)-MPMPA Accounts for Its Prolonged Post-exposure Anti-HCV Activity

David L. Wyles[1,*], Krysten A. Jones[1], Nadejda Valiaeva[1,2], James R. Beadle[1,2], Robert T. Schooley[1], Karl Y. Hostetler[1,2]

[1] *University of California, San Diego, La Jolla, USA*
[2] *Veterans Medical Research Foundation, San Diego, USA*

Background: Octadecyloxyethyl 9-(*S*)-[3-methoxy-2-(phosphonomethoxypropyl)]adenine (ODE-MPMPA) exhibits potent anti-HCV replicon activity (EC50 1–2 μM) with a high selectivity (CC50 > 150 μM). In vitro resistance selection experiments demonstrated a high barrier to resistance with selection of low fold-change variants (A/Q49L 1.3-fold, K50N 1.6-fold) and/or unfit variants that do not replicate in vitro (Q58L). Continued selection at higher concentrations (9 μM) resulted in reversion to wild type replicon sequences while maintaining a resistant cell

phenotype. To better characterize the activity of ODE-MPMPA replicon rebound and compound uptake studies were performed.

Methods: Huh-7.5.1 cells stably expressing the BM4-5 FEO replicon (1b) were seeded in 96 well plates (2500 cells/well) and exposed to ODE-MPMPA at the EC50 (1.5 μM) and EC90 (8 μM). After 48 h compound was removed and replaced with complete media. Luciferase expression was determined and replication levels expressed as a percentage of control wells. Cellular uptake studies were performed in Huh7 cells with 8 μM ^{14}C-labeled MPMPA and ODE-MPMPA.

Results: Replications levels at 48 h were 52% and 8% at the EC50 and EC90, respectively. At 96 h after compound removal replication levels had declined further (23% and 2%). At one week replication remained suppressed (57% and 2%). After exposure to 8 μM drug, intracellular concentrations of radiolabeled MPMPA at 2, 4 and 24 h were 43, 46 and 54 pmol/10^6 cells compared with 330, 970 and 3340 pmol/10^6 cells for ODE-MPMPA. At 2, 4 and 24 h, cellular levels of ODE-MPMPA were 8-, 21- and 62-fold higher than those observed with 8 μM MPMPA.

Conclusions: ODE-MPMPA displays potent and prolonged suppression of HCV replicon replication after a single exposure. Prolonged elevated intracellular concentrations of drug and metabolites lead to the delayed rebound on removal. High intracellular levels combined with the high fitness cost and/or low fold-change of resistant mutants contribute to the high resistance barrier. Studies to determine the intracellular concentrations of the active metabolite (MPMPApp) are underway.

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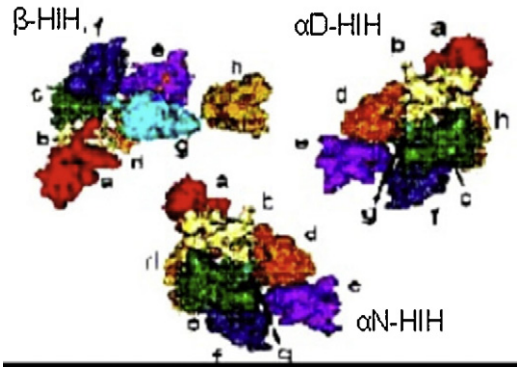
Anti-EBV Activity of Hemocyanin Isolated from Helix Lucorum

Svitlana D. Zagorodnya[1,*], Pavlina Dolashka[2], Galina V. Baranova[1], Anna V. Golovan[1], Nadiya V. Nesterova[1]

[1] *Institute Microbiology and virology NASU, Kyiv, Ukrenia*
[2] *Institute of Organic Chemistry, BAS, Sofia, Bulgaria*

The Epstein-Barr virus (EBV) is the representative of the family of *Herpesviridae*. EBV can be the agent that causes miscellaneous lymphomas as well as nasopharyngeal carcinoma, carcinoma of parotid glands, stomach adenocarcinoma and other diseases. EBV, as other herpesviruses, affects on central and peripheral nervous system. The object of the present investigation was to study the activity of hemocyanin from *Helix lucorum* (HlH) against Epstein-Barr virus. Hemocyanin was isolated from the hemolymph of Bulgarian garden snails. In contrast with other molluscan hemocyanins, three isoforms (β-HlH, α_N-HlH and α_D-HlH) (figure) with molecular mass about 450 kDa were isolated.

Cytotoxicity and antiviral activity of the isoforms of HlH were investigated in cell culture Raji. Cytotoxic concentration (300 μg/ml) was determined using the trypan blue. Samples for analysis of antiviral activity were collected in 48 h after infecting. AntiEBV activity of the samples was determined according to the level of inhibition of EBV DNA using PCR and primers to the capsid antigen. Clear dose-response effect was observed in a concentration range from 1 to 100 μg/ml, when analysis of the preparations was carried out immediately after infecting. 50% inhibition of the level of accumulation of viral DNA was observed at the lowest concentration. Proceeding from the index of selectivity, that is 300 for hemocyanins isolated from *Helix lucorum*, it is possible to make a conclusion about their availability for the further researches as of drugs, active against an Epstein-Barr virus.



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Investigation of Anti-EBV Activity of Ganciclovir in Combination with Antiflogistics

Svitlana D. Zagorodnya[1,*], Anna O. Kurova[1], Galina V. Baranova[1], O.L. Chyuko[2], Georgiy I. Danilenko[2], Nadiya V. Nesterova[1]

[1] *Institute Microbiology and virology NASU, Kyiv, Ukrenia*
[2] *Institute of Organic Chemistry NASU, Kyiv, Ukrenia*

Contamporary approaches to the treatment of herpes infections, especially Epstein-Barr virus, include the use of etiotropic medicines, as well as the holding of sensitizing therapy. The spectrum of drugs active against EBV remains quite limited and ganciclovir and acyclovir are used in medical practice. Both drugs are nucleoside analogues and consequently affect at the stages of viral DNA synthesis. Anti-inflammatory drugs are used in combination with antiviral drugs. The question of their joint effect both at the cellular level and at the level of a microorganism remains open. The goal of this investigation was to study the antiviral activity of ganciclovir against EBV in combination with anti-inflammatory drugs *in vitro*. As an anti-inflammatory drugs indomethacin, brufen and amizon were used. Indomethacin is the derivative of indol acetic acid and one of the most active non-steroidal anti-inflammatory drugs. It is strong inhibitor of prostaglandin biosynthesis also. Ibuprofen is nonsteroidal anti-inflammatory drug that has anti-inflammatory, analgesic and moderate antipyretic effect. The essential role in its mechanism of action play inhibition of the biosynthesis of prostaglandins E and F, both at central and at peripheral level. Amizon – non-opioide analgesic with a pronounced anti-inflammatory, antipyretic, interferonogenic and immunomodulatory properties. We tried to investigate how will affect at level of anti-EBV activity of ganciclovir the adding of strong antiflogistics such as, ibuprofen, indometacin and amizon. Tested compounds were added to culture cell Raji in non-toxic concentration and various proportional ratios between ganciclovir and every antiflogistics (mM) 1:3, 1:1, 3:1. Antiviral activity was estimated by PCR according to the level of inhibition of accumulation of viral DNA. We found that ibuprofen has no antiviral activity per se and inhibit ganciclovir effect. Indometacin is not active against EBV but and does not change ganciclovir activity. We confirmed our previous data that anti-EBV activity of amizon is the same as ganciclovir one. Combined action of amizon and ganciclovir did not altered effect of ganciclovir.

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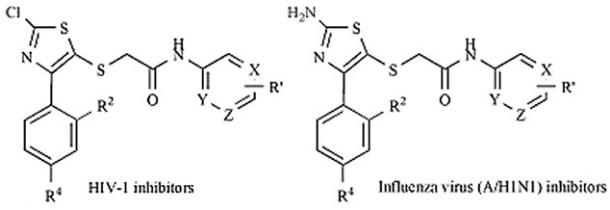
Thiazolylthioacetamides as a Novel Class of Potential Antiviral Agents

Peng Zhan[1,*], Xuwang Chen[1], Xinyong Liu[1], Christophe Pannecouque[2], Lieve Naesens[2], Erik De Clercq[2], Ailin Liu[3], Guanhua Du[3]

[1] *Department of Medicinal Chemistry, School of Pharmaceutical Sciences, ShanDong University, Ji'nan, China*
[2] *Rega Institute for Medical Research, Minderbroedersstraat, Belgium*
[3] *Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China*

In continuation of our endeavor to develop new, potent, and less toxic antiviral agents, a novel series of 2-amino/chloro substituted thiazolylthioacetamide derivatives have been synthesized and evaluated for in vitro anti-HIV activities in MT-4 cells for inhibition of the wild-type HIV-1 (strain IIIB), clinically relevant mutant HIV-1 strains, and HIV-2 (strain ROD). The bioactivity results showed that three compounds possessed potent activity against wild-type and several key mutant strains (E138K, K103N, L100I) of HIV-1 with EC50 values in submicromolar range. Based on the chemical structures, these molecules can be proposed to act as HIV-1 NNRTIs. Meanwhile, these 2-amino/chloro substituted thiazole derivatives were also evaluated for anti-influenza virus activities in Madin-Darby canine kidney (MDCK) cells infected with different strains of human influenza virus (A/H1N1, A/H3N2 and B viruses). Encouragingly, two derivatives A8g and A8h within 2-amino substituted thiazole series inhibit the influenza A/H1N1 replication with EC50 much lower than that of oseltamivir carboxylate, ribavirin, amantadine and rimantadine. However, no activity was observed for A/H3N2 and B viruses. Additionally, compounds A8g and A8h showed almost no activity in the neuraminidase (H1N1) inhibition assay, thus pharmacological studies are in progress to confirm their mechanism of action.



Newly synthesized thiazolylthioacetamide derivatives

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Antiviral Effectivity of Ceria Colloid Solutions

Nadiya Zholobak[1,*], Alexander Shcherbakov[1], Vladimir Ivanov[2], Zoya Olevinskaya[1], Nikolay Spivak[1]

[1] *Zabolotny Institute of Microbiology and Virology NASU, Kiyv, Ukrenia*
[2] *Kurnakov Institute of General and Inorganic Chemistry, Moskow, Russia*

Nanotechnology is one of the fast developing areas of industry and science. Substances having novel and unexpected properties are created basing on its principles. One of the most interesting objects of nanotechnology is nanocrystal cerium dioxide (NCD). NCD of 1–3 nm has minimal toxicity, participates in redox processes and has regenerative ability like enzymes. The mentioned properties depend on the precursor, the method of synthesis of nanoparticles, the used stabilizers, etc. We have created the NCD nanoparticles of such dimensions, stabilized them by sodium/ammonium polyacrylate (PAA) or citrate and investigated their cytotoxicity and influence on viral reproduction and cytopathic action in the cell cultures L929, EPT, VERO. The minimal diagnostic cytotoxic effect has been determined for NCD stabilized by citrate 10 mM. For PAA-stabilized nanoparticles the toxicity decreases from the mice cells to the primates ones. The antiviral activity of synthesized nanoparticles in the systems of L929/VSV and RF/HSV-1 was determined. It was shown, that in prophylactic (24 h before infecting) scheme PAA-stabilized NCD forms antiviral resistance in working concentrations 0.1–0.01 mM. In vitro citrate-stabilized nanoceria forms virus-resistant condition both in prophylactic and therapeutic (1 h after infecting) schemes, and also demonstrates a significant virucidal effect, reducing the virus titer in the investigated model systems into 2.6–4.8 lg. It is well-known, that the violation of the redox balance against viral infection is accompanied by the development of pathologic intracellular processes. The abilities to adjust ROS level and to inhibit the development of oxidative degradation are the main properties of NCD, which, in turn, affect the way of a cascade of the intracellular regulatory processes. Thus, the nanoceria ensures the survival of the infected cells. The obtained results are very interesting, because they open the prospect for further in-depth study of the synthesized aqueous sols of NCD for their future practical applications both for prevention and treatment of viral infections.

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Computer-aided Design and Evaluation of Novel Anti-CHIKV Compounds

Marcella Bassetto[1,*], Tine De Burghgraeve[2], Pieter Leyssen[2], Johan Neyts[2], Andrea Brancale[1]

[1] *The Welsh School of Pharmacy, Cardiff University, Cardiff, United Kingdom*
[2] *Rega Institute for Medical Research, Leuven, Belgium*

Chikungunya virus (CHIKV) is a mosquito-borne alphavirus with a single-stranded, positive-sense RNA genome. This pathogen is responsible for outbreaks of febrile arthralgia in humans. Recent cases of CHIKV infections have been recorded in Asia, Africa and Europe. To date, no antiviral drugs are available for the treatment of infections with this important emerging virus. The viral genome encodes four non-structural proteins (nsP), which could be considered potential antiviral targets. In particular the nspP2 is a protease involved in the processing of the non-structural polyprotein, an important step in viral replication. Based on the previously developed homology model of the CHIKV nsP2, we have performed a series of molecular modeling simulations to find a possible inhibitor of this enzyme. Initially we have carried out a virtual screening simulation using a database of 350,000 compounds, and we have identified one compound which prevented virus-induced cell death at low μM concentration. In order to optimize this hit, we carried out a series of molecular dynamics simulations on the nsP2/natural substrate and nsP2/hit complexes. Based on these studies we have selected a new series of compounds to be tested versus the CHKV. In this presentation we will discuss the results obtained and the initial SARs of these compounds.

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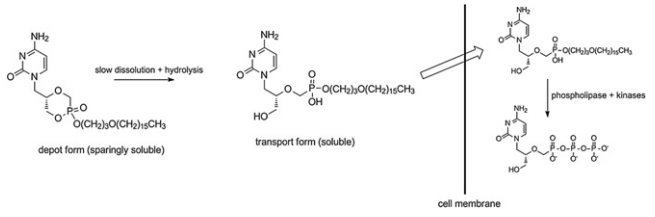
Intravitreal Alkoxyalkyl Esters of Cyclic Cidofovir for Treatment of Ocular Viral Infections

James R. Beadle[1,*], Lingyun Cheng[2], Karl Y. Hostetler[1], William R. Freeman[2]

[1] *Univ. of California, San Diego, La Jolla, USA*
[2] *Jacobs Retina Center, Univ. of California, San Diego, La Jolla, USA*

Cytomegalovirus (CMV) retinitis occurs in transplant recipients and other immunosuppressed individuals, such as AIDS patients. Since systemic treatment with the intravenous drug cidofovir (CDV, Vistide®) is hampered by significant toxic side effects, local intervention by intravitreal injection has been investigated. Intravitreal CDV injections can cause a sight-threatening drop in intraocular pressure and are not presently used for anti-CMV therapy. We are investigating sparingly soluble CDV prodrugs as an alternative local treatment for CMV retinitis. Hexadecyloxypropyl cyclic cidofovir (HDP-cCDV), a potent inhibitor of HCMV replication in vitro, forms a slow-release depot in the vitreous that does not adversely affect intraocular pressure or visual clarity. We propose that slow dissolution and hydrolysis of HDP-cCDV converts HDP-cCDV to the more soluble prodrug HDP-CDV which is then taken up by ocular tissues. Metabolism in ocular tissues and retina leads to efficient formation of the active antiviral metabolite, CDV diphosphate (figure). To determine the rate of hydrolysis, HDP-cCDV was suspended in aqueous solution at 37 °C and the rate of appearance of HDP-CDV was determined by HPLC analysis. In a second study, the ocular distribution and clearance of ^{14}C-labeled HDP-CDV was investigated after intravitreal injection (28 μg, rabbits). Through 5 weeks, the drug was detectable in vitreous, retina and ciliary body, and accumulated preferentially in the retina. Overall, our studies suggest that alkoxyalkyl esters of cyclic cidofovir could be useful for local treatment of CMV retinitis, where toxicity and a short half-life has limited the use of unmodified CDV.



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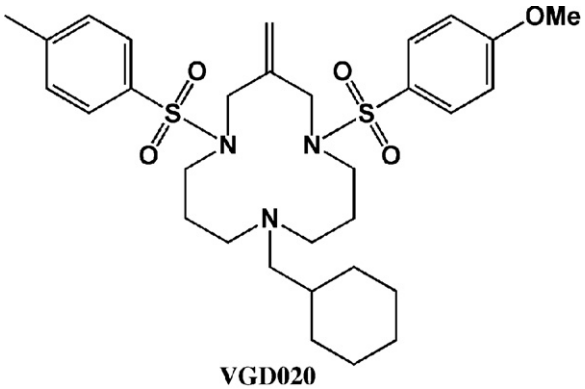
Effect of Molecular Symmetry on Potency in Novel Down-Modulators of the CD4 Receptor

Thomas W. Bell[1,*], Violeta G. Demillo[1], Florian Goulinet-Mateo[1], Rameez Ali[1], Nicholas C. Pflug[1], Chiraphorn Khan[1], Kurt Vermeire[2], Dominique Schols[2]

[1] *Department of Chemistry, University of Nevada, Reno, USA*
[2] *Department of Microbiology and Immunology, Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium*

Cyclotriazadisulfonamide (CADA) inhibits the entry of HIV into CD4+ target cells by down-modulating expression of the primary cellular HIV receptor, CD4. Many analogs have been made, and a correlation between CD4 down-modulating and antiviral activities has been observed. Most previously synthesized CADA analogs are symmetrical, with the same arenesulfonyl sidearm on both sides. Exceptions are two compounds with dansyl on one side and tosyl on the other (KKD015 and KKD016). The inactivity of a corresponding symmetrical analog with two dansyl groups suggested that decreased symmetry might increase potency in other cases. By a new synthetic route, new unsymmetrical CADA analogs were prepared and most exhibit CD4 down-modulating activity. The most potent (VGD020), shows a 12-fold increase in potency relative to CADA. The related symmetrical compounds, bearing two tosyl or two methoxybenzenesulfonyl sidearms were found to be 3–4 times less potent than VGD020. The results are consistent with a previous 3D-QSAR model showing that electron-donating groups on sidearms increase potency. They add a new, previously unexplored dimension to the SAR analysis, molecular symmetry. The observation that an unsymmetrical CADA compound can be more potent than either symmetrical analog correlates with the unsymmetrical conformation observed in crystal structures of several CADA analogs and suggests that this may be the bioactive conformation. This information may be of use in designing new analogs for use as tools to identify the cellular target of CADA compounds.



VGD020

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Tripartate Prodrugs of Hydroxy-containing Compounds

María-José Camarasa[1,*], Alberto Diez-Torrubia[1], Silvia Cabrera[1], Graciela Andrei[2], Robert Snoeck[2], Jan Balzarini[2], Sonsoles Velázquez[1]

[1] *Instituto de Química Médica (CSIC), Madrid, Spain*
[2] *Rega Institute for Medical Research, K.U. Leuven, Leuven, Belgium*

The lymphocyte surface glycoprotein dipeptidyl-peptidase IV enzyme (DPP-IV/CD26), selectively cleaves X-Pro (or X-Ala) dipeptides from the N-terminus of a variety of natural peptides. We have developed a novel enzyme-based prodrug approach that provides conjugates [peptide]–[drug] specifically cleavable by DPP-IV. The approach was applied to a variety of drugs containing a free amino group that was directly coupled with the carboxyl group of amino acids via an amide bond (bipartate prodrug). With these conjugates, it was possible to modulate the hydrolisis rate (half-life) and the physicochemical properties of the compounds by modifying the nature and length of the peptide (di- or tetrapeptides) of the prodrug moiety. (García-Aparicio et al., 2006; Diez-Torrubia et al., 2010).

Recently, we expanded, our prodrug strategy to an hydroxy-containing compound such as the highly potent and selective bicyclic nucleoside analogue (BCNA) inhibitor of Varicella Zoster Virus (VZV), named Cf1743, which exhibit a very low water solubility and poor oral bioavailability. Several of the synthesized prodrugs that contain a dipeptide moiety (cleavable by CD26), a heterobifunctional linker [released by chemical or enzymatic (esterases) hydrolysis of the ester bond] and the drug (tripartate prodrugs)

showed a high improvement in aqueous solubility together with an efficient conversion to the biologically active parent drug in the presence of DPPIV/CD26 and in serum.

We now explore the viability of this DPP-IV/CD26 prodrug approach in a variety of hydroxy-containing drugs of different nature (primary, secondary, tertiary or aromatic hydroxyl groups). A broad variety of prodrugs have been designed synthesized and evaluated for their pharmacokinetic properties including chemical and enzymatic stability (cleavage rates) and water solubility. The results indicated that the prodrugs are efficiently converted to the parent drugs, several of them showed markedly increased water solubility. Thus, the results support the wide applicability of our prodrug approach.

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Synthesis and evaluation of biological activity of new aminoadamantane amides containing hydroxycinnamoyl moiety

Maya G. Chochkova[1,*], Asya P. Georgieva[1], Galya I. Ivanova[2], Ivanka G. Stankova[1], Tsenka S. Milkova[1]

[1] *South-West University "Neofit Rilski" Department of Organic Chemistry, 2700, Blagoevgrad, Bulgaria*
[2] *Requimte, Polo da Universidade do Porto Departamento de Química Rua do Campo Alegre, 4169-007, Porto, Portugal*

In humans, oxidative stress is involved in the pathogenesis of many diseases, including such as influenza virus infections. Therefore antioxidants are those molecules potentially applicable in prevention from flu. The hydroxycinnamic acids and their derivatives are natural antioxidants with multiple mechanisms of action. The compounds are known to have a variety of activities: antiviral, antibacterial, immunostimulatory and etc. In order to obtain new potent antiviral drugs, we connect two pharmacophores with proved antiviral activity such as antiviral drugs – aminoadamantane derivatives and hydroxycinnamic acids. Amidation of hydroxycinnamic acid with amantadine or rimantadine was carried out using EDC/HOBt as peptide coupling method. The structures of the obtained amides were characterized by spectral methods (UV, ^{1}H NMR, MS). The evaluation of antiviral and antiradical activities of synthesized amides is in progress.

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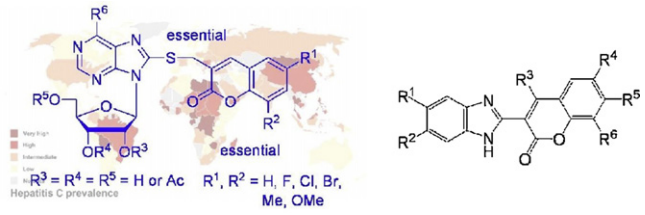
Coumarins Hybridized with Heterocycles or Ribonucleosides for Eradication of Hepatitis C Virus

Reuben Jih-Ru Hwu[1,2,*], Shu-Yu Lin[1], Shwu-Chen Tsay[1], R. Singha[1], J. Neyts[3]

[1] *National Tsing Hua University, Hsinchu, Taiwan*
[2] *National Central University, Jhongli, Taiwan*
[3] *Katholieke Universiteit Leuven, Leuven, Belgium*

Coumarin moiety conjugated with a benzimidazole moiety by a –SCH_2– linker would exhibit potent inhibitory effects on HCV. Consequently two series of analogues were synthesized to constitute new compound libraries by incorporation of heteroatoms into the benzimidazole moiety. Some of these coumarin–heterobicycle conjugates possessed appealing antiviral activities with significant selectivity index values. Lack of the sulfur atom-containing linkage led to poor biological activities. Prominent examples included coumarin conjugates containing an imidazopyridine, purine, or benzoxazole moiety they were found to inhibit HCV replication at an EC_{50} value of 6.8, 2.0, and 12 mM, respectively. Furthermore, the conjugation was extended to compounds with three components: coumarin, purine, and ribofuranose. The essential functional groups and moieties therein will be discussed.

Our laboratory has successfully established three compound libraries. Through analysis of the data therein, we came up with their structure–activity relationships (SAR). In this talk, we report our design and synthetic strategies on obtaining the targets and their SAR. These new findings provide a hybridization approach of value for drug development in the future.



Further reading

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HSV-1 and Alzheimer's Disease: The Case for Antiviral Treatment

Withdrawn

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Synthesis and Antiviral Evaluation of N^9-[2-(Phosphonomethoxy)ethyl] (PME) Analogues Derived from 8-Substituted Purines

Zlatko Janeba[1,*], Antonín Holý[1], Robert Snoeck[2], Graciela Andrei[2], Erik De Clercq[2], Jan Balzarini[2]

[1] *Institute of Organic Chemistry and Biochemistry, AS CR, Czech Republic*
[2] *Rega Institute for Medical Research, KU Leuven, Belgium*

Structure–activity relationship (SAR) studies have shown that the nature of the heterocyclic base plays a determining role in the biological activity of the acyclic nucleoside phosphonates (ANPs). This activity is connected especially with purine derivatives, the only exception being the cytosine derivative HPMPC. It was also shown that mostly compounds derived from purines containing a free amino group were active (adenine, guanine, 2-aminopurine

and 2,6-diaminopurine), owing to their proton donating/accepting capacity. Any other substitutions at C-2 or C-6 of the purine moiety led to the decrease or annihilation of the biological activity. While extensive data regarding the effect of the C-2 and C-6 substituents on the antiviral and cytostatic activity of purine ANPs were obtained, data relating to the effect of the C-8 substitution are rather scarce. Thus, various 8-substituted purine ANPs were synthesized by nucleophilic aromatic substitution reactions or various cross-couplings, starting from the corresponding 8-bromopurine derivatives. The antiviral properties of these analogues will be reported.

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Acyclic Nucleoside Phosphonates: Past, Present, and Future

Withdrawn

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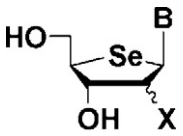
Design, Synthesis, and Anti-HCV Activity of 2′-Modified-4′-selenonucleosides

Lak Shin Jeong[1,*], Jin Ha Yu[1], Hyuk Woo Lee[1], Jung Hee Choi[1], Won Jun Choi[1,2]

[1] *Ewha Womans University, Seoul, South Korea*
[2] *Dongguk University, Ilsan, South Korea*

Hepatitis C virus (HCV) has a positive sense single strand RNA genome which is replicated to a negative strand RNA by RNA dependent RNA polymerase of NS5B. Since the inhibition of RNA dependent RNA polymerase leads to no replication of HCV, this enzyme serves as an attractive target for the development of new anti-HCV agents. Many classes of nucleoside and non-nucleoside derivatives have been synthesized as anti-RNA dependent RNA polymerase inhibitors. Among those, 2′-fluoro- and 2′-azidonucleosides have been shown to be good templates for antiviral and antitumor agents. On the basis of a bioisosteric rationale, their 2′-fluoro- and 2′-azido-4′-thio analogues were also reported to show potent anticancer and antiviral activities. Recently, we have reported the synthesis of 4′-selenonucleosides and their unusual conformations. Thus, on the basis of bioisosteric rationale, it would be of great interest to synthesize the 2′-modified-4′-selenonucleosides (X = OH, F, or N_3) and to compare their biological activity with that of 4′-oxo- or 4′-thionucleosides. In the synthesis of 2′-fluoro-4′-selenonucleosides, it was first revealed that selenium atom participated in the DAST fluorination of 4′-selenonucleosides and that conformational bias induced by bulky selenium acted as a decisive factor in the DAST fluorination. Among compounds synthesized, 2′-"UP"-fluoro-thymine analogue showed significant anti-HCV activity in a cell-based replicon assay. Asymmetric synthesis, conformational study, and antiviral activity of novel 2′-modified-4′-selenonucleosides will be presented in detail.



X = OH, F, or N_3

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Genome Specific Diagnosis of Influenza Virus Strains by Hairpin-type Peptide Nucleic Acid

Kunihiro Kaihatsu*, Nobuo Kato

Osaka University, Ibaraki, Japan

Influenza A virus is a negative-strand RNA virus that possesses 8 genome segments in the virion. The virus conserved essential genome sequences to retain the infectivity, while it mutates the other sequence parts to obtain versatility. Here we identified the highly conserved 15 base sequences on the nonstructural protein (NS) genome by CONSERV software. We designed a hairpin-type peptide nucleic acid (PNA), of which the sequence is complementary to the NS genome conserved sequence of swine-origin influenza virus A/Osaka/53/H1N1. The hairpin-type PNA effectively recognized the viral genome of the swine-origin influenza virus and inhibited the reverse-transcription in a sequence specific manner. We immobilized the hairpin-type PNA on a plate to examine if the PNA could capture and diagnose the swine-origin influenza virus. As a result, the hairpin-type PNA selectively captured the swine-origin influenza virus (pdm-H1N1) from other seasonal viruses (Fig. 1). Further, we developed a method to visualize the virus genome on the plate by naked eyes even the virus concentration was 10–100 fold lower than that of clinical samples (Fig. 2). Our method can be utilized for the diagnosis of drug-resistant or highly pathogenic viruses based on their genome sequences without PCR and fluorescence detection system.

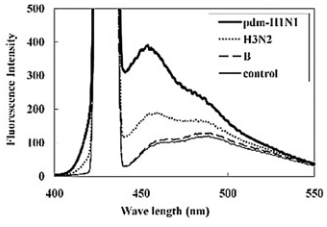

Fig.1. Genome sequence specific detection of swine-origin influenza virus by a hairpin-type PNA.

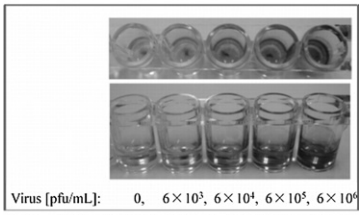

Fig.2. Detection of swine-origin influenza virus genome by naked eyes.

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Development of Novel Evaluation Method to Anti-influenza Drug Resistance using Docking Study

Norihito Kawashita[1,2,*], Masashi Yasuda[1], Yu-Shi Tian[1], Kousuke Okamoto[1], Masaya Kawase[3], Teruo Yasunaga[2], Tatsuya Takagi[1,2]

[1] *Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Japan*
[2] *Research Institute for Microbial Diseases, Osaka University, Suita, Japan*
[3] *Nagahama Institute of Bio-Science and Technology, Nagahama, Japan*

On April 2009, novel swine influenza was occurred in Mexico and was going around all over the world (pandemic). In 2009, more than 600,000 people were infected and 14,000 people were died. Some neuraminidase inhibitor such as oseltamivir is frequently used for treatment. Meanwhile, oseltamivir resistance is one of the problems for treatment recently. For example, His275Tyr (for H1N1) mutation shows oseltamivir resistance.

Against this background, if we can evaluate these kinds of drug resistance rapidly, it contributes an antiviral treatment on site and applies to the prediction of drug resistance in near future. Now we developed a novel evaluation method of antiviral drug resistance in silico.

This method is combination of docking simulation, which calculates a binding affinity between drug and protein, and Boltzmann distribution, which represents an existing probability of molecule. We thought that drug susceptive strains have more suitable docking poses than drug resistant strains. Therefore, all docking poses are converted to the existing probability and expectation value by Boltzmann distribution this can evaluate drug resistance between different complexes.

In this case, we evaluated drug resistance of influenza neuraminidase inhibitor by using structures of neuraminidase and the inhibitor (oseltamivir). We compared to ligand conformations between before and after docking to determine suitable docking pose. The expectation value of drug resistant strains is lower than susceptive strain. It was suggested that our evaluation by comparison with the expectation value of wild type be appropriate since our method agreed with the experimental data.

Our method considers a number of docking poses, the method features more accurate than the method from only top docking score. Therefore, it is useful for appropriate selection of drugs when novel mutant appears.

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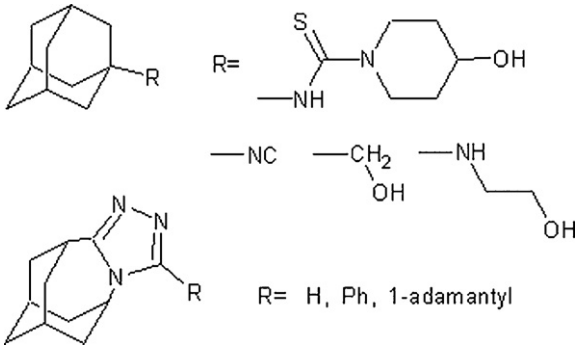
Antiviral Properties of New Cage Compounds

Yuri Klimochkin[1,*], Marat Baimuratov[1], Ekaterina Knyazeva[1], Nadezhda Baleeva[1], Marina Leonova[1], Michail Skomorokhov[1], Evgeny Belanov[2], Sergey Balakhnin[2]

[1] *State Technical University, Samara, Russia*
[2] *FSRI SRC VB "Vector", Koltsovo, Russia*

Traditionally, derivatives of adamantane are considered as potential treatments for influenza. The ability of cage structure to interrupt functions of M2 protein, acting as ion channel, allows to suggest that derivatives of polycyclic molecules can be also efficient inhibitors of the replication of other viruses with ion channels. Simultaneously, the same proteins are acting during the assembly of mature viral unit, therefore cage substances can interrupt the late stages of viral reproduction. By sequential search of the viral inhibitors, series of the new compounds has been prepared on the basis of modification of the polycyclic carbonic acids, amines, ketones, alcohols, lactames and also from adamantane alkenes and arenes. Antiviral activity of the prepared substances against the number of RNA viruses (influenza virus type A, H5N1, H1N1, H3N2 pestivirus BVDV as surrogate model of hepatitis C virus) and DNA-viruses (orthopoxviruses vaccinia, cowpox, ectromelia and variola viruses herpes virus) have been investigated on the cell cultures. In the issue sufficiently large number of compounds with antiviral action in a varying degree has been prepared. Hydroxy and nitrogen containing derivatives of the adamantane which have no other substituents in the cage unit show maximal activity against influenza viruses. The ability to suppress reproduction of pox viruses mostly belongs to cage compounds with high Log *P* values and derivatives of the heterocyclic system of the 4-azahomoadamantane.

Acknowledgment: The work is supported by the FCP «Scientific and pedagogical staff of innovative Russia in 2009–2013».



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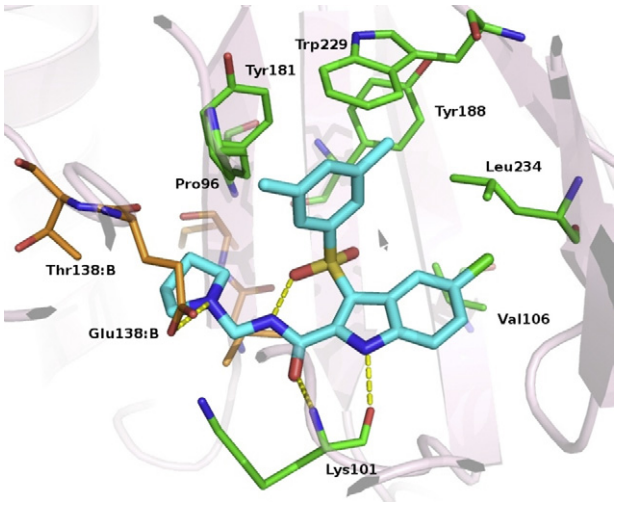
Indolylarylsulfones as HIV-1 Non-Nucleoside Reverse Transcriptase Inhibitors: New Cyclic Substituents at the Indole-2-carboxamide

Giuseppe La Regina[1,*], Antonio Coluccia[1], Andrea Brancale[2], Giovanni Maga[3], Jan Balzarini[4], Ettore Novellino[5], Romano Silvestri[1]

[1] *Sapienza University, Roma, Italy*
[2] *Cardiff University, Cardiff, United Kingdom*
[3] *CNR, Pavia, Italy*
[4] *Katholieke Universiteit, Leuven, Belgium*
[5] *Federico II University, Napoli, Italy*

New indolylarylsulfone derivatives bearing cyclic substituents at the indole-2-carboxamide linked through a methylene/ethylene spacer were potent inhibitors of the WT HIV-1 replication in CEM and PBMC cells with inhibitory concentrations in the low nanomolar range. Against the mutant L100I and K103N RT HIV-1 strains in MT-4 cells, several compounds showed antiviral potency superior to NVP and EFV. Against these mutant strains, the derivatives were equipotent to ETV. Molecular docking experiments on this novel series of IAS analogues have also suggested that the H-bond interaction between the nitrogen atom in the carboxamide chain of IAS and Glu138:B is important in the binding of these compounds. These results are in accordance with the experimental data obtained on the WT and on the mutant HIV-1 strains tested.



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Anti-HCV Drug Development

Withdrawn

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A Novel Family of Multivalent Compounds able to Interact with GP120: Anti-HIV Evaluation and Binding Analysis With Surface Plasmon Resonance

Virginia Lozano[1,*], Leire Aguado[1], Bart Hoorelbeke[2], Marleen Renders[2], María-José Camarasa[1], Ana San-Félix[1], Jan Balzarini[2], María-Jesús Pérez-Pérez[1]

[1] *Instituto de Química Médica (CSIC), Madrid, Spain*
[2] *Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium*

It is well known that lectins of different origin show interesting properties against HIV replication (Balzarini, 2006). More recent studies support a potential dual mechanism of action for the anti-HIV activity of lectins (Balzarini, 2007; François and Balzarini, in press): (1) directly, by binding to the glycans of the HIV envelope and thus blocking viral entry and (2) indirectly, by favouring deletions in the envelope glycan shield triggering the immune system to recognize previously hidden immunogenic epitopes. However lectins suffer from a number of drawbacks including their high molecular weight, peptidic nature, poor pharmacokinetics, etc. that hamper their development as potential drugs. Based on the key interactions established between lectins and the glycans of gp120, we have designed, synthesized and tested three series of 1,3,5-triazine derivatives (monomers, dimers and trimers) functionalized with aromatic amino acids. These structures are rich in recognition elements meant to mimic the interactions that lectins establish with gp120. The anti-HIV evaluation showed that dimers and mostly trimers exhibit moderate but significant anti-HIV-1 activity in the low micromolar range that is accompanied by the absence of toxicity against CEM cells. Moreover, the most active compounds were subjected to gp120 binding analysis with surface plasmon resonance. The results indicated that some trimers are able to efficiently bind to gp120 with estimated K_D values in the lower micromolar range. Thus, the collected data support the interest of this family of novel anti-HIV agents.

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Targeting HCV (+) Strand RNA Genome by a Novel PNA–neamine Conjugate

Dineshkumar Manvar*, Nootan Pandey, Virendra N. Pandey

UMDNJ-New Jersey Medical School, Dept. of Biochemistry, Newark, USA

The polycationic neamine moiety of the aminoglycoside antibiotic neomycin B was conjugated to a 15 mer peptide nucleic acid (PNA) targeting nucleotide sequence 342–356 downstream of 5′NTR in the core coding region of HCV RNA genome. The cellular uptake of this PNA–nea conjugate is highly efficient and dependent on the concentration of the conjugate in the uptake medium. At as low as 200 nM concentration of fluorescently tagged PNA–nea conjugate, nearly 80% of the cells were fluorescence positive within 6-8 h of incubation. We used MH14 cell culture system carrying stably replicating HCV subgenomic replicons for determining antiviral activity of the PNA–nea conjugate targeting coding start region of HCV core. We noted severe inhibition of HCV replication without any cytotoxic effect on MH 14 cells when anti-HCV PNA–nea conjugate was supplemented in the cell culture medium. We found both HCV replication and translation were efficiently blocked by the conjugate with IC_{50} of inhibition being 200 nM. These results suggest a potential therapeutic application for this class of novel compounds.

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Targeting the Flavivirus Helicase

Eloise Mastrangelo[1,*], Margherita Pezzullo[2], Martino Bolognesi[2], Suzanne Keptein[3], Johan Neyts[3], Boris Pastorino[4], Xavier de Lambellerie[4], Mario Milani[1]

[1] *CNR-Istituto di Biofisica, Milano, Italy*
[2] *Università di Milano, Milano, Italy*
[3] *University of Leuven, Leuven, Belgium*
[4] *Université de la Méditerranée, Marseille, France*

Environmental, demographic and ecological reasons suggest that either novel or known flaviviruses will continue to emerge, posing new threats to the human population. Additionally, therapeutic interventions present different outcomes: for example, the success of vaccination against yellow fever virus has been hampered by difficulties encountered when similar programs were launched against dengue virus (DENV). In this context, we are focusing on anti-flavivirus drugs, which should preferably be active against all four DENV serotypes and other flavivirus infections, such as yellow fever virus (YFV), Japanese encephalitis virus (JEV) and tick born encephalitis virus (TBEV). We have therefore extensively studied, among others, helicase (Hel) enzymes involved in the replication process. Starting from crystal structures of these proteins, we identified an unexploited protein site that might be mechanistically involved in Hel catalytic cycle, and which could in principle be exploited for enzyme inhibition. Targeting such new site, we performed *in silico* docking searches using a library of small molecules as a source of potential inhibitors that would bind to the

site. These procedures allowed to identify few compounds with high predicted affinity for the Hel new site. Subsequently, anti-Hel activity of the identified compounds was shown through *in vitro* enzymatic assays. More specifically, we showed unequivocally that the MM3 compound (previously known for more than 20 years for an unrelated therapeutic application) is an efficient inhibitor of flavivirus Hel. We further analyzed the effect of MM3 on Flavivirus replication in cell culture. MM3 was shown to inhibit the replication of several flaviviruses, including YFV, DENV, JEV and TBEV. Results from virus yield assays, after which viral RNA was assessed by means of quantitative RT-PCR, revealed that EC50 values for inhibition of flavivirus replication are for a number of viruses in the nM range, in particular highly potent activity was observed against the YFV (EP 09174368).

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Stereoselective Synthesis of Different Types of Nucleotide Prodrugs

Chris Meier[1,*], Rios Morales Edwuin Hander[1], Arbelo Román Cristina[1], Lindström Nadine[1], Balzarini Jan[2]

[1] *Organic Chemistry, Department of Chemistry, Faculty of Sciences, University of Hamburg, Hamburg, Germany*
[2] *Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium*

Since the discovery of cancer and antiviral diseases many efforts have been done in order to infer with their propagation. One of them uses pronucleotides as potential lipophilic nucleotide precursors to deliver phosphorylated metabolites to cells. In cases in which the phosphorus atom has four different substituents the pronucleotides are P-chiral, e.g. in *cyclo*Sal-derivatives, phosphoramidates or in the HepDirect compounds (Meier, 2006; Cahard et al., 2004; Erion et al., 2004). Since very recently, these pronucleotides could not be prepared in the form of single diastereomers due to lacking control of the stereochemistry at the phosphorus atom during synthesis. Their separation using liquid chromatography was almost impossible. However, it has been proven that the configuration at the phosphorus atom has an influence on the biological activity. For this reason the stereoselective synthesis of such compounds is of extreme relevance. Here, we present new diastereoselective syntheses of pronucleotides by using a convergent strategy and a linear strategy. Using chiral auxiliaries it was possible to prepare successfully optically active compounds with asymmetric phosphorus atoms. On these ways *cyclo*Sal-nucleotides, arylphosphoramidates and HepDirect-pronucleotides with different substitution patterns were synthesised with very high diastereomeric excesses (higher 95% *d.e.*) (Arbelo Roman et al., 2010; Rios Morales et al., 2010). In addition to this, biological activities of several compounds against HIV-1 and HIV-2 infected CEM/0 and HIV-2 infected CEM/TK$^-$ cells will be presented and discussed.

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A Comparison of the ability of wild-type and S282T mutant HCV NS5B to incorporate 2′-α-F-2′-β-*C*-methylguanosine-5′-monophosphate and 2′-α-OH-2′-β-*C*-methylguanosine-5′-monophosphate

Eisuke Murakami*, Haiying Bao, Angela M. Lam, Christine Espiritu, Shalini Bansal, Phillip A. Furman

Pharmasset, Inc., Princeton, USA

Several nucleoside/tide analogs targeting HCV RNA-dependent RNA polymerase (RdRp) in clinical development possess either a 2′-α-OH-2′-β-*C*-methyl or 2′-α-F-2′-β-*C*-methyl substitution in the sugar moiety. The S282T mutation confers resistance to certain nucleoside/tide analogs containing the 2′-α-*C*-methyl substitution including the guanosine monophosphate prodrugs, IDX-184 and INX-189. Both compounds are metabolized to a common triphosphate, 2′-α-OH-2′-β-*C*-methylGTP. PSI-352938 and PSI-353661 are prodrugs of 2′-α-F-2′-β-*C*-methylGMP analogs that are metabolized to 2′-α-F-2′-β-*C*-methylGTP. PSI-352938 and PSI-353661 demonstrate potent anti-HCV replicon activity against both the wild-type and the S282T mutant replicons. To better understand the difference in activity *in vitro* conveyed by the 2′-α-fluorine to the activity of PSI-352938 and PSI-353661 against the S282T mutant, biochemical assays monitoring incorporation of 2′-α-F-2′-β-*C*-methylGMP or 2′-α-OH-2′-β-*C*-methylGMP into a nascent RNA chain were performed under single turnover conditions where the enzyme concentration is in excess over the RNA substrate. This primer-dependent assay resembles the elongation step during RNA replication. The wild-type RdRp incorporated 2′-α-F-2′-β-*C*-methylGMP and 2′-α-OH-2′-β-*C*-methylGMP at similar efficiencies. Substrate specificity was calculated as a ratio of incorporation efficiency for the nucleotide analog to that for the natural GMP. The substrate specificity for 2′-α-F-2′-β-*C*-methylGTP was similar for wild type and S282T RdRp with less than a 2-fold difference. The ability of the S282T mutant enzyme to incorporate 2′-α-OH-2′-β-*C*-methylGMP was significantly different from that with the wild-type. The kinetic parameters for incorporation efficiency of 2′-α-OH-2′-β-*C*-methylGMP were not determined because the 2′-α-OH-2′-β-*C*-methylGTP was highly discriminated against by the S282T RdRp. Therefore, our *in vitro* data suggest that the mechanism of S282T resistance to 2′-β-*C*-methylguanosine nucleotide analogs involves discrimination against the 2′-α-OH-2′-β-*C*-methylGTP, but not the 2′-α-F-2′-β-*C*-methylGTP by the mutant enzyme during RNA elongation.

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QSAR Analysis of Anti-influenza (A/H1N1) Activity of Azolo-adamantanes

Eugene Muratov[1,2,*], Ekaterina Varlamova[1], Anatoly Artemenko[1], Victor Kuz'min[1], Pavel Anfimov[3], Vladimir Zarubaev[3], Victor Saraev[4], Oleg Kiselev[3]

[1] *A.V. Bogatsky Physical-Chemical Institute NAS of Ukraine, Odessa, Ukrenia*
[2] *University of North Carolina, Chapel Hill, USA*
[3] *Influenza Research Institute, Saint Petersburg, Russia*
[4] *Saint Petersburg State Institute of Technology, Saint Petersburg, Russia*

Keywords: Azolo-adamantanes; Influenza A/H1N1; Selectivity index

Influenza spreads around the world in seasonal epidemics, resulting in the deaths of up to 500 thousand people every year. Since vaccination and existing antivirals cannot guarantee protection against influenza, battling this virus remains important health care task that requires design and development of new drugs. Application of cheminformatics methods shortens the development time and reduces costs of antiviral drug research. The goal of the present study is computer-assisted design of novel selective agents by the means of QSAR analysis of antiviral activity of azolo-adamantanes against influenza. The dataset comprised 60 azolo-adamantanes. Thorough investigation of the relationship between antiviral activity against influenza strain A/PR/8/34 (H1N1) (EC_{50}, μM), cytotoxicity on MDCK cells (CTD_{50}, μM), and selectivity index (ratio of CTD_{50} to EC_{50}) and the structure of investigated compounds was carried out using Hierarchic QSAR Technology (HiT QSAR). Prior to development of QSAR analysis, the compounds were divided on two classes according to their activity, selectivity, and cytotoxicity: EC_{50}active < 0.1 μM < EC_{50}inactive CTD_{50}toxic < 1 μM < CTD_{50} non-toxic and SI non-selective < 10 < SI selective. Five-fold external cross-validation was used for the estimation of predictive power of obtained random forest models. We succeeded to develop predictive model of antiviral activity with cumulative classification correct rate CCR_{5FECV} = 0.8. The quality of cytotoxicity and selectivity models was somewhat lower but still acceptable (CCR_{5FECV} = 0.64–0.7). New selective anti-influenza agents were computationally designed and predicted using developed models. Six of them were recommended to synthetic and biological experiments.

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Viral Genome Dynamics During Antiviral Resistance Selection: A First Glimpse into Viral Evolution

Simone Musiu*, Jan Paeshuyse, Mathy Froeyen, Philippe Lemey, Johan Neyts

Katholieke Universiteit Leuven, Leuven, Belgium

The nature of the RNA-dependent RNA polymerase (RdRp) of most RNA viruses renders their genome prone to the accumulation of mutations. Hence the virus population exists as a complex and dynamic mutant distribution, i.e. a quasispecies. The quasispecies enable rapid adaptation of the virus to any changes in its natural environment. Also during antiviral therapy, rapid selection of drug resistant virus is facilitated by the existence of the virus as a quasispecies rather than a defined genomic sequence. We employed the bovine viral diarrhea virus (BVDV) [family of the *Flaviviridae*, genus pestivirus] as a model virus. Most, if not all of the currently know pestiviral RdRp inhibitors target a 7 Å region between F224 and E291 within the finger domain of the enzyme. Here we describe how the RdRp coding region of the viral genome evolves during *in vitro* antiviral resistance selection. To this end we employed a panel of selective inhibitors of BVDV replication i.e. LZ37 (Paeshuyse et al., 2009), AG110 [Paeshuyse et al., 2007) and BPIP (Paeshuyse et al., 2006). Resistant virus was selected (against each compound) by serially passaging (25 times) the virus in the presence of increasing concentrations of inhibitor. The entire NS5B gene was sequenced. Furthermore the sequence flexibility of the polymerase region F224–E291 was analysed. It was observed that different resistance mutations could be obtained during independent parallel resistance selection. The sequence of BPIP- and LZ-resistant BVDV clustered separately from AG110-resistant BVDV. This might indicate that there exist certain genomic constraints that impede the potential truly random nature of the quasispecies. To study this we designed a resistance selection scheme for AG110 and BPIP that allowed monitoring genomic changes in parallel. Preliminary data highlight a more complex evolutionary pattern than initially observed. The results provide a first glimpse into patterns of viral evolution during selective antiviral pressure exerted by specific pestiviral polymerase inhibitors.

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Structure-based design of small-molecules that selectively inhibit dengue virus methyltransferase

Christian G. Noble*

Novartis Institute for Tropical Diseases, 10 Biopolis Road, Singapore

Flaviviruses contain a single methyltransferase (MTase) for methylation of the 5′ cap of the Flavivirus RNA. This protein contains a single domain with both guanine N7 and ribose 2′O MTase activities. We have analysed the crystal structures of Flavivirus MTases and identified a Flavivirus-conserved hydrophobic pocket next to the binding site for S-adenosyl-methionine (SAM), the substrate of the methylation reaction. Several derivatives of S-adenosylhomocysteine (SAH), the product of the methylation reaction, were synthesised. These compounds were designed to contain substituents that extended into the hydrophobic pocket and were found to be more potent against dengue virus MTase than SAH, but were not active against related human enzymes. Crystal structures showed that these compounds bound into the hydrophobic pocket of dengue MTase, and induced conformational changes in residues lining the pocket. The structures show the specific interactions between the compounds and the dengue MTase and suggest ways to further improve their potency. Together these data show that selectivity for disease-related MTases, can be produced.

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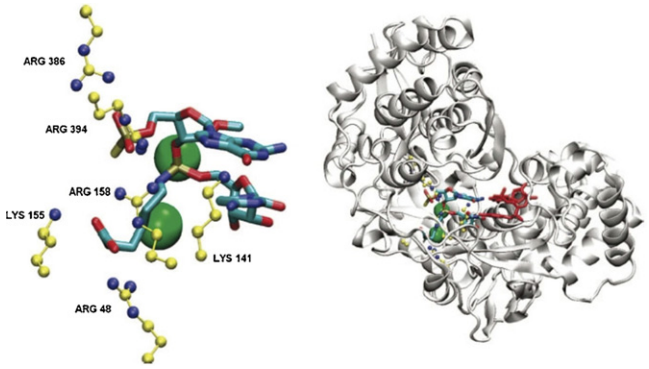
Phosphoramidate Dinucleosides as Inhibitors of Hepatitis C Virus Subgenomic Replicon and NS5B Polymerase Activity

S. Priet[1,*], I. Zlatev[2], I. Barvik[3], J. Neyts[4], H. Dutartre[1], B. Canard[1], F. Morvan[2], K. Alvarez[1]

[1] *AFMB, CNRS UMR 6098, Marseille Cedex 09, France*
[2] *IBMM, UMR 5247 CNR, Montpellier, France*
[3] *Faculty of Mathematics and Physics, Prague, Czech Republic*
[4] *Rega Institute for Medical Research, Leuven, Belgium*

The hepatitis C virus (HCV) represents one of the major viral infections in the world. The development of new therapeutic strategies is urgently needed. HCV replicates its genomic RNA thanks to its own polymerase, so-called the HCV NS5B. This enzyme thus remains a target of choice for inhibitors. We have developed GC Dinucleosides exhibiting a internucleosidic linkage with neutral, amphiphile, positively or negatively charged amino side chains. A first series named "GC 3′-OH", carrying various linkages have been previously reported as hepatitis C virus inhibitors. In order to enhance the efficacy, we synthesized a novel "GC 3′-H" series as potential chain terminators with novel neutral and bis-negatively charged amino side chains. Their inhibitory effect on HCV NS5B polymerase was evaluated in vitro and in HCV subgenomic replicon containing Huh-6 cells. As expected, 3′-H compounds are more potent than their 3′-OH counterparts to inhibit HCV polymerase activity. The most potent inhibitor, a 5′-phosphorylated dinucleotide bearing a bis-negatively charged side chain, exhibits an IC_{50} value of 8 μM in vitro and EC_{50} value of 2.6 μM in the HCV subgenomic replicon system. A molecular structure model is presented to propose an interpretation of the gain afforded by the of 3′-H-cytidine modification.



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Tick-borne Flaviviruses Infection in Non-human Primates

Yulia Rogova*, Natalia Pripuzova, Larissa Gmyl, Natalia Tereshkina, Liubov Terekhina, Mikhail Vorovich, Karina Grishina, Galina Karganova

Chumakov Institute of Polimyelitis and Viral Encephalitides Russian Academy of Medical Sciences, Institut Poliomyelita, Russia

Employment of different animal models for tick-borne encephalitis (TBE) and Omskhemorrhagic fever (OHF) infection leaded to selection of particular lines of mice, as the most susceptible model. To investigate the virus pathogenesis, the safety and the protectiveness of vaccines and drugs sometimes it is necessary to use a model close to human by its susceptibility. Monkeys are not sensitive to peripheral inoculation with neurotropic flaviviruses. Subcutaneous (s/c), intramuscular or intraperitoneal injection of TBE virus does not cause encephalitis in these animals. However, in some studies it was shown that monkeys can have clinical manifestation of TBE and OHF and pathomorphological changes in CNS similar to human. A lot of epidemiological data accumulated about a high number of seropositive people among endemic territories population is the evidence for existence of inapparent forms of TBE in humans which are about 100 times more frequent than acute forms with clinical symptoms. Thus, monkeys can be very close to humans by their sensitivity to tick-borne flaviviruses.

In our study we particularly concentrated on the development of the most appropriate and informative model using s/c inoculation of two different monkeys' species (Macaca fascicularis and Cercopitecus aethiops) with two virulent strains of TBE virus and one strain of OHF virus. We have compared animal susceptibility, TBE virus strains virulence, and terms of experiments using following parameters: body temperature curve, level and duration of viremia, clinical manifestation, virus titers and lesions in CNS and viscera. Both viruses caused inapparent infection in monkeys of both species. We found which parameters and target organs can serve as the markers of infection at the different time points after inoculation.

The assessment of different viruses and two species of monkeys allowed us to choose monkeys of genus *Macaca* as the most susceptible model to peripheral inoculation of members of mammalian tick-borne flaviviruses group.

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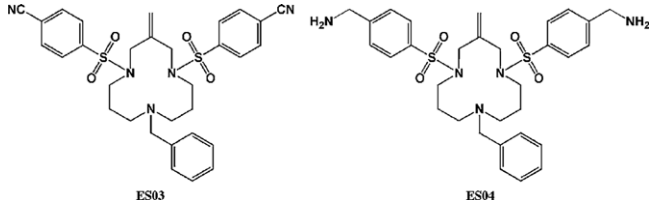
Synthesis of Novel CADA Analog Prodrugs Designed as Down-Modulators of the CD4 Receptor

Emily D. Scarbrough[1,*], Kurt Vermeire[2], Dominique Schols[2], Thomas W. Bell[1]

[1] *Department of Chemistry, University of Nevada, Reno, USA*
[2] *Department of Microbiology and Immunology, Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium*

Cyclotriazadisulfonamide (CADA) inhibits HIV replication by specifically down-modulating expression of the CD4 receptor protein on host cells. Many analogs of CADA have been synthesized in order to enhance potency, reduce toxicity, and improve physical properties, especially solubility and cell permeability (Bell et al., 2006). These analogs have also been used to develop a three-dimensional quantitative structure-activity relationship (3D-QSAR) computer model. Current studies are aimed at developing a prodrug approach involving novel CADA analog ES04. This compound is expected to have a CD4 down-modulation potency that is similar to that of CADA, according to our 3D-QSAR model. ES04 is the parent compound for prodrugs bearing dipeptide chains that are covalently bonded to the two amino groups of the aminomethylbenzenesulfonyl side arms. Cleavage of these chains by dipeptidyl-peptidase IV (Garcia-Aparicio et al., 2006) is expected to convert the prodrugs into ES04. The synthesis of ES04 involves reduction of the dicyano CADA analog ES03, which was prepared by means of a recently developed palladium-catalyzed macrocyclization method. The anti-HIV and CD4 down-modulation activities of the novel CADA compounds will also be presented.



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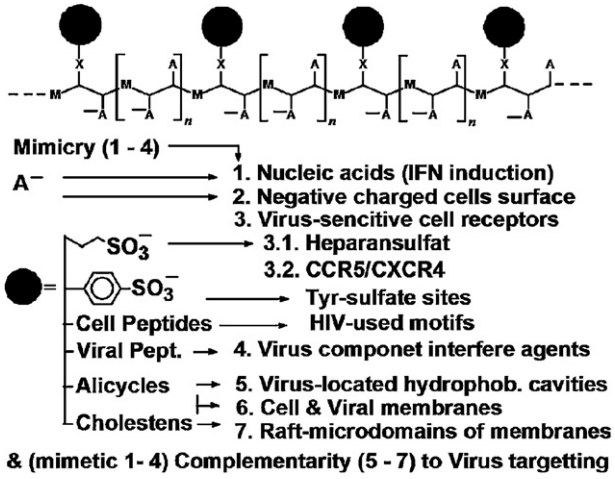
Polymer-cooperative Approach to Multi-blocking the Viruses

A. Serbin[1,2,*], E. Karaseva[1,2], O. Alikhanova[1], V. Tsvetkov[1,3]

[1] *Biomodulators RC, Health RDF, Moscow, Russia*
[2] *Topchiev Institute of Petrochemical Synthesis, RAS, Moscow, Russia*
[3] *Orehovich Institute of Biomedical Chemistry, RAMS, Moscow, Russia*

Polymeric macromolecules (nucleic acids, proteins, etc.) construct a fundament for all biologic systems including the viruses and viral pathogenesis. But small molecules (nucleotides, amino acids, etc.) cannot provide the adequate structure-functional potency. Same objective law covers the small molecular antivirals, which in principle cannot be adequate blockers for many macromolecular targets (excepting the sub-molecular small-scale sites), and fatally promote viral drug resistance (even through the super-high pre-clinical efficiency). Within this fundamental law we accumulate the research efforts for systematic development of antiviral (semi-)synthetic polymer systems (AVP), in part, based on water-soluble biocompatible polyelectrolytes. To achieve a multi-blocking antiviral activity (maximal efficiency with lowest drug resistance) the polymer-cooperative principles were studied via controlled combinations of polymeric backbone nature with various kind of virus-targeted side vectors (VT), designed through bio-mimicry and/or complementarity to anti-viral targeting (experimental routs see on the figure). This strategy leaded to number of AVP-generations, possessing expanded antiviral activity on both interferon-inducing/immune stimulating (*in vivo* – 1), and direct virus life cycle inhibiting levels (*in vitro*, as entries 2–7, or self-assembly/maturation inhibitors – 4.). The current experimental DB contains data for inhibition against influenza (A,B) HIV (various strains) *herpesviridae*, and other viruses, including viral strains resistant to approved small-molecular antivirals. To understand the polymer-cooperative effects a computational modeling was applied too.



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Synthesis and *In Vitro* Anti-influenza Activity of New Amino Acids and Peptidomimetics Derivatives of Oseltamivir and Rimantadine

Ivanka Stankova[1,*], Kiril Chuchkov[1], Michaela Schmidtke[2], Angel Galabov[3]

[1] *Department of Chemistry, South-West University "Neofit Rilski", Blagoevgrad, Bulgaria*
[2] *Jena University Hospital, School of Medicine, Department of Virology and Antiviral Therapy, Jena, Germany*
[3] *The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria*

Amantadine hydrochloride (1-adamantanamine hydrochloride, Symmetrel®) was the first adamantane derivative introduced in medicine as effective therapy against Asian A influenza virus. Among various substituents a growing interest in adamantyl derivatives is gaining prominence because of well known drugs like rimantadine, Memantine, Adapalene, Adatanserin and others in clinical trials. The pronounced central nervous system stimulating and cardiovascular effects of amantadine necessitated the search for newer more potent and less toxic agents for the control of pandemic influenza viruses. Influenza virus neuraminidase inhibitors (NAI) are an important class of antivirals for the treatment and prophylaxis of influenza. Their in vitro activity against the highly pathogenic influenza virus A(H5N1) has also led to the recommendation that they have been used for the treatment and prophylaxis of human H5N1 infections. A new series of oseltamivir and rimantadine with unnatural amino acids and peptidomimetics was designed and examined for antiviral activity in vitro against influenza A virus. The esters were synthesized from the amino acids 4-F-phenylalanine (R,S) and glycine containing a thiazole and thiazolyl-thiazole ring and oseltamivir and rimantadine following a two-step procedure. Derivative of oseltamivir with 4-F-phenylalanine (R) inhibited markedly the influenza virus-induced cytopathic effect at non cytotoxic concentrations (selectivity index = 455). The remaining compounds were considerably less effective.

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A Computational Approach to Search Active Peptides as Membrane Fusion Inhibitors of HIV-1

Yu-Shi Tian[1,*], Norihito Kawashita[1,2], Chris Verathamjamras[1], Kousuke Okamoto[2], Teruo Yasunaga[2], Masanori Kameoka[2], Tatsuya Takagi[1,2]

[1] *Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Japan*
[2] *Research Institute for Microbial Diseases, Osaka University, Suita, Japan*

Gp41 acts as one of main factor glycoproteins in the membrane fusion step of HIV-1 infection. This event has been well studied and artificial peptides those mimic the heptad repeats (HR) have been proved active in anti-HIV treatments. For example, one of the peptides is the famous drug named T20, or enfuvirtide was approved as the first membrane fusion inhibitor. However T-20 resistant isolates appeared during clinical use. Another peptide named C34, which is designed according to the most common isolate of HIV-1, has also been well studied. Due to the potential to enhance the activities of anti-retroviral effects, many groups made efforts in modifying the sequence. However the modifications were limited

to several site points within the peptide, such as inserting EK or ER motif to increase the helicity of the peptide. We here present our method used in screening of HIV-1 sequence database *in silico*. The energy of peptides those are the truncated parts (aa. 628–631) of the isolates uploaded in Los Alamos HIV-1 database were assessed. Peptides exhibiting low energies were selected as candidates firstly. Subsequently, modifications were introduced according to both the information of strong modifications previously reported and alanine scan computation. At last KYK peptides were developed. Several of these peptides were active in several different isolates, including T-20 resistant isolates. Our KYK peptides are under further studies. This concise method applied here can be used in many similar cases in which peptide inhibitors play import roles as drugs.

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The 3D-Screen Technology, an Innovative Cell-based Assay to Identify Modulators that Alter Target Protein Conformation: Example with HCV

Isabelle Valarche*, Amaya Berecibar, Mehdi Lahmar, Luc Batard, Aurelie Martin, Annick Piessens, Celine Fernagut, Caroline Freslon-Evain, Philippe Guedat, Majid Mehtali

Vivalis, Saint Herblain, France

All proteins exert their biological function through a defined tridimensional structure that determines their precise interactions with one or several specific molecular partners (proteins, DNA, etc.). Binding of natural or synthetic ligands to a given target protein induces dramatic or subtle conformational changes that influence the interaction of this protein with its cellular partners, resulting in specific modulation of the biological responses. Based on this fundamental biological principle we developed the 3D-Screen technology, an innovative human-cell based assay designed to identify small molecules that alter conformation of target proteins. Since knowledge of the biological function of the protein is not required, this highly sensitive technology can be applied to an extensive range of therapeutic targets. Moreover, screening for conformational alterations in the natural cellular environment rather than for functional alterations using purified target protein provides access to promising modulators acting through original mechanisms of action such as allosteric modulators. This technology is based on the specific recognition of the target native conformation by a short peptide sequence (3D-Sensor) that activates the expression of a reporter gene. Alteration of the target conformation prevents interaction with the 3D-Sensor, eliminating the expression of the reporter gene. In an effort to identify new classes of HCV polymerase inhibitors, high throughput 3D-Screen-based screening was performed in the hepatoma Huh-7 cell line. Optimization of the resulting hits led to compounds with potency in low nanomolar range on genotypes 1a and 1b and active against NS5B variants resistant to known inhibitors. Interestingly, activity of the series was improved by two logs on the 3D-Screen and replicon assays without modifying activity either of recombinant polymerase or of NS5B in the context of the in vitro replication complex. These results suggest a novel mechanism of action that is currently under investigation.

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Synthesis and Antiviral Activity of 3-Methoxy-2-(phosphonomethoxy)propyl Nucleoside Esters Against HCV and HIV-1

Nadejda Valiaeva[1,2,*], James R. Beadle[1,2], David L. Wyles[1], Robert T. Schooley[1], Karl Y. Hostetler[1,2]

[1] *University of California, San Diego, La Jolla, USA*
[2] *Veterans Medical Research Foundation, La Jolla, USA*

Novel therapies for hepatitis C virus (HCV) and HIV infection are greatly needed. We previously identified octadecyloxyethyl 9-(S)-[3-methoxy-2-(phosphonomethoxy)propyl]adenine (ODE-(S)-MPMPA) a potent inhibitor of HCV (EC_{50} = 1.43 μM) and HIV replication in vitro (EC_{50} = 0.03 μM). ODE-(S)-MPMPA has greatly reduced cytotoxicity compared with the analog having a 3-hydroxypropyl group, ODE-(S)-HPMPA, in Huh7 cells the CC_{50} values were 150 μM vs. 35 μM. To identify additional potent antivirals, we synthesized of MPMP-analogs of guanine, 2,6-diaminopurine, and cytosine by reaction of the corresponding nucleobase with (*R*)- or (*S*)-methyl glycidyl ether. These derivatives then reacted with alkoxyalkyl p-toluenesulfonyloxymethyl phosphonates to provide the final compounds after acidic deprotection. Antiviral evaluation of the new compounds showed that although ODE-(S)-MPMPA is the most active anti-HCV compound in genotype 1b or 2a replicons, all MPMP-purine nucleosides possess antiviral activity in a EC_{50} range between 10 and 25 μM for both (S) and (R) isomers and have the same low cytotoxicity. All MPMP-purine nucleosides are very active against HIV in MT-2 cells in vitro, with some compounds having EC_{50} values less than 10 pM. However, the ODE-(S)-MPMP pyrimidines did not show significant activity against either HCV or HIV. We also evaluated various 3-alkoxy substituents of the acyclic side chain. The ethoxy and isopropoxy analogs of (R,S)-MPMP-adenine were synthesized and evaluation of their antiviral activity shows that the methoxy group is the preferred substituent.

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Herpes Simplex Virus Thymidine Kinase Inhibitor GLS122E and Its 6-Deoxy Prodrug GLS361B (Sacrovir™)—Potential for Preventing Viral Disease Recurrence *In Vivo*

Ivan B. Yanachkov[1,*], Edward J. Dix[2], Milka I. Yanachkova[1], Sofya Dvoskin[1], Wei-Chu Xu[1], Bryan M. Gebhardt[3], George E. Wright[1]

[1] *GLSynthesis, Inc., Worcester, USA*
[2] *Assumption College, Worcester, USA*
[3] *Louisiana State University Eye Center, New Orleans, USA*

After initial infection herpes simplex viruses (HSV) establish a latent state in neural ganglia, from which future reactivation and recurrence of acute infection may occur. The current anti-HSV drugs suppress the acute infection, but do not control virus reactivation, especially subclinical reactivation and resulting transmission. HSV types 1 and 2 express virus-specific thymidine kinases (TK), which are likely required for virus proliferation in non-replicating (neural) cells and thought to be essential for reactivation from latency. Cell culture and animal model experiments have shown that HSV TK inhibitors suppress virus reactivation from established latent infections, and, therefore, could be developed as drugs to target recurrent infections and, possibly, subclinical reactivation and virus transmission. The guanine analog N^2-(3-(trifluoromethyl)phenyl)guanine (mCF_3PG, GLS122E) is a potent, specific, non-substrate HSV 1 and 2 TK inhibitor, which has been

shown to inhibit reactivation of virus from latently infected murine trigeminal ganglia cocultured on Vero cells. Here we report that IV treatment with a nanoparticle formulation of mCF_3PG also suppresses reactivation of latent ocular infection in mice. Further development of mCF_3PG, however, is restricted by its low water solubility and lack of oral bioavailability (in mice). We synthesized and studied the 6-deoxy analog of mCF_3PG – 2-((3-(trifluoromethyl)phenyl)amino)purine (GLS361B, Sacrovir™) – as a possible prodrug. Sacrovir™ has higher water solubility than mCF_3PG and is converted to mCF_3PG by human liver cytosol. Sacrovir™ has modest oral absorption in mice but is converted rapidly into mCF_3PG and a second oxidized metabolite. It is oxidized to the same metabolites after IP injection into guinea pigs. Further studies to enhance the oral bioavailability of Sacrovir™ and to determine its ADME properties in different species are underway.

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Anti-HBV Activities of Novel 2′,3′-C-substituted beta-L-nucleoside Analogues

Xiao-Xiong Zhou[1,*], Staffan Torssell[1], Olov Wallner[1], Piaoyang Sun[1], Tim Shaw[2], Zhuhui Huang[3], Charlotte Larsson[4], Bjorn Kull[4]

[1] *Novadex Pharmaceuticals AB, Huddinge, Sweden*
[2] *Victorian Infectious Diseases Reference Laboratory, Melbourne, Australia*
[3] *Southern Research Institute, Frederick, USA*
[4] *Actar AB, Stockholm, Sweden*

Hepatitis B virus (HBV) infection is a medical challenge of global proportions. Today there are some 300 million people chronically infected with the virus and about 1 million patients die from HBV-related liver diseases each year. Nucleoside/nucleotide HBV pol inhibitors have been used widely for the treatment of HBV infection. Due to the persistence of the HBV infection, a prolonged treatment of years are often required, which puts a high demand on the safety profile of a successful therapy. Besides, the development of resistance has been observed with the current therapies, which is fueled by the high rate of viral replication and the low fidelity of the viral polymerase. There remains a strong need for new, potent and safe pharmaceutical agents to treat HBV, and particularly the new therapeutics that are useful in treating the resistant HBV infections. A series of novel 2′,3′-C-substituted beta-L-nucleosides have been synthesized and evaluated. The lead compound showed a good anti-HBV activity and safety property. The further studies on those 2′ and 3′-modified beta-L-nucleosides are on-going.

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Invitation to the Twenty-Fifth International Conference on Antiviral Research, Sapporo, Japan on April 16–19, 2012

Dear Friends and Colleagues

The 25th International Conference on Antiviral Research (ICAR) will be held in Sapporo, Japan, at the Royton Sapporo and co-hosted by the International Society for Antiviral Research (ISAR) and the Japanese Association for Antiviral Therapy (JAAT).

The conference will begin on Monday, April 16th and conclude with the Conference Banquet in the evening on Thursday, April 19th.

ICAR is a unique interdisciplinary multi-national meeting which covers all virus types and brings together those involved in basic virology, drug design and synthesis, mechanistic and structural studies, assay design, in vitro evaluation, animal models and clinical trials. For each of these areas there will be invited world leading plenary speakers who will deliver definitive overview lectures. There will also be oral presentations from submitted abstracts selected by our expert panel of reviewers, and two poster sessions.

A clinical symposium will be held during the meeting program and will feature a selection of key and timely updates on the progress of antiviral agents in clinical development which have been selected to cover the interests of conference attendees. We will also hold mini-symposia with presentations by internationally known antiviral researchers. The topics of these symposia will be selected closer to the time of the conference to ensure that they will be of the highest relevance.

As we have done at previous ICARS, the prestigious 'Elion' and 'Prusoff' Awards from ISAR will be given to individuals in recognition of their major contributions in the field. Additionally, we will also make several Poster Awards, to graduate students and junior researchers.

Sapporo is an outstanding venue for our conference and is a popular tourist destination for visitors from all over the world.

We hope you will join us for a memorable 25th ICAR in Sapporo in April 2012.

ISAR and JAAT Conference and Program Committees.

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Future Conferences

2012 April 16 – April 19 Sapporo, Japan

2013 TBD

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Review

Crimean-Congo hemorrhagic fever: Current and future prospects of vaccines and therapies☆

Maryam Keshtkar-Jahromi[a], Jens H. Kuhn[b], Iva Christova[c], Steven B. Bradfute[d], Peter B. Jahrling[b], Sina Bavari[d,*]

[a] Union Memorial Hospital, University of Maryland, Baltimore, MD, USA
[b] Integrated Research Facility at Fort Detrick, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Fort Detrick, Frederick, MD, USA
[c] National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria
[d] United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD, USA

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ABSTRACT

Crimean-Congo hemorrhagic fever (CCHF) is a tick-borne disease caused by CCHF virus (CCHFV), a nairovirus in the family *Bunyaviridae*. CCHF occurs sporadically in a number of countries in Asia, the Middle East, southeastern Europe and Africa. Patients may develop subclinical to severe hemorrhagic disease, with fatal outcomes in a substantial percentage of cases. Transmission usually occurs through contact with viremic livestock or patients or bites by infected ticks. The number of reported cases has increased in recent years, possibly due to global climatic change and human perturbations of biocenoses that may have led to the migration of tick vectors. There is currently no FDA-approved vaccine or specific antiviral therapy for CCHF. The classification of CCHFV as a WHO Risk Group IV pathogen and the lack of suitable animal models has caused progress in developing new prophylactic and therapeutic measures to be slow. Ribavirin is active against CCHFV *in vitro*, but its efficacy for human therapy has not been definitively demonstrated by clinical studies. CCHF-immunoglobulin is also in use, but without clear evidence of efficacy. In this article, we review the development of prophylaxis and therapy for CCHF and discuss future prospects for vaccine and drug development.

Contents

Abbreviations: ALT, alanine aminotransferase; CCHF, Crimean-Congo hemorrhagic fever; CCHFV, Crimean-Congo hemorrhagic fever virus; CCL, chemokine (C-C motif) ligand; CFA, complement-fixation assay; ELISA, enzyme-linked immunosorbent assay; FDA, US Food and Drug Administration; IFN, interferon; IL, interleukin; mAb, monoclonal antibody; NIAID, US National Institute of Allergy and Infectious Diseases; PKR, protein kinase R; RT-PCR, reverse transcription polymerase chain reaction; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor; VLP, virus-like particle; WHO, World Health Organization.

☆ Disclaimer: This work was partially supported by the Defense Threat Reduction Agency, Transformational Medical Technologies project number 0048-09-RD-T (to SB). JHK performed this work as an employee of Tunnell Consulting, Inc., a subcontractor to Battelle Memorial Institute under its prime contract with NIAID, under Contract No. HHSN272200200016I. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the US Army, the US Department of Health and Human Services or of the institutions and companies affiliated with the authors.

* Corresponding author at: United States Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, Frederick, MD 21702, USA. Tel.: +1 301 619 4246; fax: +1 541 754 3545.
E-mail address: sina.bavari@us.army.mil (S. Bavari).

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1. Introduction

Crimean-Congo hemorrhagic fever virus (CCHFV), a member of the genus *Nairovirus* in the family *Bunyaviridae*, causes what today is referred to as Crimean-Congo hemorrhagic fever (CCHF) (Haenni et al., 2005). The disease was first recognized towards the end of World War II, when some 200 Soviet military personnel and peasants fell ill in devastated western Crimea (Chumakov, 1945). However, in 1956, a disease very similar to CCHF befell a child in the Belgian Congo (today the Democratic Republic of the Congo). Between 1956 and 1965, this virus was isolated (Simpson et al., 1965, 1967) and demonstrated to be identical to that causing the Soviet cases (Casals, 1969; Woodall, 2007).

CCHF usually develops after humans have been bitten by ixodid ticks or have been in contact with infected animals (most often livestock) or humans, or their tissues, excreta or secreta (Ergonul and Whitehouse, 2007). The disease occurs in four phases, designated the incubation, prehemorrhagic, and hemorrhagic periods and convalescence (Ergonul, 2007). After an incubation period of 1–9 days, patients present with a nonspecific influenza-like syndrome that typically lasts less than one week (Mardani and Keshtkar-Jahromi, 2007). In some cases, the hemorrhagic period develops rapidly, beginning between the third and fifth day of illness. Circulatory shock and disseminated intravascular coagulation may occur in severe cases (Ergonul, 2007; Mardani et al., 2003; Swanepoel et al., 1989). The clinical symptoms of children and adolescents do not differ from those of adults. In Iran, children seem to be more vulnerable to severe disease than adults, whereas in Turkey lethal cases of CCHF have rarely been observed among children (Sharifi-Mood et al., 2008; Tezer et al., 2010).

The diagnosis of CCHF has generally been based on the detection of anti-CCHFV IgM or a four-fold increase in antibody titer from paired serum samples. However, patients who die during the first four days of disease do not develop antibodies and therefore elude serologic diagnosis (Ergonul, 2006; Vorou et al., 2007). In that case, RT-PCR is recommended, as this test can be highly specific, sensitive, and rapid (Ergonul, 2006). Together, this means that depending on the familiarity of clinicians with the disease, the point in the course of illness and the severity of the patient's condition, CCHF may be difficult differentiate from febrile illnesses.

Today, it is clear that CCHFV is one of the most widely spread tick-borne viruses of medical importance. It is endemic to Asia, Eastern Europe, the Middle East, and central and southern Africa, and it causes human disease in increasing numbers per year in many countries, including Turkey and possibly the successor nations of the Soviet Union (Ergonul and Whitehouse, 2007). Nevertheless, CCHF is a sporadic, uncommon illness even in those countries with a rather rich history of cases (Kazakhstan, Russia, South Africa). Few physicians have had hands-on experience with CCHF patients, medical response times tend to be prolonged because of delayed diagnosis, and the gathering of epidemiological data is hindered by the rural infrastructure in outbreak areas.

WHO classifies CCHFV in WHO Risk Group IV, meaning that maximum biocontainment facilities are recommended for any research that uses infectious virus. In the USA, CCHFV is classified as a Select Agent, a Risk Group 4 Pathogen, and a NIAID Category C Priority Pathogen because of the absence of efficacious prophylactic or treatment regimens. While the classification as a Priority Pathogen has released funds to study CCHFV, the requirement to work with it under Biosafety Level 4 conditions has limited the number of researchers with access to the pathogen. CCHFV is considered a potential biological weapon (Bronze et al., 2002). However, the same technical challenges that hinder progress in the molecular characterization of the virus and the development of effective prophylaxes for or vaccines against it most likely preclude its development as a mass-casualty weapon (Borio et al., 2002). On the other hand, CCHF is of interest to military forces, because it is endemic in many areas where soldiers may be deployed, either for conflicts or humanitarian aid. The fatal infection of an American soldier in southern Afghanistan in 2009 was a grave reminder of the risk posed by this disease (Carter, 2009).

2. Vaccines

Initial attempts to develop vaccines date back to the 1960s, when Soviet scientists advocated the immunization of local populations because of CCHFV endemicity. Nonspecific preventive measures such as tick eradication had proved to be expensive, inefficient and in many instances impractical (Badalov et al., 1969). Researchers of the Soviet Institute of Poliomyelitis and Viral Encephalitides developed an experimental CCHF vaccine based on brain tissue from infected newborn laboratory mice and rats. Brain tissue suspensions were inactivated by formaldehyde and heat treatment to obtain safe, noninfectious preparations. The efficacy of the vaccine was tested using the complement fixation (CF) technique and titrated against immune sera collected from CCHF convalescent patients or experimentally infected animals (Tkachenko et al., 1970). The immunogenicity of the vaccine was evaluated by serial intraperitoneal injections into newborn rats at 7-day intervals. Antibodies of all classes could be detected one week after the first booster injection (Tkachenko et al., 1970). The vaccine did not have adverse affects on the limited number of humans who volunteered to be vaccinated.

In 1970, this inactivated vaccine was approved by the Soviet Ministry of Health for CCHF prophylaxis (Tkachenko et al., 1970). In the same year, serum samples were collected from some 2000 healthy people before vaccination, two weeks after a 2nd injection and two weeks, one month, three to four months, and six months after a 3rd injection. The sera were tested for the presence of anti-CCHFV antibodies using CF and agar gel diffusion and precipitation techniques. Low levels of antibodies ($\leq$5.7% one month after the third injection) were detected, and 42% of samples tested positive using both tests. Neutralizing antibodies developed within 1–4 weeks after the 3rd injection, but titers decreased 3–6 months later (Tkachenko et al., 1971). Approximately 1500 people vaccinated

in 1970 were revaccinated once in 1971, resulting in an increased seroresponse at one month and six months, which correlated with the number of original injections (Tkachenko et al., 1971; Vasilenko, 1973). Unfortunately, no data regarding the protective efficacy of the vaccine have been published.

In 1974, the Soviet vaccine was licensed in Bulgaria and used in CCHFV-endemic areas of the country for military personnel and medical and agricultural workers over 16 years of age. Recently published data from the Bulgarian Ministry of Health suggest a four-fold reduction in the number of reported CCHF cases over a 22-year time period (1953–1974: 1105 cases; 1975–1996: 279 cases) (Christova et al., 2010). Since 1997, less than 20 cases have been reported annually to the ministry, though more have probably occurred. Notably, none of the vaccinated military personnel have contracted CCHF, and none of the vaccinated laboratory personnel working with CCHF virus became infected even after occasional exposures by needle pricking. Although there are no published data regarding the total number of vaccinated civilians who have developed CCHF, these data nevertheless suggest that the vaccine is efficacious (Christova et al., 2010). Of course, it is also possible that case reporting has changed since vaccination was instituted, or that populations have changed their behavior and reduced their tick exposure. It is also possible that the epidemiology of CCHF and the ecology of CCHFV have changed over the years in Bulgaria without any intentional intervention. Regrettably, there are no data regarding the incidence of CCHF in the same time frame in neighboring countries, preventing further analysis of vaccine efficacy.

In a recent Bulgarian study, significant antibody responses were observed in vaccinated volunteers who were regularly immunized at 2-years intervals for the last 15–20 years. Antibody levels were measured before each new booster dose and 2, 3 and 4 weeks later. Using CF assays, titers of 1:2 to 1:16 were detected before re-vaccination, followed by a 2–4-fold increase 2–3 weeks after revaccination. Consistent, high antibody titers were detected by ELISA before and after revaccination. The antibody response before the 3rd injection was either zero, borderline positive, or weakly positive, but a significant increase was detected by both CFA and ELISA thereafter (I. Christova, unpublished data).

As described by the manufacturer, the Bulgarian vaccine consists of *Active substance*: inactivated CCHFV antigen; and *Auxiliary substances*: aluminum hydroxide, thiomersal, sodium hydrogen carbonate, sodium chloride, phenol red as an indicator, tobramycin, and water (Anon., 2008). A dose of 1 mL subcutaneously is followed by a second injection 30–45 days later, re-immunization one year later and then every five years. The vaccine is designed for persons over 16 years of age. For persons who have been treated with CCHF-specific immunoglobulin, 30 days should pass before immunization. Corticosteroid usage or immunosuppressive treatment may render vaccination unsuccessful. Vaccination may be accompanied with local reactions or fever. It is not deemed necessary for persons who have recovered from CCHF. This vaccine requires the use of maximum containment facilities to generate virions for inactivation. There is also concern about using vaccines grown in mouse brains due to possible autoimmune and allergic responses induced by myelin basic protein (Hemachudha et al., 1987; Jelinek, 2008). Additional vaccine platforms should therefore be considered.

Modern vaccine development foresees the establishment of DNA vaccines, recombinant viral protein-based vaccines, and virus-like particle vaccines. However, research has been severely hindered by the absence of CCHF animal models. For instance, American scientists have developed a DNA vaccine containing the CCHF genome M segment and have shown that it elicited neutralizing antibodies in mice, as well as antibodies that immunoprecipitated M-segment expression products (Spik et al., 2006). However, they were unable to evaluate the vaccine's protective efficacy.

3. Animal models

Animal models of CCHF have until recently been limited to intracranial or intraperitoneal infection of newborn mice or rats with the suckling mouse-passaged IbAr 10200 or Hodzha CCHFV strains (Smirnova, 1979; Smirnova et al., 1973; Tignor and Hanham, 1993). Although the use of these animals has generated some interesting results, newborn mice are unusually susceptible to a wide array of pathogens, and their usefulness as disease models is questionable. However, CCHFV-infected adult mice, rats, guinea pigs, hamsters, rabbits, sheep, calves, donkeys, nonhuman primates, and other adult animals exhibit low to undetectable viremia and clear the infection without overt signs of illness (Fagbami et al., 1975; Shepherd et al., 1989; Smirnova, 1979).

Recently, two models of lethal CCHFV infection have been reported in adult mice with defective interferon responses (Bente et al., 2010; Bereczky et al., 2010). In the first model, knockout mice lacking the signal transducer and activator of transcription-1 (STAT-1) protein died 3–5 days after infection with low doses of the mouse-passaged CCHFV strain IbAr 10200, and developed thrombocytopenia and leukopenia, as well as increased serum ALT levels, which are also found in lethal human infections, supporting the validity of STAT-1 KO mice as a model of human CCHF (Bente et al., 2010; Joubert et al., 1985; Swanepoel et al., 1989). The animals also developed characteristic histopathologic lesions, including necrosis in the livers and lymphocyte depletion in the spleens, but interstitial pneumonia and intestinal hemorrhage, found in lethal human infections, were not apparent. Analysis of immune cells suggested activation of macrophages, dendritic cells, and NK cells in lethal infection, although there was no comparison with these cells in infected wild-type mice. Infected mice also had elevated serum IL-6, IL-10, and TNF-α concentrations, which correlate with findings in some human cases (Ergonul et al., 2006; Papa et al., 2006). The model was also used to demonstrate that ribavirin therapy could prevent lethal infection.

The second model employs knockout mice lacking the cell-surface IFN-α/β receptor ($IFNAR^{-/-}$), in which infection with CCHFV strain IbAr 2000 was lethal within 2–4 days (Bereczky et al., 2010). Viral replication was highest in the liver and spleen, and up to 10,000-fold higher in $IFNAR^{-/-}$ mice than in immunocompetent mice. Enlarged, hemorrhagic livers were found in $IFNAR^{-/-}$ mice infected with lower (10^5, 10^3 and 10^1 focus-forming units), but not higher (10^6 focus-forming units), doses of virus.

Both mouse models can potentially serve as platforms for studies aiming to analyze the pathogenesis and treatment of CCHF, such as inflammatory cytokine production or the effectiveness of immune serum therapy. Vaccine studies, which were not feasible in newborn mice, might also be applicable using these platforms. Whether or not CCHFV can be adapted for studies in other animals, it is possible that suppression of type I interferon responses would allow for lethal infection. The use of humanized mice may also be useful for further model development (Brehm et al., 2010). It is now necessary to focus on the further characterization of these new models, since at least in the USA, two well-characterized animal models reflecting human disease parameters should ideally be available for the approval of any drug or vaccine for human use, under the FDA "Animal Rule."

Although it appears that mouse models can be developed further to satisfy the stringent FDA requirements for drug approval, the current lack of a CCHF model in higher mammals, such as nonhuman primates, raises the question how new drugs and vaccines could be tested in human populations without the use of the "Animal Rule." Could the evaluation of new treatments be ethically justified within international collaborations in countries with less stringent rules for drug testing? If so, which countries would participate in such trials, and under what circumstances? Is CCHF so "exotic" a disease

that the development of new therapies can be postponed until a satisfactory animal model has been found? The initiation of such discussions is an urgent matter that should not be further delayed.

4. Treatment

Most CCHF patients receive only supportive therapy. Although ribavirin has been employed for over 25 years for the prophylaxis and therapy of CCHF, its efficacy remains controversial. Below we summarize all major published reports in the English-language literature of the clinical use of ribavirin for CCHF, and then comment on the need for further investigation of its therapeutic benefit. We then briefly review experience with the use of specific immunoglobulin for the treatment of CCHF in the former Soviet Union and Bulgaria and the potential development of monoclonal antibody therapies.

4.1. Supportive therapy

CCHF patients must be monitored closely for effective support. Measurement of the complete blood count, serum electrolyte levels, and coagulation indices is crucial, and transfusions with blood products must be carefully considered, based on individual deficits. Medical staff should be aware of the possibility of life-threatening hemorrhages; non-steroidal anti-inflammatory drugs are therefore contraindicated, and intramuscular injections should be avoided (Ergonul, 2006). In Turkey, two cases of CCHF complicated by hypothermia and severe hemorrhagic manifestations were recently treated successfully with only fresh frozen plasma, crystalloid, and colloid infusions, indicating that supportive measures may be sufficient even in severe cases (Yilmaz et al., 2009).

4.2. Ribavirin

4.2.1. Activity in vitro and in laboratory animals

Ribavirin is a purine nucleoside analogue with broad-spectrum antiviral activity. Indirect (inosine monophosphate dehydrogenase inhibition, immunomodulatory effects) and direct mechanisms (interference with mRNA capping, polymerase inhibition, lethal mutagenesis) have been proposed to explain its antiviral activity (Graci and Cameron, 2006). The efficacy of ribavirin against CCHFV was first described *in vitro* in 1989, when the drug markedly reduced viral yields of variants from Europe, Asia, and Africa (Watts et al., 1989). Although some isolates appeared to be more sensitive than others, CCHFV proved in general to be more sensitive to ribavirin than a bunyaviral relative, Rift Valley fever virus (genus *Phlebovirus*). A recent study confirmed that ribavirin inhibited CCHFV replication according to plaque-reduction assays, and no significant differences in drug sensitivity of different viral isolates were observed (Paragas et al., 2004). In a study in suckling mice using the mouse-passaged IbAr 10200 CCHF strain, ribavirin significantly reduced lethality, increased the mean time to death, reduced viral replication in the liver and prevented infection of brain and heart tissues (Tignor and Hanham, 1993). The protective activity of ribavirin has also been demonstrated in CCHFV-infected STAT-1 knockout mice (Bente et al., 2010).

4.2.2. Ribavirin efficacy in CCHF patients

4.2.2.1. Observational studies. The first reported clinical use was during a nosocomial outbreak in a South African hospital in 1985 (Table 1) (van de Wal et al., 1985). Six of 9 healthcare workers with penetrating injuries from CCHFV-contaminated needles were treated with intravenous ribavirin and interferon-α. Of the 3 untreated individuals, 1 developed mild disease and the 2 others developed severe CCHF. In contrast, 4 of the 5 ribavirin-treated healthcare workers remained asymptomatic and did not develop measurable anti-CCHFV antibodies, while the fifth suffered only a mild illness. However, another 42 individuals who had been exposed to contaminated blood remained healthy without treatment, making it unclear whether ribavirin therapy actually had an effect.

In 1995, the use of oral ribavirin was reported in 3 severe nosocomial CCHF cases in Pakistan (Fisher-Hoch et al., 1995). All patients became afebrile within 48 h of the initiation of treatment and recovered completely. In another nosocomial outbreak in Pakistan, ribavirin was used to treat 1 of 2 secondary CCHF cases, who survived (Athar et al., 2003). During another Pakistani CCHF outbreak, 9 patients were treated with oral ribavirin, and 4 survived (Smego et al., 2004). In 2003, in a nosocomial outbreak in a tertiary care hospital in Pakistan, ribavirin was administered to workers involved in the care of the index case (Bangash and Khan, 2003). The secondarily infected patients recovered completely, and none of the 11 health care workers became ill. Finally, it was reported that 6 and 10 CCHF patients in the Golestan Province of Iran and in Istanbul, Turkey, respectively, survived after being treated with ribavirin (Jabbari et al., 2006; Midilli et al., 2007).

4.2.2.2. Historical comparisons. In 2003, Iranian researchers compared case fatality rates among 139 suspected and 69 confirmed CCHF patients, based on oral ribavirin treatment (Mardani et al., 2003). For those with confirmed disease, the survival rate was 69.8% for treated patients and 41.7% for untreated cases, while for patients with suspected CCHFV, it was 88.4% for treated and 54.2% for untreated cases. The efficacy of oral ribavirin was determined to be 80% among confirmed and 34% among suspected CCHF patients.

Ozkurt et al. (2006) compared 22 CCHF patients treated with oral ribavirin to a historical cohort of 38 untreated patients. The case fatality rate was 9.0% in treated and 10.5% in the untreated group, a difference that was not statistically significant ($p = 0.85$). The recovery period was shorter in the treated group, but the need for blood products was the same, and the mean hospitalization time and total hospital expenditure did not differ between the groups. However, the groups were not matched for disease severity.

In another study in Iran in 2006, Alavi-Naini et al. evaluated the efficacy of oral ribavirin, and found that 37 (15.7%) of 236 treated patients and 63.2% of 19 untreated patients died (Alavi-Naini et al., 2006). The efficacy of treatment was determined to be 75% and the relative chance of recovery in the treated group was 2.29 times higher than for untreated cases.

Recently, 218 Turkish CCHF patients were retrospectively evaluated for clinical outcome based on oral ribavirin treatment (Elaldi et al., 2009). The case-fatality rate was 9/126 (7.1%) in the treated group and 11/92 (11.9%) in the untreated group. The average interval between disease onset and ribavirin administration was not significantly different among fatal and nonfatal cases in the treated group (4.4 days vs. 5.8 days; $p = 0.11$), and there was no significant difference in the clinical outcome of patients treated within 3 days of disease onset, compared to those treated later ($p = 0.14$). However, this study was criticized for faults in statistical analysis and study design (Ergonul, 2009).

4.2.2.3. Non-randomized clinical trials. In a study of 35 CCHF patients reported from Turkey in 2004, in which 30 patients had severe and 5 mild illness, the overall case-fatality rate was 2.8% (Ergonul et al., 2004). Oral ribavirin was given to 8 patients with severe disease, all of whom survived, whereas the case-fatality rate was 4.5% in the 22 patients with severe disease who did not receive the drug.

In 2009, Tasdelen Fisgin et al. evaluated the efficacy of oral ribavirin in Turkish CCHF patients: 21 cases treated within 4 days after the appearance of symptoms and 20 treated beginning 5 days or longer after disease onset (Tasdelen Fisgin et al., 2009). Eleven

Table 1
Summary of literature published since 1985 on the efficacy of ribavirin therapy of CCHF.

Country	Treated/total cases	Study type	Prophylaxis or treatment	Reference
South Africa	6/9	Observational	Prophylaxis	van de Wal et al. (1985)
Pakistan	3/3		Treatment	Fisher-Hoch et al. (1995)
Pakistan	2/2		Treatment	Athar et al. (2003)
Pakistan	12/12		Prophylaxis	Bangash and Khan (2003)
Pakistan	9/9		Treatment	Smego et al. (2004)
Iran	6/6		Treatment	Jabbari et al. (2006)
Turkey	10/10		Treatment	Midilli et al. (2007)
Iran	61/69		Treatment	Mardani et al. (2003)
Iran	236/255	Historical comparison	Treatment	Alavi-Naini et al. (2006)
Turkey	22/60		Treatment	Ozkurt et al. (2006)
Turkey	126/218		Treatment	Elaldi et al. (2009)
Turkey	10/50		Treatment	Bodur et al. (2011)
Turkey	8/30	Non-randomized clinical trial	Treatment	Ergonul et al. (2004)
Turkey	9/25		Treatment	Cevik et al. (2008)
Turkey	41/52		Treatment	Tasdelen Fisgin et al. (2009)
Iran	184/184	Comparison to evaluate timing	Treatment	Metanat et al. (2006)
Iran	63/63		Treatment	Izadi and Salehi (2009)
Iran	155/155		Treatment	Sharifi-Mood et al. (2009)
Turkey	64/136	Randomized clinical trial	Treatment	Koksal et al. (2010)

patients were untreated. At days 5–10 after disease onset, the mean platelet counts of the patients who were treated early in their illness were significantly higher than those of patients treated late, and at days 7–9, they were significantly higher than those of the untreated patients. The case fatality rate among early treated patients (5%) was lower than late treated (10%) and untreated patients (27%), but the difference was not statistically significant.

In one of the few studies to evaluate the efficacy of intravenous ribavirin, Cevik and colleagues compared outcomes in 9 severely ill patients, compared to 16 untreated controls (Cevik et al., 2008). They found no statistically significant differences between the 2 groups in terms of case fatality rate, mean duration of hospitalization or the need for blood products, and they accordingly concluded that treatment had no beneficial effect. However, as in the case of the study by Tasdelen Fisgin et al., this report should be interpreted cautiously, as it lacks the statistical power to reach a definite conclusion.

Turkish scientists recently published a retrospective case–control study of the effect of oral ribavirin on viral load and disease progression, which included 10 patients who received the drug for 10 days and 40 who received only supportive therapy (Bodur et al., 2011). There was no significant difference in viral load between the case and control groups at the time of hospital admission. During follow-up, no statistically significant differences were found in the decrease in viral load, reduction in liver enzyme concentrations, increase in platelet count or the case fatality rate. These results suggest that oral ribavirin had no positive effect, but one should note that the number of treated patients was small.

4.2.2.4. Efficacy at different time points. In a case-control study in 2006 in Iran, 84% of the 89 patients who were treated with oral ribavirin beginning within the first 72 h of illness onset recovered from the disease, whereas the survival rate of the 95 patients whose treatment began after 72 h was 74.8% (Metanat et al., 2006). In another Iranian study, in which 47 of 63 treated patients survived, those treated individuals who survived infection received their initial therapy 24 h earlier on average than treated patients who died (Izadi, 2009). The interval between the onset of disease or hemorrhage and the initiation of ribavirin administration was the most important variable correlated with survival (Izadi and Salehi, 2009).

In 2009, researchers in the Sistan and Baluchistan Provinces of Iran described a significant difference in recovery among 32 people treated with ribavirin between 2005 and 2007, compared to 123 people treated in 1999–2004 (Sharifi-Mood et al., 2009). All patients from 2005 to 2007 were treated within the first 72 h of illness onset, whereas only 79% of those from 1999 to 2004 were treated within that time frame. The case fatality rate among the 2005–2007 cases (3%) was significantly lower than in the 1999–2004 group (22%) ($p = 0.001$). Although these data support the early administration of ribavirin, other confounding variables, such as the time of diagnosis and the type of supportive clinical measures should be considered when evaluating the efficacy of therapy.

4.2.2.5. Randomized clinical trials. One hundred thirty-six Turkish CCHF patients were randomized, such that Group A ($n = 64$) received oral ribavirin and supportive therapy, while group B ($n = 72$) received only supportive therapy (Koksal et al., 2010). The two groups were matched for baseline demographic features. There was no statistically significant difference between the two groups in the incubation period, clinical presentation, laboratory findings, time of hospitalization, requirement for platelet infusions, time needed for normalization of platelet counts or survival. Not only did the ribavirin-treated patients not survive longer than the control group, but also their leukocyte counts remained abnormal for a longer period of time.

4.2.3. Ribavirin therapy: conclusions

As the variety of conclusions reached by the publications summarized above has shown, the efficacy of ribavirin for the prophylaxis and therapy of CCHF is still an open question. Early anecdotal reports described the recovery of severely ill patients treated with the drug, but more recent studies, including a large randomized clinical trial, have found few or no differences in the course and outcome of illness of treated and untreated patients. Unfortunately, underlying variation in patient populations and the failure of investigators to match cases and controls for disease severity, stage of illness at initiation of treatment and other factors have limited the value of many studies. Because the use of ribavirin is now well established in most countries where CCHF is endemic, there may be ethical objections to performing placebo-controlled trials. Researchers should therefore consider how ribavirin therapy might be further evaluated without violating ethical guidelines. In the meantime, it appears justifiable to continue to administer the

drug to suspected cases of CCHF in endemic areas until its efficacy has been definitively determined.

4.3. Specific immunoglobulin

4.3.1. Intramuscular anti-CCHF immunoglobulin

The idea of treating CCHF patients with specific immunoglobulin was first proposed by Chumakov in 1944 (Chumakov, 1945). In 1967, however, Leshchinskaya reported the lack of efficacy of intramuscular injections of convalescent serum in Soviet CCHF patients (Leshchinskaya, 1967). In 1970, the same researchers treated 61 patients with 80 mL of convalescent serum injected intramuscularly once or twice daily up to day 4 following the onset of hemorrhagic symptoms, and compared them to 88 untreated patients matched in disease severity (Leshchinskaya and Martinenko, 1970). No statistically significant differences in fever duration or lethality were observed, and virus was isolated from the blood even in patients who had received sera for 2 or 3 days. For future studies, the researchers recommended administering convalescent sera by the intravenous route, at earlier time points, for longer duration, in volumes greater than 200 mL and with higher immunoglobulin titers. Between 1964 and 1968, 98 patients were inoculated with convalescent sera during the first 2–3 days of disease, resulting in a better outcome compared to treatment after the 4th day (Lazarev, 1969).

In 1980, a CCHF patient in a nosocomial outbreak in Dubai who was treated with 300 mL of convalescent serum survived his illness, and his convalescent period was shorter than that of other survivors (Suleiman et al., 1980). During another nosocomial outbreak in South Africa in 1985, hyperimmune serum was used to treat 5 patients (van Eeden et al., 1985). Two patients received 1, and 3 patients received 2 injections of 250 mL of serum intravenously, in addition to supportive therapy. Four patients showed symptomatic improvement for at least 12 h after the first dose, and again after the second dose. The five patients who received serum all survived. In contrast, two untreated patients who died showed no antibody response at the time of death, suggesting that immune serum therapy could be of decisive importance for survival. The authors suggested that massive and continuous infusion of hyperimmune serum, continuing for 48–72 h, would more effectively influence the disease outcome.

In Bulgaria, specific intramuscular human immunoglobulin (*CCHF-Bulin*), derived from the plasma of convalescent patients, has been used since 1975 for the post-exposure prophylaxis of persons who have been in contact with suspected CCHF cases. Such individuals receive 3 mL of immunoglobulin, whereas patients with suspected CCHF receive 6 mL, and confirmed cases receive 6–9 mL on days 1–5, or until a therapeutic effect is achieved (Anon., 2008). Case–control studies of the efficacy of immunoglobulin prophylaxis and therapy have not been published.

4.3.2. Intravenous anti-CCHF immunoglobulin

Intravenous anti-CCHF immunoglobulin (CC*HF-Venin*) was tested in 1989 in 7 CCHF patients with severe hemorrhagic manifestations in Bulgaria (Vassilenko et al., 1990). Thirty milliliters were administered together with 30 mL of *CCHF-Bulin* and other general supportive measures. All 7 patients recovered quickly, without side effects, and their leukocyte, platelet, and coagulation abnormalities returned to normal. Unfortunately, this study did not include a control group, and as for *CCHF-Bulin*, data on the efficacy of *CCHF-venin* based on case–control studies are lacking.

4.3.3. Conclusions: immunoglobulin therapy

None of the studies described above have proven the efficacy of specific immunoglobulin for the post-exposure prophylaxis or treatment of CCHF, so these products should be further evaluated in well designed clinical trials. To avoid depriving patients of potentially beneficial therapy, one study group might receive ribavirin, while a second group would receive ribavirin plus immune globulin. Such an investigation might be carried out collaboratively between countries that have supplies of CCHF-specific immunoglobulin, such as Bulgaria, and those that experience a higher incidence of disease, such as Turkey or Iran. Alternatively, countries with a higher incidence of CCHF might prepare their own stocks of immune serum and evaluate them as described above.

4.3.4. Monoclonal antibodies

Monoclonal antibodies (mAbs) were first used for CCHFV identification in 1987 (Blackburn et al., 1987). Scientists are now attempting to develop anti-CCHFV mAbs for the treatment of patients. mAbs specific to both the Gn and Gc surface glycoproteins were generated to evaluate their neutralization and protection properties; mAbs to Gc, but not Gn, neutralized CCHFV in SW-13 cell cultures (Bertolotti-Ciarlet et al., 2005). However, only a subset protected mice after passive immunization, whereas some non-neutralizing mAbs to Gn protected mice from lethal CCHFV challenge. It was concluded that neutralization depends not only on the properties of the antibody, but also on host factors and mechanisms such as antibody-dependent, cell-mediated cytotoxicity.

5. Summary

The only available and probably somewhat efficacious CCHF vaccine is an inactivated antigen preparation currently used in Bulgaria. More modern vaccines are under development, but the sporadic nature of the disease even in endemic countries suggests that large trials of vaccine efficacy will be difficult to perform. Finding volunteers may prove challenging, given the growing resistance of populations to vaccines against contagious diseases such as measles or poliomyelitis. The number of people to be vaccinated and the length of time they would have to be followed to confirm protection would have to be carefully defined. Alternatively, many scientists appear to believe that treatment of CCHF with ribavirin is more practical than prevention, but some recently conducted clinical trials appear to counter assumptions of drug efficacy. Immunoglobulin preparations have been used for more than 30 years to prevent and treat CCHF in Bulgaria, but few data have been published, and their efficacy remains unproven. Although recent developments in antibody engineering have raised hopes for novel mAb therapies, this approach remains in its infancy. Research now relies on two recently developed mouse models for the evaluation of antibodies, antivirals and other forms of prophylaxis and therapy, but the predictive value of testing in these immunodeficient animals has not been defined. Given these many obstacles to progress, CCHF will clearly remain a major challenge to the infectious disease community for the foreseeable future.

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Review

New opportunities in anti-hepatitis C virus drug discovery: Targeting NS4B

Roopa Rai*, Jerome Deval**

Alios BioPharma, 260 E. Grand Ave., South San Francisco, CA 94080, USA

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ABSTRACT

Current therapy for chronic hepatitis C virus (HCV) infection constitutes a combination of pegylated interferon alfa-2a or alpha-2b and ribavirin. Although successful for many patient populations, this regimen has numerous limitations, including non-response, relapse, poor tolerability and long duration of treatment. To address these shortcomings, new small molecule agents are advancing in clinical development. Most of the current clinical candidates act by directly inhibiting key enzymes in the viral life-cycle: the NS5B polymerase, or the NS3/4A protease. Less well-studied, the non-structural 4B (NS4B) protein has recently emerged as an alternative target for Direct-acting Antiviral Agents (DAAs). NS4B is a 27-kDa membrane protein that is primarily involved in the formation of membrane vesicles – also named membranous web – used as scaffold for the assembly of the HCV replication complex. In addition, NS4B contains NTPase and RNA binding activities, as well as anti-apoptotic properties. This review summarizes the current understanding of the structure and functions of NS4B, an essential component of the replication machinery of HCV. In this literature and patent review, we report the recent developments in anti-NS4B drug discovery. These advances open the possibility for future combination therapies with other DAAs.

Contents

1. Introduction

1.1. Burden of HCV infection and unmet medical need

Hepatitis C virus (HCV) infection is a serious public health concern that affects 170 million people worldwide (Shepard et al., 2005), for which no vaccine is available. Among those infected, approximately 20–30% develop severe liver disease, such as chronic hepatitis, liver cirrhosis, or hepatocellular carcinoma (Alter and

Abbreviations: HCV, hepatitis C virus; kDa, kilo Dalton; NS4B, non-structural protein 4B; NTPase, nucleoside triphosphatase; ssRNA, single-stranded RNA; DAA, Direct-acting Antiviral Agent; ER, endoplasmic reticulum; GT, genotype; AH, amphipatic helix; ATP, adenosine triphosphate; ADP, adenosine diphosphate; Arg, arginine; Ala, alanine; Cys, cysteine; GFP, green fluorescent protein; IFN, interferon; UTR, un-translated region; TM, trans-membrane; IRES, internal ribosome entry site.

* Corresponding author. Tel.: +1 650 635 5507; fax: +1 650 872 0584.
** Corresponding author. Tel.: +1 650 635 5524; fax: +1 650 872 0584.
E-mail addresses: roopa.rai@hotmail.com (R. Rai), jdeval@aliosbiopharma.com, jeromedeval@hotmail.com (J. Deval).

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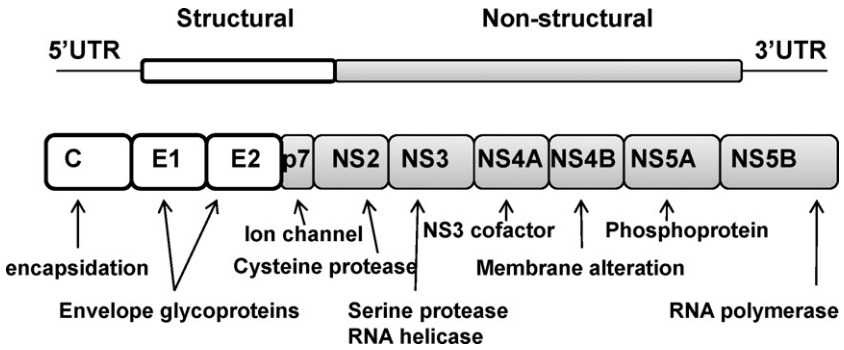


Fig. 1. Organization of the HCV genome. The RNA genome encodes a single open reading frame, flanked by the 5′ and 3′ un-translated regions (UTRs). The polyprotein of approximately 3000 amino acids is processed by host and viral proteases, and results in ten functional structural and non-structural proteins.

Seeff, 2000). The combined use of the nucleoside analog ribavirin and pegylated interferon alpha is the current standard of care. However, success in treatment depends largely on the viral genotype. For instance, the rate of viral clearance upon current standard of care is only ~50% with genotype 1, the most prevalent circulating strain in Western Europe and North America. Additionally, this drug combination has also been associated with severe side effects such as fatigue, nausea and depression, therefore precluding treatment for many individuals (Russo and Fried, 2003; Zeuzem et al., 2000). All of these issues justify the need to develop novel, more efficacious and safer anti-HCV drugs.

1.2. New targets, promising compounds

In recent years there has been significant breakthrough in identifying essential functions within HCV replication that can be directly targeted for antiviral therapy. The HCV RNA genome encodes a polyprotein that is processed into ten smaller polypeptides, including the capsid protein (C), the envelope proteins (E1 and E2), an ion channel (p7), and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Fig. 1). The first proteins to be clinically validated as therapeutic targets for Direct-acting Antiviral Agents (DAAs) were the NS3/4A protease and the NS5B polymerase. These two proteins can be inhibited with small molecules (De Francesco and Rice, 2003; Lemon et al., 2010). Similarly to what has been achieved for HIV, the advanced development of protease and polymerase inhibitors brings hope for new treatment options in the next few years. This is exemplified with Telaprevir, and Boceprevir (two NS3/4A protease inhibitors) and RG7128 (a nucleoside NS5B polymerase inhibitor) that have reached advanced clinical trials (Beaulieu, 2009; Kwong et al., 2008). NS3/4A protease and NS5B polymerase are non-structural proteins with well defined enzymatic functions. In addition, there has been a concerted effort to elucidate the specific functions of other non-structural proteins and to determine whether targeting them could lead to novel antiviral therapies. Towards this end and currently in Phase I clinical trials, NS5A-binding molecules have proven themselves to be particularly potent in suppressing HCV replication, both *in vitro* and *in vivo* (Gao et al., 2010; Lemm et al., 2010).

The less well characterized NS4B protein is also emerging as another potentially attractive target for antiviral drug discovery. Although no anti-NS4B molecule has been shown to be efficacious in clinical trials yet, there is an increasing body of evidence from *in vitro* studies that small molecules could suppress HCV replication by altering one of the recently described functions of NS4B. This review provides a comprehensive summary of the biological roles of NS4B within the HCV life cycle. We also report the current public information, from both research articles and patents, related to the inhibition of NS4B for the development of novel DAAs. The implications of using NS4B functional assays to discover and develop novel HCV inhibitors are also discussed.

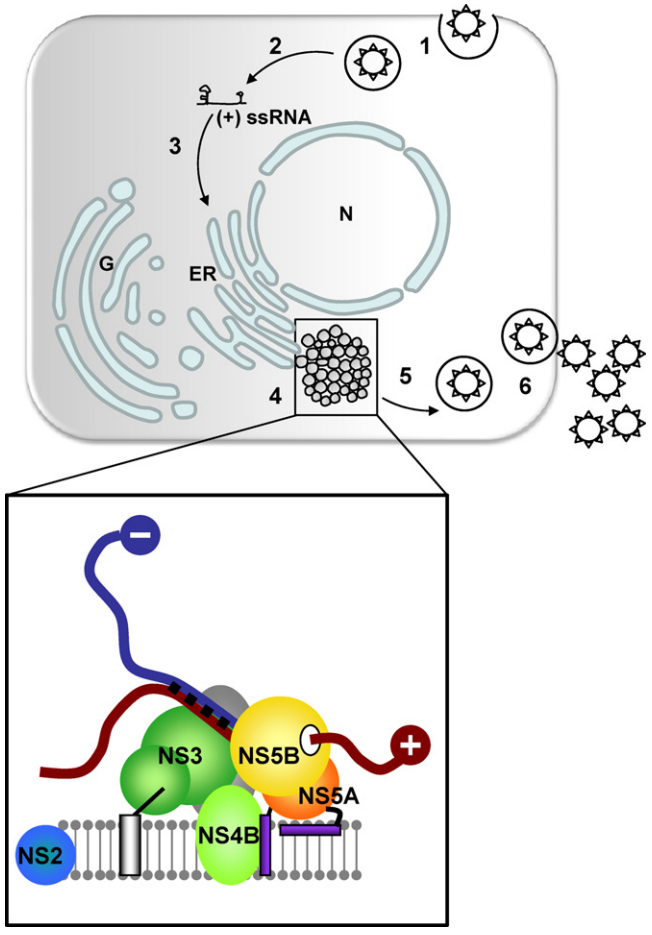


Fig. 2. The HCV life cycle. Following (1) virus attachment and endocytosis, (2) the positive single-stranded RNA ((+) ssRNA) is released by membrane fusion or uncoating. (3) The viral RNA is then recognized by the translation complex through the internal ribosome entry site (IRES). Polyprotein translation and processing take place within the endoplasmic reticulum (ER). (4) Expression of the viral proteins induces lipid rearrangements that support viral RNA synthesis, as well as (5) virion assembly and (6) release. Inset: amplification of viral (+) strand RNA using the (−) strand intermediate occurs through the formation of a replication complex formed by NS5B, the RNA polymerase, as well as the other non-structural proteins.

2. Structural and functional characterization of NS4B

2.1. Structural organization and surface topology

The replication cycle of HCV is depicted in Fig. 2. HCV infects hepatocytes by endocytosis (Dubuisson et al., 2008). After endosomal fusion, the HCV genome made of single stranded RNA (ssRNA)

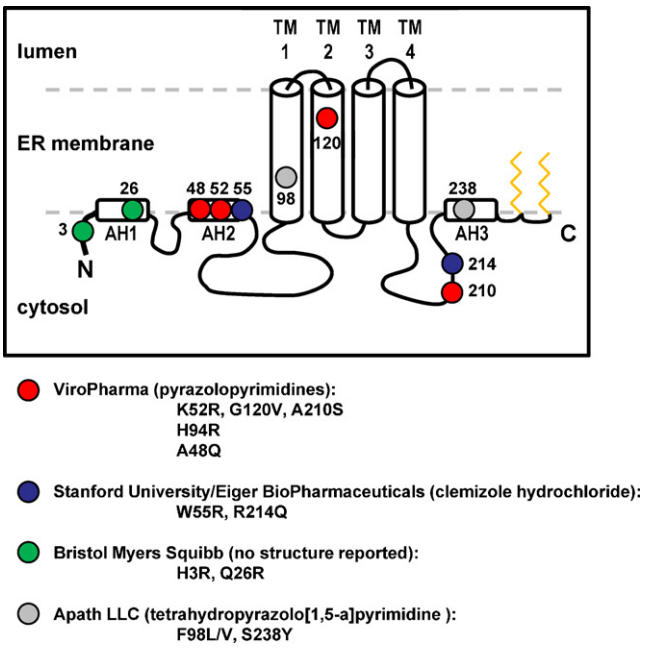


Fig. 3. Surface topology of NS4B and mapping of resistance mutations within NS4B. NS4B is a membrane protein that contains four transmembrane (TM) segments and three amphipatic helices (AH). The two palmitoylation sites are represented in yellow at the C-terminus of the protein. Amino acid changes associated with drug resistance are depicted in circles, with color coding for the different compound classes.

is translated in the cytoplasm to enable the formation of the structural and non-structural proteins (for complete review: Dubuisson, 2007). The non-structural proteins, including NS4B, are associated with the membrane of the ER to form the replication complex that amplifies viral RNA.

NS4B is a hydrophobic protein of 27 kDa, released from the polyprotein precursor after cleavage by the serine protease NS3/4A. During viral replication, NS4B co-localizes with all the other non-structural proteins to the ER in a specific membrane microdomain with lipid raft-like properties (Aizaki et al., 2004; Hugle et al., 2001). When expressed individually, NS4B is also found as an integral part of the ER (Hugle et al., 2001). In the same study it was shown that the majority of the protein is oriented towards the cytoplasm (Fig. 3). Based on computational prediction from primary amino acid structure analysis, NS4B is expected to contain at least 4 trans-membrane domains, of which only two were confirmed experimentally by introducing N-glycosylation sites along the protein sequence (Lundin et al., 2003). Additional anchorage points to the ER membrane are provided by lipid modifications (palmitoylation) on two cysteine residues (cysteines 257 and 261) at the extreme C-terminal end of the protein (Yu et al., 2006). These two residues are believed to facilitate oligomerization of NS4B, as demonstrated by site directed mutagenesis experiments.

In addition to the trans-membrane domains, several N- and C-terminal amphipatic helices have been identified. Elazar et al. (2003) was the first to report membrane association at amino acid residues 6–29, although these findings could not be reproduced in a subsequent study (Gouttenoire et al., 2009a). Two other helical regions have recently been identified: AH2 between positions 42 and 66, and AH3 between positions 229 and 253 (Gouttenoire et al., 2009a,b) (Fig. 3). In each case, alanine substitution of the conserved residues on the hydrophobic helix side abrogated membrane association and disrupted the formation of a functional replication complex.

2.2. Biological functions of NS4B in HCV replication

Early on, cytoplasm alterations in hepatic tissues from HCV-infected chimpanzees were documented (Pfeifer et al., 1980). Such alterations are caused by the presence of an intracellular membranous web during HCV replication, which can be detected by electron microscopy (Egger et al., 2002; Gosert et al., 2003). These membranes consist of aggregates of membrane vesicles that are believed to be derived in part from the ER. Expression of NS4B alone is sufficient to induce these morphological changes (Egger et al., 2002; Lundin et al., 2003), and it is now recognized that one of the main roles of NS4B is to reorganize intracellular membranes into distinct membranous structures at the site of viral replication inside infected cells. Thus, NS4B has been proposed to play a structural role in RNA replication by serving as the scaffold for replication complex assembly (for complete review: Gouttenoire et al., 2010). Membrane alteration is a commonly observed feature among plus-strand RNA viruses, and there have been speculations that it provides an expandable membrane source for virion formation, in addition to preventing the activation of host defense (Aizaki et al., 2004; Miller and Krijnse-Locker, 2008).

It is well recognized that, in cell culture experiments, adaptive mutations located in NS4B can increase RNA replication (Elazar et al., 2004; Lindstrom et al., 2006; Lohmann et al., 2003; Lundin et al., 2003). Moreover, it has recently been shown that NS4B can also modulate the production of infectious particles, therefore pointing towards a role of the protein in virus assembly and release (Jones et al., 2009).

NS4B also contains an ATP/GTPase function. The first report of a nucleotide-binding motif appears in a patent issued in 1999 (Delvecchio et al., 1999). The authors report an ATP-binding consensus sequence G*x*x*G*x*G*K (Walker A motif, where *x* indicates any amino acid) contained within amino acids 1712–1972 of the HCV polyprotein, that corresponds to the NS4B region. This motif, together with another downstream D*xx*G region, is perfectly conserved across all genotypes of HCV and cannot be modified without impairing HCV RNA replication (Einav et al., 2004). The described conserved regions are responsible for the hydrolysis of ATP to ADP, as well as GTP to GDP (Delvecchio et al., 1999; Einav et al., 2004). Overall, the slow intrinsic conversion rates for both substrates suggest that the NS4B NTPase function might require interaction with a protein partner to reach full activation (Thompson et al., 2009). Recently, Einav et al. (2008a) reported that NS4B also specifically recognizes the 3′ terminus of the negative strand of the HCV RNA genome. The authors employed a microfluidic affinity device to measure RNA binding. They identified key arginines at the C-terminal region of NS4B (positions 192–193 and 247–248) that are required for RNA binding and HCV replication. Double mutations from Arg-Arg to Ala-Ala at any of these two positions reduced the binding affinity of NS4B to RNA. While novel and exciting, the precise role of this newly discovered function for NS4B in viral replication remains to be further elucidated. Notably, the biochemical functions that were described are reminiscent of the protein 2C from the picornavirus family, a protein that has been associated with cellular membrane rearrangements (Samuilova et al., 2004, 2006).

Although less well supported, NS4B has been associated with a number of additional functions. This includes cell transformation, a function that has been proposed to provide a mechanism for the development of hepatocellular carcinoma (Park et al., 2000). This anti-apoptotic effect has been recently linked to the NTPase activity of NS4B (Einav et al., 2008b). Also, NS4B has been shown to interact with NS5B, that might modulate the RNA polymerase activity of the latter protein (Piccininni et al., 2002). Finally, NS4B can adopt an oligomerization state (Yu et al., 2006). The main oligomerization determinants were mapped at the N-terminus of the protein,

Table 1
Compounds identified as NS4B binding molecules.

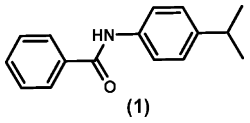
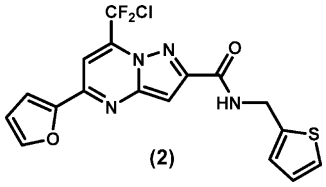
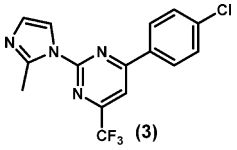
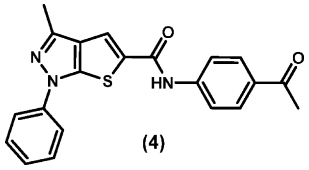
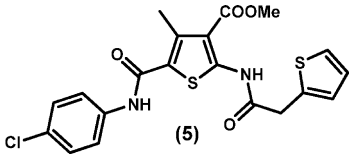
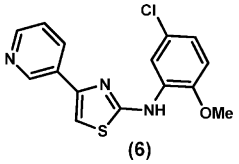
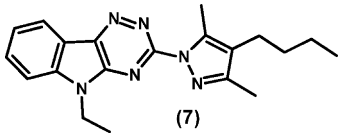
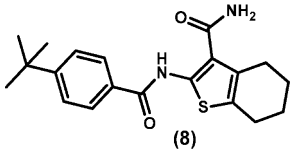
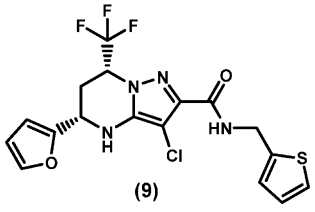
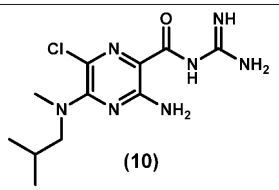
Patent #	Compound class (Example#, Table# in patent)	Structure	EC_{50} (μM)	CC_{75} (μM)
ViroPharma US 2007/0269420	Phenyl benzamide (Example 4, Table 1)	 (1)	2.8	100
	Pyrazolo pyrimidines (Example 3, Table 2)	 (2)	0.63	>100
	Trifluoromethyl pyrimidine (Example 1, Table 3)	 (3)	2.2	>100
	Thienopyrazole (Example 1, Table 4)	 (4)	3.2	50
	Aminothiophenes (Example 1, Table 5)	 (5)	0.7	75
	Phenylthiazolylamines (Example 1, Table 6)	 (6)	1.5	50
	Triazinoindoles (Example 1, Table 7)	 (7)	1.13	20 (CC_{50})
	Tetrahydrobenzothiophenes (Example 1, Table 8)	 (8)	0.87	n/a
Apath, LLC WO 2010/096115	Tetrahydropyrazolopyrimidine-2-carboxamides: 6 compounds specifically claimed (#AP 80978)	 (9)	0.45	100 (CC_{50})

Table 2
Compounds identified as inhibitors of vesicle formation.

Patent #	Compound class (Example# in patent)	Structure	EC_{50} (μM)	CC_{50} (μM)
Stanford University WO 2010/039195;	Amiloride analogs 38 compounds claimed (Compound 10 specifically claimed as C4)	 (10)	3	>5

within the AH2 helix region (Welker et al., 2010). In addition, the C-terminal palmitoylation also contributes to this function through Cys-261, which might be critical for HCV genome replication (Yu et al., 2006).

3. NS4B as an emerging target for HCV therapy

3.1. The pyrazolopyrimidines and other ViroPharma Inc. compounds

One of the first reports of an inhibitor of NS4B comes from a patent issued by ViroPharma Inc. (Chunduru et al., 2007). Using an NS4B-expressing cell line, they identified small molecules inhibiting the anti-apoptotic effect of NS4B. Compound selectivity was measured by the capacity to induce cell-death only in cells expressing NS4B, and binding to the target was confirmed by quenching of intrinsic fluorescence from tryptophan residues contained within the purified NS4B. Using this method, the authors identified eight chemical families or compound classes that were able to inhibit HCV replication. Representative examples from each compound class (Compounds 1–8) are shown in Table 1 These compounds were evaluated to bind to NS4B with a K_d in the low micromolar to sub-micromolar range. Compound 7 from the triazino indole series was used for further target validation. Huh7 hepatic cells containing genotype 1b HCV replicon were passaged seven times in the presence of 5 μM of Compound 7. The resulting cells were ten-fold less susceptible to the compound while a control cell line passaged in parallel in the absence of compound remained fully susceptible to compound inhibition. Furthermore, the mutations that resulted were located in NS4B (K52R, G120 V, A210S), while no amino acid changes were observed in the NS5B region. These mutations were not reintroduced back into the wild-type replicon backbone to confirm the observed phenotype. Among the other chemical families, the pyrazolopyrimidine and the aminothiophene series appear to be the most attractive with sub-micromolar binding affinities, significant replicon activity and good safety window.

The pyrazolopyrimidine (Compound 2, Table 1) was further studied by Genelabs (now part of GSK) in collaboration with Stanford University (Bryson et al., 2010). Using an NS4B-GFP fusion construct expressed in Huh7.5 cells, Compound 2 was shown to alter the sub-cellular distribution of NS4B. It was reported to have a replicon EC_{50} of 0.3 and 0.6 μM in genotype 1b and 1a, respectively. More importantly, its role as an inhibitor of NS4B function was supported through resistance studies. Genotype 1b replicon cells were grown in the presence of Compound 2 at concentrations up to 5 μM, and the most common mutation selected under drug pressure was H94R within NS4B. The H94R mutation, when reintroduced by mutagenesis into the wild-type NS4B replicon construct, conferred a 37-fold potency loss to Compound 2. In another publication by the Stanford group, the mechanism of action of Compound 2 was further defined to disrupt the interaction between helix AH2 and the membrane surface (Cho et al., 2010). In this article, a series of mutations conferring resistance against Compound 2 were selected by passaging cells containing HCV replicon in the presence of the drug. Another resistance-associated mutation at position 48 (A48Q) which mapped to the second amphipatic alpha helix (AH2) is reported (Fig. 3). Interestingly, Compound 2 was not as potent against genotype 2a (EC_{50} >50 μM) as it was against 1b (EC_{50} = 0.3 μM), suggesting it interacts with non-conserved residues in NS4B.

Recently, new compounds related to the pyrazolopyrimidines series (Compound 9, Table 1) were discovered by Apath LLC as anti-HCV NS4B inhibitors (Slomczynska et al., 2010). The Apath patent claims analogs of the tetrahydropyrazolo[1,5-a]pyrimidine scaffold which results from a partial saturation of ViroPharma's pyrazolopyrimidine core. The patent exemplifies 6 compounds; Compound 9 (AP 80978) was the most potent with 1b replicon EC_{50} of 0.45 μM; it had an EC_{50} <2 μM against the genotype 1a replicon. Resistance selection experiments identified a change at position 98 (F98L/V) and 238 (S238Y) that were associated with a loss of sensitivity to Compound 9. However, only the phenotype of the mutation F98L/V was confirmed by site directed mutagenesis. Although Compound 9 was not active against genotype 2a, chimeric replicons containing the genotype 1b con-1 sequence inserted into a 2a background at positions 53–218 regained sensitivity to the drug.

3.2. Inhibition of vesicle formation by an amiloride analog

One of the functions of NS4B is to cause membrane aggregation to form the so-called membranous web. Helix AH2 expressed alone also induces lipid vesicle aggregation that can be monitored by fluorescence microscopy. Furthermore, mutations within AH2 (A51E and W55D, 1b replicon) abrogate HCV replication, thus suggesting that anti-HCV small molecules might be able to interfere with the AH2 function. An assay was therefore developed by Dr. Glenn's group at Stanford University to screen for compounds inhibiting AH2-mediated vesicle formation (Cho et al., 2010). The authors identified a class of pyrazine compounds as inhibitors of vesicle formation, whose activity was confirmed by dynamic light scattering measurements. This class of compounds was further claimed in a patent application (Glenn et al., 2010), in which 38 compounds are claimed and exemplified with a specific claim to Compound 10 (C4), shown in Table 2 Compound 10 is an amiloride analog (3-amino-N-carbamimidoyl-6-chloro-5-(isobutyl(methyl)amino)pyrazine-2-carboxylate) that is active against both genotype 1b and 2a, with an EC_{50} of ~3 μM (Cho et al., 2010).

3.3. The discovery of clemizole hydrochloride (Table 3, Compound 11)

If the interaction between NS4B and the HCV RNA is essential for virus replication, preventing this interaction would constitute a novel and potentially attractive avenue for drug discovery. With this objective in mind, the microfluidic RNA binding assay described earlier was used to screen a restricted library of pharma-

Table 3
Compounds identified as RNA binding inhibitors.

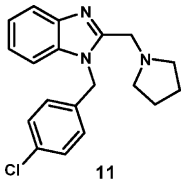
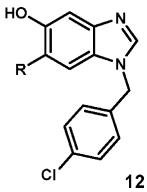
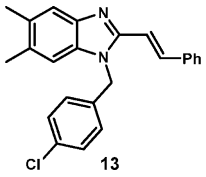
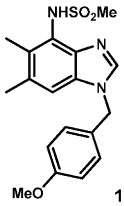
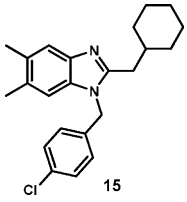
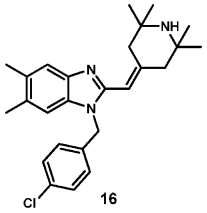
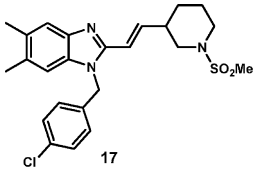
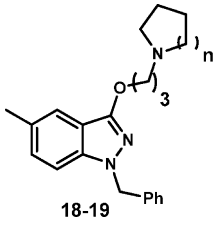
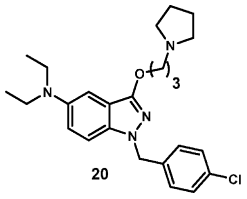
Patent #	Compound class (Example# in patent)	Structure	EC_{50} (μM)	CC_{50} (μM)
Stanford University US 2010/0028299	Clemizole	 11	>20 (GT 2a)	>20
	Clemizole analog Compound 60 (R=H) Compound 62 (R=OH)	 12	<5 (GT 2a)	>5
	Clemizole analog Compound 145	 13		
	Clemizole analog Compound 106	 14		
Stanford University WO 2010/107739	Clemizole analog Compound EBP468	 15	8	>25
	Clemizole analog Compound EBP871	 16	2.7	na
	Clemizole analog Compound EBP550	 17	9.3	>25
Stanford University US 2010/0015093	Indazole series Compound 193 ($n=1$) Compound 199 ($n=2$)	 18-19	<5 (GT 2a)	>5

Table 3 (*Continued*)

Patent #	Compound class (Example# in patent)	Structure	EC_{50} (μM)	CC_{50} (μM)
Stanford University WO 2010/107742	Indazole series Compound EBP534	 20	3.3	>25

cologically active compounds against a purified NS4B-GFP fusion protein (Einav et al., 2008a). One of these small molecules, clemizole hydrochloride (Compound 11, Table 3), was able to inhibit RNA binding with an IC_{50} of 24 nM, that translated to an EC_{50} of 8 μM against the infectious virus of genotype 2a (JFH1). Interestingly, clemizole hydrochloride might not be active against genotype-1 viruses (EC_{50} >20 μM) (Choong et al., 2010a; Einav et al., 2010c). Prolonged passaging of cells harboring the HCV replicon in the presence of clemizole hydrochloride resulted in the selection of resistant clones containing mutations located either at the 3′ UTR or within the NS4B gene. When introduced to the replicon system, mutations W55R and R214Q conferred a 2.2- and 5-fold resistance to clemizole hydrochloride, respectively (Fig. 3). Surprisingly, these mutations increased binding affinity between NS4B and the 3′-terminal RNA sequence. Clemizole hydrochloride is a well-characterized H1 histamine receptor antagonist that was introduced to the US market in the late 1950s as Reactrol/Allercur. The molecule has a wide safety margin, and was therefore taken forward in 2009 into Phase 1B clinical trials in HCV-infected patients. The first ongoing open-label study consists of four weeks of oral treatment with 100 mg BID of clemizole hydrochloride administered immediately prior to the initiation of treatment with standard of care therapy (PEG-IFN + ribavirin) in treatment-naïve subjects chronically infected with HCV genotype 1 or 2.

Clemizole analogs with improved anti-NS4B activity have also been claimed and exemplified in a patent (Einav et al., 2010b). In this patent, several clemizole analogs inhibit HCV replication with EC_{50} (genotype 2a) below 5 μM. It was noted that the pyrrolidinomethyl substitution at the 2-position of the benzimidazole ring (which is apparently required for the H1 antagonism property of clemizole) is not important for its anti-NS4B activity (Table 3, Compounds 12–14). Furthermore, the 4-chlorobenzyl substituent is the most exemplified at the 1-position of the benzimidazole. Analogs of clemizole were further claimed in a recent patent by the Stanford group (Choong et al., 2010a). In this patent, selected compounds are reported with genotype 1b replicon potency of 1.8–12 μM, a clear improvement over clemizole. Furthermore, the hERG activity of some of these compounds was tracked since clemizole-like compounds contain an aromatic core and a pendant tertiary amine that can block hERG K+ channel (Cavalli et al., 2002). Selected compounds from this patent are shown in Table 3; Compound 15 with a 1b replicon EC_{50} of 8 μM is clean in the hERG assay (IC_{50} >10 μM). Compounds 16 and 17 incorporate a piperidine ring whose basicity is masked either sterically or through derivatization as a sulfonamide–strategies commonly used to improve the hERG profile of leads.

3.4. The clemizole-related indazole series

Additional clemizole-related molecules with improved *in vitro* potency have recently emerged. A first patent describes an indazole core replacing the benzimidazole of the clemizole family of compounds (Einav et al., 2010a). Compounds 18–19 (Table 3) are selected compounds from this patent that are claimed and exemplified. A second follow-up patent covering additional indazole compounds reports low micromolar activity in the genotype 1b replicon assay with a good safety window as assessed by cell viability (Choong et al., 2010b). The hERG IC_{50} for selected compounds is reported; these compounds lack a window between antiviral potency and hERG activity. An example (Compound 20) shown in Table 3 has 1b replicon EC_{50} of 3.3 μM and a hERG IC_{50} of 2.8 μM.

3.5. Other efforts in finding anti-HCV compounds targeting NS4B

In 2008, scientists from Genelabs presented their NS4B program at Cambridge Health Institute Conference (Roberts, 2008). Lead compounds identified through a replicon screen were inactive when tested against NS5B polymerase and NS3/4A protease. Resistance screening with selected compounds identified mutated amino acid changes that occur in a well-defined region of NS4B that were subsequently confirmed to decrease compound potency in a transient HCV replicon assay. Optimization of this series of compounds by the Genelabs group has led to compounds that are potent – EC_{50} <50 nM in replicon assays using genotype 1b and 1a – and have antiviral activity in combination with agents currently approved or under clinical development. While specific structures were not revealed, the pharmacokinetic profile and biological characterization of this class of compounds offered hopes for clinical development.

Similarly, Bristol Myers Squibb (BMS) recently reported the result of a HCV replicon screening campaign that led to the discovery of new molecules with EC_{50} values against HCV genotype 1a and 1b replicons of around or below 1 μM, respectively (Sheaffer et al., 2008). Mutations selected by drug pressure mapped to the N-terminal region of NS4B (H3R and Q26R), and provide evidence for target identification (Fig. 3). Interestingly, the BMS group observed cellular rearrangements of lipid droplets in cells incubated with their compounds, implying that cellular determinants are also involved in the mechanism of action of these inhibitors.

4. Conclusion: current and future developments

In recent years, there has been tremendous progress made in understanding the complex role of NS4B in the replication cycle of HCV. As we described, the identification of unique functions such as vesicle aggregation, cell transformation, NTPase activity, and RNA binding has helped in developing new assays to screen for potential inhibitors. In the absence of clinical proof of concept, it is still difficult at this point to assess the most "druggable" functions of NS4B. For example, although the NTPase activity of NS4B has been described now for over 10 years, it is still not clear whether a small molecule could specifically inhibit this catalytic activity, and how such inhibition would translate at the level of virus replication. On the other hand, the membranous web formation seems like a tractable function to target for the discovery of new NS4B inhibitors, at least based on the

convergence of assays and methods reported so far that would be amenable to drug discovery. Using the assays we described, a number of publications and patents report on the inhibition of NS4B with small molecules, the majority of which constitute early pre-clinical studies. Therefore, the presented molecules can be considered as early leads and do not contain to this point all of the drug-like properties required for successful clinical development.

The furthest advanced anti-NS4B molecule is clemizole hydrochloride, an old drug that has previously been clinically approved as an antihistamine. It will be interesting to monitor the clinical progression of clemizole hydrochloride in the currently ongoing Phase Ib study. Preliminary data show that HCV infected patients might positively respond to clemizole hydrochloride when added to the current standard of care (Choong et al., 2010a). *In vitro* studies also predict that clemizole hydrochloride could be synergistic with protease inhibitors, and additive with interferon, ribavirin, nucleoside and non-nucleoside NS5B polymerase inhibitors (Einav et al., 2010c). If clemizole hydrochloride or any other anti-NS4B compound further demonstrates efficacy in human clinical trials, evaluating their combination with other clinical candidates will considerably broaden the potential treatment options for HCV-infected patients.

Conflict of interest

The authors are both employed by Alios BioPharma.

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Review

Arenaviruses and hantaviruses: From epidemiology and genomics to antivirals

R.N. Charrel[a,*], B. Coutard[b], C. Baronti[a], B. Canard[b], A. Nougairede[a], A. Frangeul[b], B. Morin[b], S. Jamal[b], C.L. Schmidt[e], R. Hilgenfeld[e], B. Klempa[c,d], X. de Lamballerie[a]

[a] Unité des Virus Emergents UMR190, Université de la Méditerranée, Marseille, France
[b] Architecture et Fonction des Macromolécules Biologiques, Université de la Méditerranée, Marseille, France
[c] Institute of Virology, Slovak Academy of Sciences, 84505 Bratislava, Slovakia
[d] Institute of Virology, Helmut-Ruska-Haus, Charité University Hospital, D-10098 Berlin, Germany
[e] Institute of Biochemistry, Centre for Structural and Cell Biology in Medicine, University of Lübeck, Lübeck, Germany

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ABSTRACT

The arenaviruses and hantaviruses are segmented genome RNA viruses that are hosted by rodents. Due to their association with rodents, they are globally widespread and can infect humans via direct or indirect routes of transmission, causing considerable human morbidity and mortality. Nevertheless, despite their obvious and emerging importance as pathogens, there are currently no effective antiviral drugs (except ribavirin which proved effective against Lassa virus) with which to treat humans infected by any of these viruses. The EU-funded VIZIER project (Comparative Structural Genomics of Viral Enzymes Involved in Replication) was instigated with an ultimate view of contributing to the development of antiviral therapies for RNA viruses, including the arenaviruses and bunyaviruses. This review highlights some of the major features of the arenaviruses and hantaviruses that have been investigated during recent years. After describing their classification and epidemiology, we review progress in understanding the genomics as well as the structure and function of replicative enzymes achieved under the VIZIER program and the development of new disease control strategies.

Contents

* Corresponding author.
E-mail address: remi.charrel@univmed.fr (R.N. Charrel).

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1. Introduction

Life-threatening RNA viruses emerge regularly, and often in an unpredictable manner. Yet, the very few drugs available against known RNA viruses have sometimes required decades of research for development. At present, very long delays between molecule identification and commercial application are common, and this situation is unlikely to change in the immediate future at least in part owing to the constant upgrade of safety procedures and regulations. Can we generate preparedness for outbreaks of the, as yet, unknown viruses? Here we address these questions for arenaviruses and hantaviruses, two groups of zoonotic RNA viruses that include several major human pathogens, and for which novel viruses have repeatedly been discovered during the last decade. To understand the current situation, we present a comprehensive review covering various aspects of epidemiology, evolutionary relationships, diseases in humans, and the means currently available for preventing and combating human infections. For each group of viruses, the final paragraph deals with objectives and achievements conducted by all partners within the VIZIER European research project.

The VIZIER (VIral enZymes InvolvEd in Replication) (http://www.vizier-europe.org/) project was set up to develop the scientific foundations for countering this challenge to society. VIZIER studied the most conserved viral enzymes (that of the replication machinery, or replicases) that constitute attractive targets for drug-design. The aim of VIZIER was to determine as many replicase crystal structures as possible from a carefully selected list of viruses in order to comprehensively cover the diversity RNA viruses, and generate critical knowledge that could be efficiently utilized to jump-start research on any emerging RNA virus. VIZIER was a multidisciplinary project involving (i) bioinformatics to define functional domains, (ii) viral genomics to increase the number of characterized viral genomes and prepare defined targets, (iii) proteomics to express, purify, and characterize targets, (iv) structural biology to solve their crystal structures, and (v) pre-lead discovery to propose active scaffolds of antiviral molecules.

2. Arenaviruses

2.1. Taxonomy and epidemiology

The eighth edition of the Report of the International Committee for Taxonomy of Viruses (ICTV) indicates that the *Arenaviridae* family contains a single genus *Arenavirus* including 22 viral species (Salvato et al., 2005). However, several new viruses (1 south American virus [Chapare], 4 north American arenaviruses [Skinner Tank, Big Brushy Tank, Tonto Creek, Catarina], and 4 African viruses [Kodoko, Morogoro, Merino Walk, and Lujo viruses]) were discovered very recently and therefore are not yet included in the ICTV list. On the basis of their antigenic properties, the arenaviruses form two groups: the Tacaribe serocomplex (New World) and the Lassa-Lymphocytic choriomeningitis serocomplex (Old World) (Salvato et al., 2005). Nucleocapsid antigens are antigenically similar for most arenaviruses, and quantitative relationships show the basic split between Old World and New World viruses. Individual viruses are immunologically distinct in neutralization assays, which depend on the specificity of epitopes contained in the envelope glycoproteins (Salvato et al., 2005).

Specific rodents are the principal hosts of the arenaviruses, the only exception to date being Tacaribe virus which was isolated from *Artibeus* fruit-eating bats. Each virus species is closely associated with a specific vertebrate species. Thus, the distribution of the host dictates the distribution of the virus. Lymphocytic choriomeningitis virus (LCMV) is the only arenavirus to exhibit a worldwide distribution due to its close association with *Mus musculus*, which has been disseminated on all continents mostly by association with human activities. Other arenaviruses are distributed either in the New World or in Africa. Humans usually become infected through contact with infected rodents, or via inhalation of infectious rodent excreta or secreta. The domestic and peridomestic behavior of rodent reservoir hosts in general is a major contributory factor to viral transmission from rodent to human. However, in most cases, transmission of arenaviruses to humans occurs following recreational or agricultural incursions into environments providing critical habitat for rodent hosts. Additionally, professionals handling infected rodents in the field or laboratory are at increased risk of infection (Sewell, 1995).

Perturbation of the environment due either to human activities (modern farming practices), or natural ecological changes (flooding, storms) may result in behavioral changes of the reservoir hosts. Such changes have been implicated in the emergence of human disease caused by arenaviruses, such as Machupo virus infection cases consecutive to flooding in the El Beni region of Bolivia. Lassa, Junin, Machupo, Guanarito, and Sabia viruses are known to cause severe hemorrhagic fever, in western Africa, Argentina, Bolivia, Venezuela, and Brazil, respectively (Peters et al., 1996). Lassa virus is believed to cause up to 300,000 annual infections with 30% morbidity and 16% mortality (McCormick et al., 1987; McCormick and Fisher-Hoch, 2002). These five viruses are included in the Category A Pathogen List, and considered Biosafety Level 4 (BSL-4) agents in the United States and in Western Europe.

LCMV, the family prototype, was first isolated in 1933 during serial passage in monkeys of human material obtained from a fatal infection in the first documented epidemic of St. Louis encephalitis (Armstrong and Lillie, 1934). LCMV is an agent of acute central nervous system disease (Barton and Hyndman, 2000), congenital malformations (Barton et al., 1993), and has recently been identified in organ-transplantation recipients (CDC, 2005; Fischer et al., 2006; Amman et al., 2007) and immunocompetent patients (Charrel et al., 2006; Emonet et al., 2007). Clusters of fatal infections in organ-transplanted patients have recently been reported

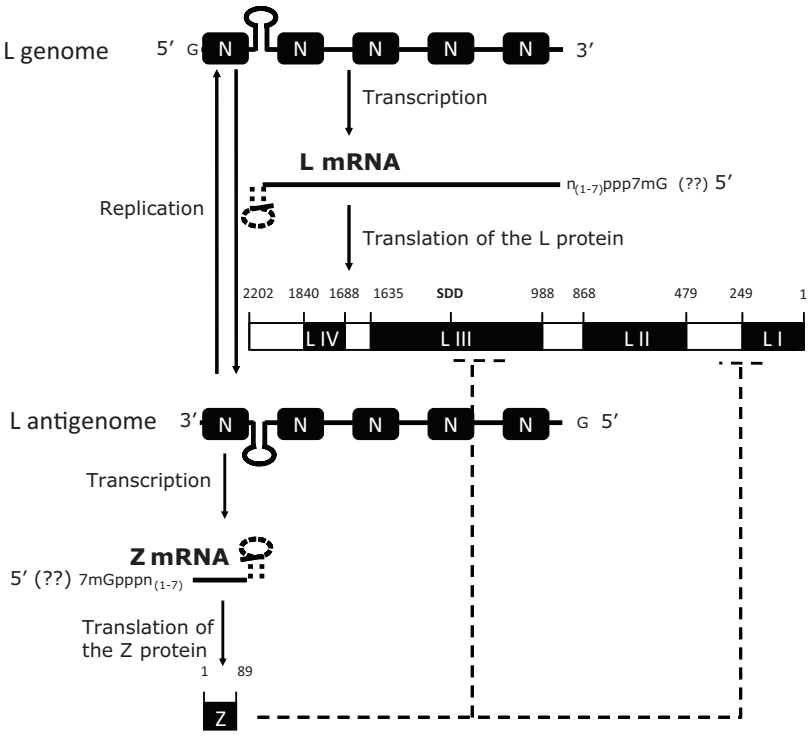

Fig. 1. Schematic representation of the replication and transcription mechanism of the Large L RNA genome of arenaviruses, leading to the translation of the Z and L proteins (adapted from Meyer and Southern, 1993). N is the Nucleoprotein interacting with both genome and antigenome. The L protein is shown with its four conserved domains LI, LII, LIII and LIV, where LIII is carrying the canonical SDD motif. Capping elements (n(1-7)ppp7mG) have been found on the N mRNA and an extra G is found at the 5′ of the genome and antigenome (Garcin and Kolakofsky, 1990). The amino acids positions correspond to the boundaries designed within VIZIER for the Parana virus L protein. The dashed lines illustrate the interaction between Z and L (adapted from Wilda et al., 2008) with the expected interacting regions within the L sequence.

and may constitute a new example of an emerging disease associated with a novel situation related to advances in medicine (Fischer et al., 2006; CDC, 2005, 2006, 2008; Palacios et al., 2008; Amman et al., 2007). Very little is known about the health consequences of human infection by other arenaviruses. Flexal and Tacaribe viruses have caused febrile illness in laboratory workers (Peters et al., 1996). Whitewater Arroyo virus may have been associated with three fatal cases of infection in California (CDC, 2000). Exposure to Pichinde virus has resulted in seroconversion without symptoms (Buchmeier, unpublished results). Tacaribe virus is believed to have caused a single case of a febrile disease which resolved with mild meningitis (J. Casals, unpublished data).

2.2. Genome organization and replication strategy

Arenaviruses possess single-stranded bi-segmented RNA genomes. Each of the two RNA segments encode two non-overlapping reading frames of opposite polarity: the viral RNA-dependent RNA polymerase (L protein) and a zinc-binding matrix protein (Z protein) for the large (L) genomic segment (~7200 nucleotides); the nucleocapsid protein (NP) and the glycoprotein precursor (GPC), secondarily cleaved into the envelope proteins G1 and G2, for the small (S) genomic segment (~3500 nucleotides). The genes on both S and L segments are separated by an intergenic non-coding region with the potential of forming one or more hairpin configurations. The 5′ and 3′ untranslated terminal sequences of each RNA segment possess a relatively conserved reverse complementary sequence spanning 19 nucleotides at each extremity.

The genome organization and ambisense nature of the arenavirus gene transcription strategy result in these viruses displaying characteristics of both (−) and (+) RNA viruses (Fig. 1). For RNA replication, the viral polymerase binds at the 3′ end of the templates, traverses the template RNA from end to end, and synthesizes a full-length complementary RNA. The 5′ ends of the S-derived subgenomic mRNAs extend beyond the ends of the genomic RNA templates (Garcin and Kolakofsky, 1990; Raju et al., 1990; Meyer and Southern, 1993). These non-templated extensions are variable in length (1–7 nucleotides) and terminate with 5′ cap structures. The process of "cap snatching" has been well documented for influenza viruses and bunyaviruses, but a similar mechanism for cap acquisition by arenaviruses has not been confirmed yet (Dias et al., 2009; Jin and Elliott, 1993). The arenavirus subgenomic mRNAs terminate within the intergenic noncoding region, and they are not polyadenylated (Singh et al., 1987; Southern et al., 1987). In many cases, it appears that transcription termination occurs on the distal side of the intergenic stem-loop structure so that the 3′ termini of the mRNA could be stabilized by the formation of terminal hairpin structures (Franze-Fernández et al., 1987; Meyer and Southern, 1993).

From the perspective of the replication machinery, the L protein (RdRp) must be adapted to synthesize RNA from an RNA template embedded into an RNP as well as from a naked RNA molecule. The L protein carries the SDD motif suggesting it is encodes the RdRp activity. It has been demonstrated that the L protein in association with the Nucleoprotein can replicate and transcribe the Tacaribe genome (Lopez et al., 2001). Two key residues within the Lassa virus domain III were shown to be involved in transcription but not replication (Hass et al., 2008). In the structural model proposed by Hass and collaborators, these residues would be positioned at the intersection of the palm and thumb of the polymerase. The priming reaction is likely to be different according to which RNA template is used. Indeed an original feature of this reaction is a resulting non-templated G molecule at the 5′ end of non-capped genomes and antigenomes (Garcin and Kolakofsky, 1990) whereas there may be a peculiar, though not unique to *Arenaviridae*, "cap snatching" or

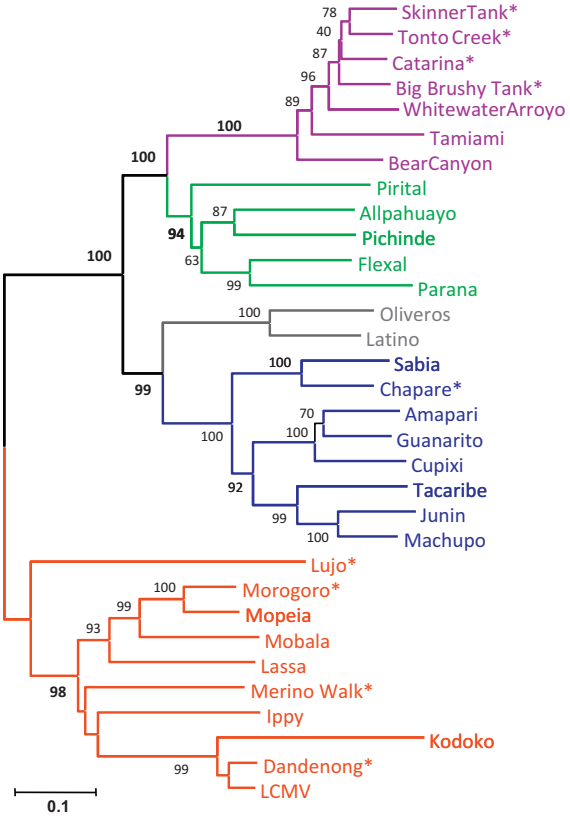

Fig. 2. Phylogeny of arenaviruses based on the analysis of complete amino acid sequences of the nucleoprotein (see Charrel et al., 2008). Phylograms were obtained by a combination of the pairwise distance method, neighbor-joining algorithm for tree reconstruction, and bootstrap analysis using 500 pseudoreplications. Evolutionary lineages are colored: red, Old World arenaviruses; green, clade A new world arenaviruses; blue, clade B new world arenaviruses, grey, clade C new world arenaviruses, purple, recombinant new world arenaviruses. *, newly discovered arenaviruses not included in the ICTV list.

primer/realignment mechanism in the case of mRNA. This latter mechanism has also been reported in influenza virus (Shaw and Lamb, 1984) and bunyavirus (Bouloy et al., 1990).

2.3. Recently discovered arenaviruses

Several arenaviruses have been discovered during the past three years in the Americas and in Africa. Their relative phylogenetic relationships with other members of the *Arenaviridae* are depicted in Fig. 2. Chapare virus was recently isolated from a fatal human case of hemorrhagic fever in the Chapare River region close to Cochabamba in Bolivia (Delgado et al., 2008). To date, there is no evidence for other cases in the same region or elsewhere. There is currently no information concerning its vertebrate host. Morogoro virus was isolated from *Mastomys sp* trapped in Tanzania (Vieth et al., 2007). This virus is most closely related to Mopeia virus regardless of the gene used for analysis (Charrel et al., 2008).

Dandenong virus was identified through a metagenomic approach, and then isolated from tissue specimens collected from a fatal case of infection in a patient who underwent kidney transplantation in Australia from a donor who had just completed a three-month visit to the former Yugoslavia, where he had travelled in rural areas (Palacios et al., 2008). Catarina, Skinner Tank, Tonto Creek and Big Brushy Tank viruses were recently discovered in the southwestern regions of the United States of America from Neotoma rodents (woodrats) (Cajimat et al., 2007, 2008; Milazzo et al., 2008). They appear most closely related to Whitewater Arroyo virus, another North American arenavirus. Kodoko virus was detected, but not isolated, in tissues of *Mus Nannomys minutoides* trapped in Guinea. Partial sequence analysis indicates that Kodoko virus roots LCMV (Lecompte et al., 2007).

Partial sequences of Pinhal virus (*Calomys tener* from Brazil) were obtained from Genbank with minimal information, the reason for which it has not been included in the phylogeny presented in Fig. 2; based on these partial genetic data, Pinhal virus appears to be most closely related to Oliveros virus (Charrel et al., 2008). Most recently, five cases of viral hemorrhagic fever (VHF) were reported to have been caused in South Africa by a novel arenavirus, provisionally named Lujo virus, which is most closely related to the Old World group viruses (Briese et al., 2009). Merino Walk virus (MWV) was isolated from a rodent, *Myotomys unisulcatus*, collected in South Africa, in 1985; MWV has been recently genetically and antigenically characterized and is proposed as a novel species within the Old World arenavirus complex (Palacios et al., 2010).

2.4. Evolutionary relationships among the arenaviruses

The first phylogenetic studies of arenaviruses were based on analysis of partial NP gene sequence in the S RNA segment. There was a strong correlation with their antigenic relationships (Bowen et al., 1996, 1997). Four major phylogenetic lineages were identified: the Old World lineage, the three New World lineages (Fig. 2). The Old World (Lassa-LCM serocomplex) lineage comprises seven viruses: LCM, Lassa, Mopeia, Mobala, Ippy, Morogoro and Kodoko virus. New world arenaviruses were subdivided into three lineages, designated A, B, C. Lineage A includes five South American viruses, Pirital, Pichinde, Flexal, Parana, Allpahuayo. Lineage B includes eight South American viruses, Sabia, Junin, Machupo, Guanarito, Amapari, Tacaribe, Cupixi and Chapare virus. Lineage C comprised two South American viruses: Oliveros and Latino.

Efforts to determine full-length genomes initiated in the early 2000s showed that Whitewater Arroyo virus, indigenous to North America, was a recombinant virus resulting from recombination between ancestors belonging to lineages A and B. Additional viruses indigenous to North America were also found to be recombinants since they possess a common ancestor with Whitewater Arroyo virus (Charrel et al., 2001). Accordingly a fifth lineage, A^{rec}, was described and includes viruses possessing a genome organization resulting from recombination in the S RNA segment: Whitewater Arroyo, Tamiami, Bear Canyon, Catarina, Skinner Tank, Big Brushy Tank, and Tonto Creek (Charrel et al., 2002, 2003; Archer and Rico-Hesse, 2002; Cajimat et al., 2007, 2008; Milazzo et al., 2008). This was the first time recombination had been described in the family *Arenaviridae*.

Since reassortant (inter-segmental recombination) arenaviruses had been generated experimentally (Lukashevich, 1992), sequences were analyzed to investigate whether or not field viruses may be the result of reassortment, but to date there is no evidence to support this possibility (Charrel et al., 2008). With the accumulation of complete genome sequences phylogenetic analyses were progressively updated. The most comprehensive study, based on the comparative analysis of complete sequences of the four genes for the largest set of viruses, was published recently (Charrel et al., 2008). In addition to placing new viruses in the family, the new data support and extend the previous analyses.

2.5. Arenavirus infections of humans

There are a variety of syndromes associated with arenavirus infection. In most cases, they depend on the virus causing the infection. Viral hemorrhagic fever can be caused by Lassa and Lujo viruses in Africa, or by Junin, Machupo, Sabia, Guanarito and Chapare viruses in South America (Peters et al., 1996; Delgado et al., 2008; Briese et al., 2009). It is likely that these viruses can also

cause less severe forms of infection, even non-symptomatic infections, but these aspects are poorly understood, and the relative proportions are unknown.

Infection by LCM virus can result in acute central nervous system disease and congenital malformations (Barton and Hyndman, 2000; Barton et al., 1993); it has recently been described as an important cause of fatal infection in organ transplantation recipients, and immunocompetent patients (CDC, 2005; Fischer et al., 2006; Amman et al., 2007; Charrel et al., 2006; Emonet et al., 2007; Palacios et al., 2008). LCM virus is currently the arenavirus for which the largest amount of data has been accumulated on pathogenesis, seroepidemiology, and array of clinical symptoms. However, its role remains obviously underestimated because of the very limited number of laboratories able to perform diagnosis (Asnis et al., 2010; Barton, 2010; De Ory et al., 2009).

Flexal and Tacaribe viruses have caused febrile illness in laboratory workers (Peters et al., 1996). Whitewater Arroyo virus may have been associated with 3 fatal cases of infection in California (CDC, 2000). Exposure to Pichinde virus has resulted in numerous seroconversions among humans without any noticeable clinical significance (Buchmeier, unpublished results). Tacaribe virus is believed to have caused a single case of a febrile disease with mild CNS symptomatology (J. Casals, unpublished data). Very little is known about the health consequences of infection with the other arenaviruses. The lack of data is mostly due to the absence of investigations in regions where these viruses circulate.

2.6. Vaccines

A live attenuated Junin virus vaccine (Candid#1) was produced in the early 1990s. Its efficacy was proven in a double-blind trial in 15,000 agricultural workers at risk of natural infection. Subsequently, more than 100,000 people were immunized in Argentina. A prospective study showed that Candid #1 vaccine efficacy was greater or equal to 84%, and no serious adverse effects were detected (Maiztegui et al., 1998). This vaccine was licensed in 2006 for use exclusively in Argentina, whereas in the USA Candid #1 remains only as an investigational new drug and studies addressing long-term immunity and safety have not been conducted. The current availability within the USA of a Candid #1 Master Virus Seed is uncertain and re-importation of Candid #1 vaccine from Argentina is likely to meet unsolvable obstacles due to foot-and-mouth-disease virus (FMDV) activity in several geographic regions of Argentina, and potential lack of FDA-compliant documentation (Emonet et al., 2011).

Currently there is no definitive evidence that immunity conferred by Candid#1 vaccine is protective against the other South American arenaviruses causing viral hemorrhagic fevers.

Several attempts to produce a satisfactory Lassa virus vaccine have been made during the past 30 years, some of which show significant promise. The most advanced projects include (i) a replication-competent vaccine based on attenuated recombinant vesicular stomatitis virus vectors expressing the Lassa viral glycoprotein showed that a single intramuscular vaccination elicited a protective immune response in nonhuman primates against a lethal challenge (Geisbert et al., 2005); (ii) ML29, a recombinant Lassa/Mopeia virus vaccine demonstrated protection against Lassa virus challenge in guinea pigs and Rhesus macaques (Lukashevich et al., 2008); (iii) a yellow fever 17D vaccine expressing Lassa virus glycoprotein precursor protected guinea pigs against fatal Lassa fever (Bredenbeek et al., 2006). Recently, a recombinant LCMV/Vesiculovirus vaccine was described that is attenuated, prevents lethal challenge with LCMV in mice, elicits rapid and long-lived cell-mediated immunity against lethal challenge with wild-type LCMV, confers rapid and long-lived cell-mediated protection against overwhelming systemic infection and liver disease, and presents no detectable gain in pathogenicity after propagation in immunodeficient hosts (Bergthaler et al., 2006).

Despite the urgent need for vaccines against arenaviruses and numerous scientific attempts to develop safe, effective and acceptable vaccines, there is currently no WHO-approved vaccine available.

2.7. Therapy of arenavirus infections

2.7.1. Immune plasma

The emergence of Argentine hemorrhagic fever in the 1950s with a mortality rate from 15 to 30% was first combated with immune sera or gamma globulins obtained from convalescent patients. The efficacy of this treatment was established through a double blind placebo-controlled trial organized from 1974 to 1978 that showed that case-fatality rate among cases treated with normal plasma was 16.5% while the rate in those patients treated with immune plasma was 1.1% (Maiztegui et al., 1979). However, alternative forms of treatment/prophylaxy need to be investigated because (i) immune plasma therapy lacks efficacy if given after 8 days of evolution (Enria et al., 1984; Enria and Maiztegui, 1994), (ii) there is a risk of transfusion-associated diseases, (iii) 10% of patients treated with immune plasma develop a late neurological syndrome (Maiztegui et al., 1979; Enria et al., 1985), (iv) the maintenance of adequate stocks of immune plasma is difficult due to the reduced number of cases.

2.7.2. Antiviral drugs

To date, ribavirin (1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is the only antiviral approved and commercially available for arenaviruses. It has proved to be efficient in reducing fatality rates if administered at the early stage of infection Lassa fever virus (McCormick et al., 1986). Recently, there has been intense activity centered on molecules with potential antiviral activity against arenaviruses. High-throughput screening of molecules for their antiviral effects is being increasingly performed by both public laboratories and private companies. From 2003 to 2006, six articles reporting testing of molecules with antiviral efficacy on arenaviruses were published (Albiol Matanic and Castilla, 2004; Asper et al., 2004; Bolken et al., 2006; Castilla et al., 2005; Gunther et al., 2004; Uckun et al., 2005). To date, the most promising molecules are those which interfere with virus membrane fusion through the interaction of the G2 fusion subunit with the signal peptide, with an IC_{50} below 10 nM (Larson et al., 2008; York et al., 2008; Lee et al., 2008). N-substituted acridone derivatives showed *in vitro* efficacy against Junin virus with excellent selectivity indices. The inhibitory effect was exerted via blocking virus replication (Sepúlveda et al., 2008).

T-705 (6-fluoro-3-hydroxy-2-pyrazinecarboxamide), a substituted pyrazine derivative offered significant protection against Pichinde virus-infected hamsters when administrated orally after virus challenge (Gowen et al., 2007, 2008). An essential step in antiviral development is an appropriate animal model to document efficacy of molecules against arenaviruses; several models have been proposed and are summarized by Gowen and Holbrook (2008). Recently, MHC class I knock-out mice were shown to be susceptible to Lassa virus and thus could constitute a good animal model for Lassa virus infection (Flatz et al., 2010, PMID 20360949). AG129 IFN-α/β and -γ receptor-deficient mice were also successfully used to validate the antiviral of MY-24 against Tacaribe virus (Gowen et al., 2010).

2.8. The VIZIER quest for new antiviral drugs against arenaviruses

For arenaviruses, domain design was performed from the little genomics data available at the outset of the project. A total

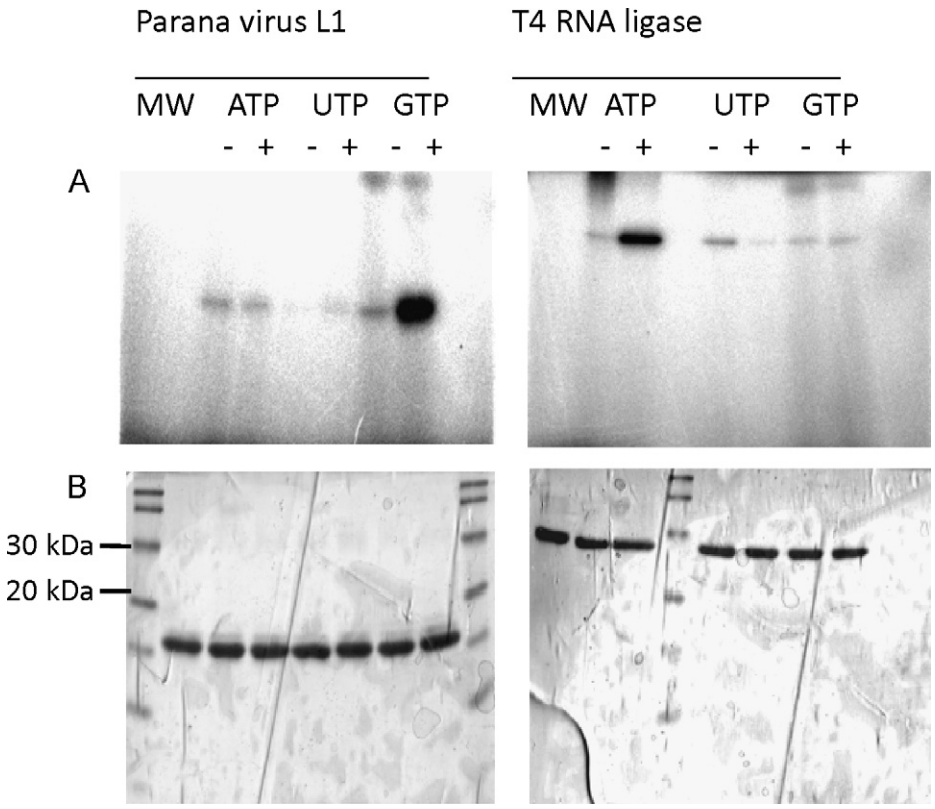

Fig. 3. NTP binding assay of Parana virus L1 domain (New England Biolabs). The experimental procedure was as described previously (Egloff et al., 2002). Briefly, 2 μg of protein was incubated in 10 μl Tris 10 mM NaCl 50 mM, pH 7.5, with either 0 or 50 μM (1 μCi) of [α-32P]ATP, [α-32P]UTP or [α-32P]GTP. The mixture was then UV-irradiated 3 min using a UV lamp (40 W, λ = 254 nm) at a 12 mm distance. Samples were then boiled before SDS PAGE electrophoresis. Wet and dried gels were analyzed by Coomassie blue staining (Panel B) or using a FujiImager to visualize radiolabelled products (Panel A), respectively. The T4 RNA ligase was used as a positive control for specific ATP binding.

of six full-length L RNA segments were sequenced and available from public databases at the outset of VIZIER. Efforts during VIZIER project led to the complete sequence characterization for a total of 15 additional arenaviruses listed in the ICTV catalogue, but also for new arenaviruses not yet listed in the ICTV, such as Morogoro virus (Charrel et al., 2008). So, VIZIER achievement in the field of genomics has to be considered as a major contribution not only to the project objectives, but also for the scientific community.

2.8.1. The search for a soluble sub-domain of the arenavirus L protein

No rational antiviral strategy has been followed to target specifically the replication machinery, probably because of our current meager knowledge of the L protein structure and function. The bottleneck for structural and functional studies is an appropriate production yield of pure recombinant L protein. To date, functional studies related to the L protein have been derived from replicon studies (Hass et al., 2008), or a minigenome transfected into infected cells (Lee et al., 2000). The arenavirus L protein plays an essential role in genome transcription and replication (Lopez et al., 2001). It consists of 2220 amino-acid residues. Because of its size, expression in heterologous expression systems remains problematic. Recently, the design of smaller subdomains has been successfully implemented to improve expression yields compatible with the requirements to obtain biochemical and structural data. For example, a random cloning approach based on deletion library screening selected a soluble, functional and crystallizable fragment of the influenza PB2 subunit (Guilligay et al., 2008).

Efficient domain design can also be achieved by combining bio-informatic and experimental data, as exemplified by the strategy used to produce crystallizable fragments of a pestivirus RdRp (Choi et al., 2004). Both random and rational strategies were available in the VIZIER pipeline (Coutard et al., 2008; Manolaridis et al., 2009; Gorbalenya et al., 2010). Extensive sequence analysis based on homologous proteins from similar viruses or different strains of Lassa virus led to the prediction of four domains encompassing residues 1–250, 493–887, 1007–1653, and 1757–1909, which were termed regions LI to LIV, respectively (see Fig. 1) (Vieth et al., 2004). A biological function was predicted exclusively for region LIII as the putative RNA-dependent RNA Polymerase, RdRp. For Tacaribe virus, both domains LI and LIII were recently shown to be involved in the interaction with the Z protein (Wilda et al., 2008). Taken together, these data led to the rational design of 4 domains for protein expression, the latter being shown in Fig. 1 for the Parana virus L protein.

Lassa virus domains LI and LIV were cloned into the pIVEX2.4d vector and successfully over-expressed using cell-free translation. The data indicated that these domains can fold independently. Crystals of LI domain from Lassa virus were obtained but exhibited only poor diffraction. Several alternative expression constructs for this domain, with different domain boundaries, were prepared in order to improve the quality of the crystals. These design efforts did not only rely on theoretical predictions (secondary structure, homology to corresponding domains of other arenaviruses), but also on limited proteolysis of larger fragments and subsequent analysis of the resistant stable domain cores. Experimental findings showed that significant improvement of the crystals could be achieved.

The domains LI to LIV were cloned for 14 other viruses (Parana, LCM, Ippy, Mopeia, Guanarito, Pirital, Allpahuayo, Bear Canyon, Cupixi, Amapari, Machupo, Sabia, Latino, Oliveros) to bring sequence diversity and improve thus the success rate of expression, crystallization, or crystal diffraction (Coutard and Canard, 2010).

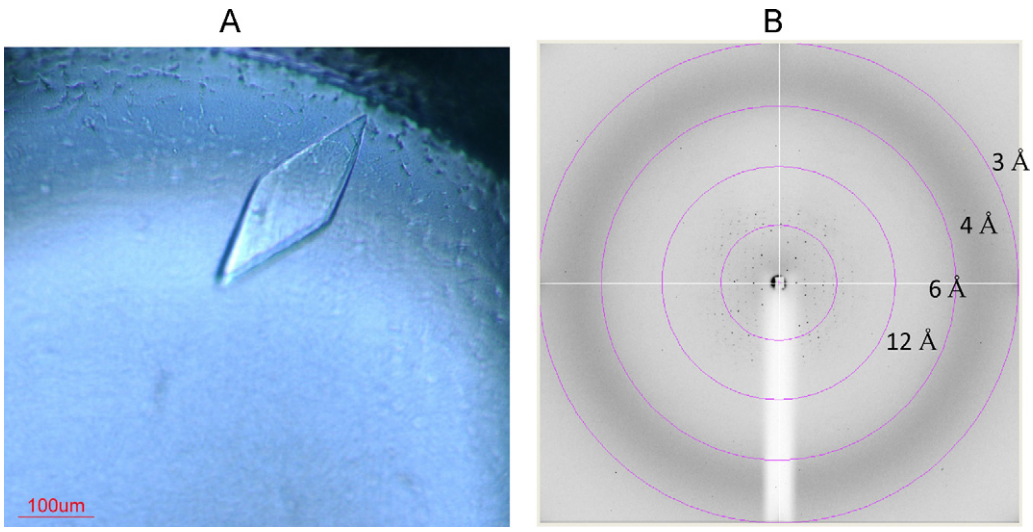

Fig. 4. Crystallization and crystal diffraction of the Parana virus L1 Domain. Panel A: Crystals of Parana virus L1 Domain were obtained from a two step screening procedure. Initial crystals hits were grown from commercial screens using the vapor diffusion technique in $(NH_4)_2PO_4$ 500 mM, Imidazole 100 mM pH 8.0. Crystals were then refined in a secondary screening procedure by adding two parts of the described crystallization solution to one part of screening solutions (as described in Jabafi et al., 2007). The best crystals were obtained in a solution made of 2 part of $(NH_4)_2PO_4$ 500 mM, Imidazole 100 mM pH 8.0 and one part of NH_4Cl 1.75 M. Panel B: Diffraction pattern of the obtained crystals. 3, 4, 6 and 12 Å resolution limits are indicated by purple circles.

The expression procedure followed a previously described protocol (Berrow et al., 2006) for expression in E. coli. Only 3 constructs were expressed in the soluble fraction. All of them correspond to an LI domain. When purified, only the domain of Parana virus was stable in a non-denaturing buffer (CHES 10 mM, NaCl 50 mM, pH 9). Surprisingly, the protein mass observed on SDS-Page stained with Coomassie blue was 5 kDa lower than expected (23 kDa instead of 28 kDa). The Mass Spectrometry analysis confirmed that about 50 amino acids were missing at the C-terminal end of the protein, probably resulting from proteolytic cleavage. Although the protein did not exhibit the expected mass, wide range functional and crystallization screenings were launched. In parallel, a smaller construct (1–196 aa) for Parana virus L was re-cloned as well as for 13 other arenavirus LI domains.

2.8.2. Preliminary functional data for the N-terminal end of the Parana virus L protein

The structure prediction of the Parana virus LI domain using PHYRE (Kelley and Sternberg, 2009) suggested that it could be a nucleotide binding domain. A nucleotide binding screening was assayed for the Parana LI domain, and the results are shown in Fig. 3. The Parana LI domain was found to bind GTP specifically, under experimental conditions requiring UV-cross-linking. In comparison, none of the tested conditions provided any significant binding of ATP or UTP These results represented an interesting starting point to elucidate progressively the LI function since (i) the 3′ end of the genome and antigenome is a G, (ii) an extra G was shown to be added at their 5′ end (Garcin and Kolakofsky, 1990), and (iii) mRNAs are capped following a probable cap-snatching pathway indicating that at least a G or ^{7m}GTP binding site may exist in the L protein (Fig. 1). The involvement of the LI domain in these three stages of viral replication/transcription was thus investigated.

2.8.3. Crystallization of the Parana virus LI protein domain

Both 1–196 aa and 1–249 aa constructs were used for crystallization trials. No improvement was observed with the shorter construct, compared to the degraded protein resulting from the long one. Nevertheless, despite many crystallogenesis efforts, X-ray diffraction could not be improved below a 6 Å resolution (Fig. 4). However, the strategy based on homologue screening with the 13 other arenavirus LI domains led to the crystallization of the cognate LCM virus LI domain, for which the crystal structure was determined at a 2.7 Å resolution (Morin et al., 2010).

2.8.4. Structural and functional study of LCM virus LI domain

The LCMV LI Domain consists of 4 β-strands surrounded by seven α-Helices. This organization was found to be related to the recently discovered N-terminal domain of influenza PA protein (Dias et al., 2009). Together with the conserved amino acid motif PD. . .(D/E)XK, the domain was proposed to be a type II endonuclease. This function was confirmed using enzymatic assays. Following mutational analysis and a cell-based replicon system, endonuclease knock-out was associated to a transcription-null phenotype, suggesting that this domain is involved in functional RNA production, probably in the cap snatching process. Sequence analysis suggested that the endonuclease was conserved among *Arenaviridae* and *Bunyaviridae*. This finding was concomitantly confirmed by the crystal structure determination of the La Crosse virus endonuclease (Reguera et al., 2010). Moreover, the structure determination of both LCMV and La Crosse virus endonucleases showed that they are very closely related to that of influenza virus. From an antiviral design point-of-view, it means that the ongoing efforts to design influenza endonuclease inhibitors may directly benefit to the neglected *Arenaviridae* and *Bunyaviridae* virus family. Indeed, 2,4-dioxo-4-phenylbutanoic acid, a potent inhibitor of the influenza endonuclease, is also active on La Crosse virus endonuclease and has been crystallized in its active site (Reguera et al., 2010).

2.8.5. Lassa virus Z protein

Arenaviral Z proteins are small zinc-binding proteins of 90–103 amino-acid residues. For LCM virus and Lassa virus, the Z proteins were shown to be the driving force for the budding of virus from infected cells (Strecker et al., 2003, 2006). The expression of Lassa virus Z protein alone in Vero cells is sufficient for the production and release of lipid-enveloped virus-like particles (Strecker et al., 2003). N-terminal myristoylation of the Lassa virus Z protein has been demonstrated to be essential for the interaction of the protein with host-cell membranes (Strecker et al., 2006). The arenaviral Z proteins have also been reported to interact with several host-cell proteins including the promyelotic leukemia protein (Borden et al., 1998a), the nuclear fraction of the ribosomal protein P0 (Borden et al., 1998b), and eIF4E (Campbell Dwyer et al., 2000).

For some arenaviruses, the Z protein has been reported to be involved in regulation of transcription and RNA replication (Strecker et al., 2003). Recently, the Z protein of Tacaribe virus has been shown to inhibit the polymerase activity of the L protein (see below; Wilda et al., 2008). The core of the Z protein contains a RING domain of about 60 residues, which is able to bind two zinc ions (Salvato and Shimomaye, 1989). The so-called late domains (PTAP and PPPY) at the C-terminus of the proteins are also required for the formation and release of virus-like particles (Strecker et al., 2003).

Within the VIZIER project, the Lassa Z protein was expressed with an N-terminal His tag in E. coli and purified by Ni-NTA chromatography. The findings suggest that the addition of metal ions to the growth medium affected the solubility and the oligomerization state of the protein. Z protein from non-metal-supplemented cultures consisted of a mixture ranging from monomers to polymers. In contrast to the observations reported by Garcıá et al. (2006), we were able to produce a stable and soluble, metal ion-free form of the Lassa virus Z protein. The addition of Zn^{2+} or Co^{2+} resulted in a narrower oligomer-distribution with a maximum corresponding to 12- to 14-mers (according to gel filtration and Dynamic Light Scattering). The metal content was determined by Atomic Absorption Spectroscopy (AAS) and by Proton-Induced X-ray Emission-spectroscopy (PIXE). The maximum metal-ion content was determined as two and three Zn^{2+} per protein, by AAS and PIXE, respectively. The nature and the location of a possible third metal-binding site are currently unknown. Studies to characterize the binding sites of ^{57}Fe-substituted Lassa virus Z protein by Mößbauer spectroscopy are in progress. Homogeneous protein fractions (according to Dynamic Light Scattering) yielded small crystals, which are currently being improved.

3. Hantaviruses

3.1. Taxonomy and epidemiology

Hantaviruses form a unique genus (*Hantavirus*) within the *Bunyaviridae* family. Twenty-three species are currently recognized by the ICTV but many recently discovered novel hantaviruses remain to be included. On the other hand, proposed new stringent species definition criteria may lead to a reduction in the number of species (Maes et al., 2009a,b). Hantaviruses differ from other bunyaviruses in one important ecological aspect: they are not transmitted by arthropod vectors. In common with the arenaviruses, their natural hosts are mainly rodents and the hantaviruses also have a strict association with specific natural hosts. Thus, by analogy with the arenaviruses their distribution is determined by that of their vertebrate host (Schmaljohn and Hjelle, 1997). They are dispersed and infect hosts via aerosolized rodent excreta and in the case of their natural hosts they produce an asymptomatic persistent infection. In contrast, infected humans may develop hemorrhagic fever with renal syndrome (HFRS) in Asia and Europe, or hantavirus cardiopulmonary syndrome (HCPS) in the Americas.

Hantaan virus (HTNV) and Seoul virus (SEOV) are prominent examples of hantaviruses that cause HFRS in Asia. Approximately 150,000 HFRS cases are estimated to occur worldwide annually, more than 90% being reported from Asia where the most severe cases with fatality rates reaching 15% are recorded (Kariwa et al., 2007). The most common European hantavirus is Puumala virus which causes a mild form of HFRS often called *nephropathia epidemica.* Severe HFRS is also seen in Europe and is caused by Dobrava-Belgrade virus (DOBV) (Krüger et al., 2001; Klempa et al., 2008). Sin Nombre virus (SNV) from North America and Andes virus (ANDV) from South America are the most important representatives of viruses causing HCPS. Thus far, ANDV is the only known hantavirus that displays human-to-human transmission (Padula et al., 1998; Martinez et al., 2005; Ferres et al., 2007).

3.2. Genome structure and replication strategy

Hantaviruses are lipid-enveloped, spherical viruses of about 80–110 nm in diameter. They have a genome organization typical of the *Bunyaviridae* family, consisting of three negative-stranded RNA segments: a small (S) segment (~1700–2100 nucleotides) encoding the viral nucleocapsid (N) protein, a medium (M) segment (~3700 nucleotides) encoding the envelope glycoproteins G1 and G2 (co-translationally cleaved from a glycoprotein precursor), and a large (L) segment (~6500 nucleotides) encoding the viral RNA-dependent RNA polymerase (L protein). The L protein acts as a replicase, transcriptase, endonuclease and possibly, RNA helicase. The 5′ and 3′-terminal sequences of all three genome segments are genus-specific, highly conserved and display reverse complementarity to each other, thus being capable of forming panhandle structures. They are thought to play a role in viral transcription and in the proposed prime-and-realign mechanism of replication (Plyusnin et al., 1996; Maes et al., 2004; Khaiboullina et al., 2005).

3.3. Recently discovered hantaviruses

Over the past three years hantavirus research has passed two important milestones. The first was the discovery of hitherto unknown endemic hantaviruses in Africa and the second was the discovery of a group of novel, phylogenetically very distinct hantaviruses in non-rodent hosts.

Although several studies in African populations have shown the presence of antibodies that cross-react with Eurasian hantaviruses, no indigenous hantaviruses in African rodents were known until 2006 when the first genetic evidence for the presence of hantaviruses in Africa was reported. The first African hantavirus was found in the African wood mouse (*Hylomyscus simus*) trapped in a forest habitat in Guinea, West Africa, and named Sangassou virus (SANGV) after the village where the animal had been trapped (Klempa et al., 2006). A second African hantavirus was found in Guinea soon after and its discovery was even more surprising than the first one, thus initiating the search for hantaviruses in non-rodent hosts. Very divergent hantavirus sequences were found in Therese's shrew (*Crocidura theresae*). It was named Tanganya virus (TGNV), and is only distantly related to other hantaviruses, reflecting the fact that it was found in a shrew instead of a rodent (Klempa et al., 2007). Although HFRS and HCPS are not recognized diseases in Guinea, or Africa in general, the pathogenic potential of SANGV, TGNV, and probably other, yet undiscovered African hantaviruses should not be underestimated. Hantavirus-associated disease may be confused with other severe diseases or may be unrecognized because of limited health-care conditions.

Interestingly, Thottapalayam virus (TPMV) found in 1971 in Asian house shrew (*Suncus murinus*) was for decades considered to be the single exception of a hantavirus with a non-rodent reservoir. However, shortly after the discovery of the shrew-associated TGNV several other unique shrew-borne isolates of hantavirus were reported. These include the detection of Camp Ripley virus in the northern short-tailed shrew (*Blarina brevicauda*) (Arai et al., 2007), Ash River virus in the masked shrew (*Sorex cinereus*), Jemez Springs virus in the dusky shrew (*Sorex monticolus*) in the United States (Arai et al., 2008a), Cao Bang virus (CBNV) in the Chinese mole shrew (*Anourosorex squamipes*) in Vietnam (Song et al., 2007a), Seewis virus in the Eurasian common shrew (*Sorex araneus*) in Switzerland (Song et al., 2007b), and Imjin virus (MJNV) in the Ussuri white-toothed shrew (Crocidura lasiura) in Korea (Song et al., 2009). Most recently, the spectrum of hantavirus hosts was further extended to moles when Asama virus was found in the Japanese shrew mole (*Urotrichus talpoides*) trapped in Japan (Arai et al., 2008b) and Oxbow virus in the American shrew mole (*Neurotrichus gibbsii*) trapped in the United States (Kang et al., 2009).

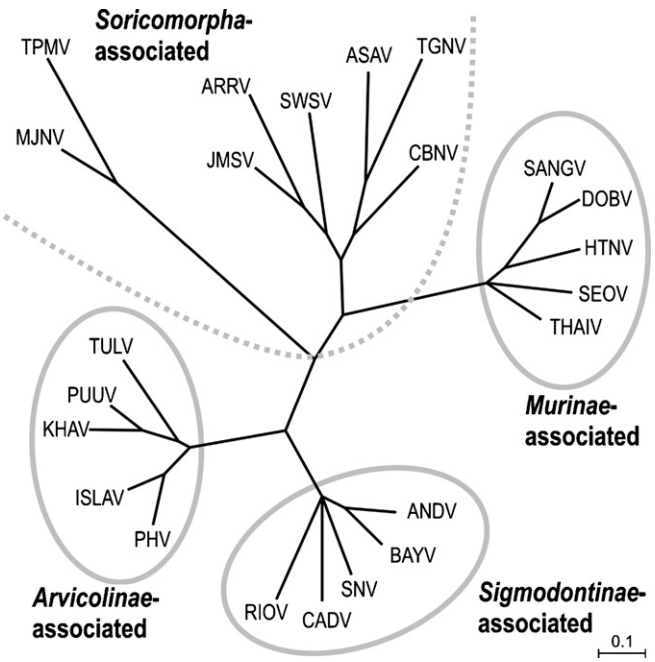

Fig. 5. Maximum likelihood phylogenetic tree of hantavirus representatives illustrating relationships between the three major groups of rodent-borne hantaviruses (indicated by grey ellipsoids) and novel shrew- and mole-associated hantaviruses (indicated by interrupted grey curve) based on partial N protein amino acid sequences (147 aa, position 217–365). The tree was computed with the TREE-PUZZLE package by using the JTT evolutionary model. The scale bar indicates an evolutionary distance of 0.1 substitutions per position in the sequence. SANGV, Sangassou virus; HTNV, Hantaan virus; SEOV, Seoul virus; DOBV, Dobrava-Belgrade virus; THAIV, Thailand hantavirus; PUUV, Puumala virus; TULV, Tula virus; KHAV, Khabarovsk virus; ISLAV, Isla Vista virus; PHV, Prospect Hill virus; SNV, Sin Nombre virus, ANDV, Andes virus; BAYV, Bayou virus; RIOV, Rio Segundo virus; CADV, Cano Delgadito virus; TPMV, Thottapalayam virus; MJNV, Imjin virus; TGNV, Tanganya virus; CBNV, Cao Bang virus; ARRV, Ash River virus; JMSV, Jemez Springs virus; SWSV, Seewis virus; ASAV, Asama virus.

3.4. Evolutionary relationships among the hantaviruses

Hantavirus species are strongly associated with one (or a few closely related) specific rodent species as their natural reservoir hosts, mainly rodents but, as recently reported, also insectivores (order *Soricomorpha*; shrews and moles). This close association is mirrored also in their phylogeny; rodent-associated hantaviruses form three major evolutionary clades corresponding to the three subfamilies of their rodent hosts. HTNV, SEOV, DOBV, as well as the recently discovered African SANGV are examples of *Murinae*-associated hantaviruses, PUUV and Tula virus (TULV) belong to the *Arvicolinae*-associated hantaviruses while SNV and ANDV are members of the group called *Sigmodontinae*-associated hantaviruses. Interestingly, the *Soricomorpha*-associated viruses do not seem to be monophyletic. At least with the currently available limited sequence data, there seem to be at least two main clusters of shrew- and mole-borne viruses but without a clear association to their hosts (Fig. 5).

The obvious similarities of hantavirus and rodent phylogenies have been the basis for the hantavirus-rodent co-evolution and co-speciation concept (Plyusnin et al., 1996). However, recent phylogenetic analyses and evolutionary rate calculations as well as recent findings of shrew- and mole-associated hantaviruses suggest that this concept will have to be re-evaluated (Ramsden et al., 2008, 2009). Moreover, high diversity and wide geographic distribution of the recently discovered hantaviruses together with the fact that insectivores and mice are very divergent in evolutionary terms make it reasonable to predict that other groups of mammals will be found to carry yet undiscovered hantaviruses in the near future (Henttonen et al., 2008; Klempa, 2009).

3.5. Hantavirus infections of humans

Both hantavirus diseases, HFRS and HCPS, are acute febrile infections. The initial symptoms are very similar and include abrupt onset of high fever, malaise, myalgia, back and abdominal pain, and other flu-like symptoms. HFRS is mainly characterized by renal failure and hemorrhages, varying from small petechiae to severe internal bleeding and the disseminated intravascular coagulation syndrome. On the other hand, pneumonia and cardiovascular dysfunction are characteristic for HCPS.

Common factors of hantavirus infections include increased permeability of the microvascular endothelium and thrombocytopenia. The complex pathogenesis of HFRS and HCPS is currently assumed to be a multifactoral process which includes T-cell mediated endothelial damage, immune effectors, and β_3-integrin dysfunction-mediated increase of vascular permeability (Krüger et al., 2001; Maes et al., 2004; Khaiboullina et al., 2005; Gavrilovskaya et al., 2008).

3.6. Vaccines

Different rodent brain and cell culture-derived inactivated vaccines are used locally in South Korea and China but the methods of their preparation are considered unacceptable for human vaccination in most western countries (Piyasirisilp et al., 1999). Hantavax®, a formalin-inactivated suckling mouse brain marketed in Korea demonstrated capacity to elicit neutralizing antibodies in only about half of the recipients (Cho et al., 2002; Sohn et al., 2001). Field trials have not been conducted, and a case–control study was inconclusive because of insufficient statistical power (Park et al., 2004). In China, HTNV- and SEOV-inactivated vaccines have been produced in cell cultures, and elicited neutralizing antibody responses in about 50% and 80% of recipients receiving three doses, respectively. An inactivated, bivalent vaccine (HTNV and SEOV), was also developed and tested in a total of 1100 persons in China: more than 90% of vaccines developed neutralizing antibodies to both HTNV and SEOV after 3 doses (Dong et al., 2005).

A variety of techniques, including recombinant proteins, virus-like particles and chimeric viruses as well as DNA vaccines have been investigated and found protective in animal trials (reviewed in Maes et al., 2009b). In the most recent approach, adenovirus vectors expressing hantavirus proteins protected hamsters against lethal challenge with ANDV (Safronetz et al., 2009).

To avoid some of the problems associated with cell culture or rodent brain-derived vaccines, two molecular vaccines for HFRS have been tested in humans. The first was a recombinant vaccinia virus (VACV)-vectored vaccine expressing the M and the S segments of HTNV (Woo et al., 2005). Low rate of neutralizing antibodies to HTNV were observed, and this vaccine has not been pursued. Recently, plasmid DNA delivered by gene gun has been developed and tested in animals (Hooper et al., 1999; Spik et al., 2008).

3.7. Therapy of hantavirus infections

3.7.1. Immune plasma

At present, there have been no published reports of controlled clinical trials of immunotherapy for HFRS or HCPS (Jonsson et al., 2008).

3.7.2. Antiviral drugs

Currently, no approved antiviral drug is available for specific treatment of the hantavirus diseases. The only antiviral drug which has been shown to have *in vitro* activity and to some extent also in

vivo activity against some hantaviruses is ribavirin (Severson et al., 2003). The efficacy of ribavirin therapy given to HTNV-infected suckling mice showed that the ribavirin-treated mice had a higher survival rate than the placebo control group (Huggins et al., 1986). Ribavirin was tested for efficacy in HFRS patients in China and shown to have a statistically significant beneficial effect if initiated early in the disease course (Huggins et al., 1991). More recently, results of a clinical study using intravenous ribavirin A in a total of 38 individuals enrolled between 1987 and 2005 were supportive of an efficacy demonstrated by a decreased occurrence of oliguria and decreased severity of renal insufficiency (Rusnak et al., 2009).

On the other hand, the results of trials in patients suffering from HCPS yielded disappointing results (Chapman et al., 1999; Mertz et al., 2004). Two double-blind, placebo-controlled efficacy trials have been performed in persons with HCPS in the cardiopulmonary phase (Chapman et al., 1999; Mertz et al., 2004). The majority of the patients were in the cardiopulmonary stage when they enrolled, and treatment with ribavirin had no clinical benefit, suggesting that its efficacy may depend on the phase of infection and the severity of disease when treatment is initiated and calling attention to the need for early intervention. The major problem is that progression to respiratory failure, shock and death typically occurs within hours of presentation in the cardiopulmonary phase, leaving little time for the study intervention to have an effect.

Recently, a diverse series of 3-substituted 1,2,4-triazole-beta-ribosides were prepared and one compound with antiviral activity was identified, 1-beta-D-ribofuranosyl-3-ethynyl-[1,2,4] triazole (ETAR). It showed promising antiviral activity against HTNV and ANDV. ETAR did not increase mutation frequency of the HTNV genome, which suggests it has a different mechanism of action from ribavirin. Mechanism and metabolism studies identified its activity as being primarily due to inosine monophosphate dehydrogenase inhibition with reduction of GTP pools, which was combined with residual complementary activity possibly affecting the L protein. Although ETAR protected suckling mice from infection with HTNV only to a degree comparable to the efficacy of ribavirin it is a promising scaffold for antiviral drug development (Chung et al., 2008).

Recently, other antiviral strategies have been evaluated such as the use of cyclic peptides which bind $\alpha_v\beta_3$ integrin as a virus receptor and thereby blocked SNV and HTNV infection of Vero E6 cells (Larson et al., 2005; Hall et al., 2007) or multivalent cyclic peptides presented on nanoparticles which specifically prevented SNV infection *in vitro* (Hall et al., 2008).

3.8. The VIZIER quest for antiviral drugs against hantaviruses

The main achievements of the VIZIER Program in the field of hantaviruses were obtained in the virus discovery and genomics part of the project. Identification of Sangassou virus as the first African hantavirus (Klempa et al., 2007) dramatically extended the dogmatic view about geographic range, evolution, and epidemiology of hantaviruses. Tanganya virus (Klempa et al., 2007) as the second African hantavirus was, moreover, the second shrew-borne hantavirus ever found (after TPMV, known for decades but considered exceptional) and initiated the recent boom of novel shrew- and mole-borne hantaviruses. Moreover, the novel genetic lineage of Dobrava virus causing severe HFRS cases in southern European Russia, and the Dobrava strain causing HFRS outbreaks in central European Russia were identified and genetically characterized (Klempa et al., 2008).

Unfortunately, the final goal of the VIZIER project, obtaining crystal structures, was not reached for hantavirus L protein domains. Nevertheless, intensive efforts were made in the field of hantavirus L protein domain predictions, expression, and solubility problems. Altogether, 85 expression constructs were prepared involving 20 different domain predictions/constructs for seven different hantavirus strains. The main effort was focused on the LI domain of still unknown function and the polymerase domain by itself. Different domain prediction approaches were used. First of all, tools developed within the consortium were used, such as Viralis and VaZyMoLo. Altogether, twelve soluble proteins of five different viruses were prepared, including two constructs of the LI domain, three constructs of the LII domain, and one construct of the N protein.

4. Conclusions

Although many studies are under way to discover and further investigate antivirals active against pathogenic arenaviruses and hantaviruses, there will be a significant delay before drugs may be available for patient treatment in hospital settings (approach #1). Therefore, the option to test licensed drugs for their efficacy against arenaviral and hantaviral infections should be considered and encouraged actively (approach #2). While on the face of it, this approach may appear unorthodox, it could provide the indisputable advantage of drastically reducing the delay between drug discovery and possible use as a treatment for patients. These two approaches should not be considered competitive, but complementary. They should be combined to bring active molecules against highly pathogenic viruses onto the market.In the case of both arenavirus and hantavirus protein constructs, we observed that the same constructs prepared using different viruses showed different expression levels for unknown reasons. Therefore the option of increasing the number of targets, on a purely empirical basis, may be efficient. Accordingly, using biodiversity, specifically point mutations selected in strains circulating in nature to overcome solubility, crystallization and diffraction problems showed to be an efficient strategy. Practical evidence demonstrated that increasing the number of viruses or strains, including a large diversity of clinical strains with minimal genomics heterogeneity, may be a successful option to obtain diffracting crystals. Usage of the wide spectrum of naturally occurring variants of the same viral protein to overcome solubility problems is therefore a very important lesson that can be taken from the VIZIER effort on hantaviruses. Although the obtained soluble proteins did not reach the crystallization stage during the VIZIER project, they provide a solid basis for further development in the near future.

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