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BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

Increasing the Accumulation of Modular Nanotransporters in Mouse Tumors by Attaching Polyethylene Glycol to These Nanotransporters with the Possibility of Its Release into the Tumors

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Abstract—Previously, polypeptide constructs—modular nanotransporters (MNTs)—were created to deliver biologically active molecules into the nuclei of melanoma cells. In the present work, polyethylene glycol (PEG) molecules were attached to them at the N-terminal cysteine, both with the possibility of their subsequent cleavage at the hydrolysis site of tumor-specific proteases, and without this site (non-detachable PEG). All MNT variants labeled with the radioisotope ^{111}In were administered to mice with inoculated Cloudman S91 melanoma. The kinetics of radioactivity distribution in the mouse body was studied using single-photon emission computed tomography. Analysis of the obtained data using a compartmental mathematical model allowed us to establish that the attachment of PEG to MNT increased its lifetime in the blood and significantly increased its accumulation in the tumor. Addition of a PEG detachment site by tumor-specific protease led to a strong retention of this MNT in the tumor. The data obtained can serve as a basis for the creation of new effective antitumor drugs.

Keywords: modular nanotransporters, melanoma, polyethyleneglycol, single-photon emission computed tomography, tumor-specific proteases, compartment model

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Polypeptide constructs called modular nanotransporters (MNTs) are among the promising systems for delivering locally acting drugs to a given compartment of target cells [1]. These recombinant MNTs with a molecular weight of 60–80 kDa consist of several modules connected by peptide bonds, which are parts of natural or modified proteins and peptides. The ligand module of MNTs allows them to bind to endocytosed receptors on the surface of target cells and, due to receptor-mediated endocytosis, enter endosomes; the endosomolytic module causes the formation of pores in the membranes of endosomes, thereby ensuring the release of MNTs from endosomes into the hyaloplasm; the entry, for example, into the cell nucleus, is ensured by a module with an amino acid sequence of nuclear localization signal; and the carrier module, to which the transported "cargo" can be attached, helps to impart the desired conformation to the entire molecule [1]. Previously, it was shown that an intravenously administered MNT containing α-melanocyte-stimulating hormone (αMSG) as a ligand module, which can deliver attached photosensitizer bacteriochlorin *p* molecules to the nuclei of B16-F1 melanoma cells, can not only significantly decelerate tumor growth but also cause a more than twofold increase in the average lifespan of tumor-bearing mice [1]. However, an intravenously administered substance will accumulate not only in the target tumor but also in off-target organs and tissues, such as the liver and kidneys [2], as well as will be absorbed by immune system cells [3]. It is known that such a non-target accumulation can be reduced, simultaneously with a decrease in their immunogenicity and an increase in the lifetime of the administered substances in the blood, by attaching polyethylene glycol (PEG) molecules to them [4, 5]. We have previously shown that an MNT with αMSH conjugated with PEG with a molecular weight of 40 kDa showed the lowest accumulation in the liver and, hence, the longest lifetime in the blood [6]. In the present work, in order to clarify how the attachment of PEG and the possibility of its cleavage in the tumor affects the accumulation of MNT in the tumor, we studied the kinetics of this accumulation for ^{111}In-labeled MNTs with αMSH and MNTs conjugated to PEG without the possibility of cleavage (MNT-PEG_n) and with the possibility of cleavage (MNT-PEG) by tumor-specific proteases.

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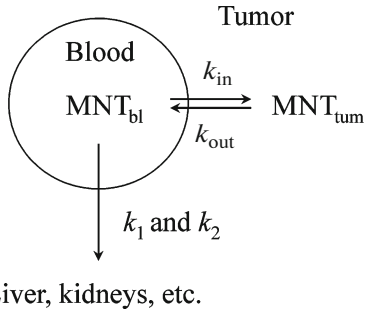

Fig. 1. Schematic diagram of a mathematical model describing the accumulation of MNT, MNT-PEG, and MNT-PEG_n in mouse tumors. The description of the model and the parameters used is given in the text.

In this work, we used the same MNT (73.1 kDa) as in the previous work [6], with the structure DTox–HMP–NLS–αMSH, where DTox is the endosomolytic module, the translocation domain of diphtheria toxin; HMP is the carrier module, a hemoglobin-like protein of *E. coli*; NLS is the optimized nuclear localization signal of the large T-antigen of the SV40 virus; and αMSH is the ligand module, α-melanocyte-stimulating hormone. This MNT targets melanoma cells characterized by increased expression of melanocortin receptors. For site-specific attachment of a maleimide derivative of PEG with a molecular weight of 40 kDa (Nanocs Inc., United States), a cysteine residue was inserted by genetic engineering methods into the amino acid sequence of this MNT at the N-terminus upstream of the DTox module (MNT-PEG_n). In another MNT variant (MNT-PEG), which is also subject to PEGylation, an LSGRSDNH site, which can be cleaved by tumor-specific proteases, such as urokinase-type plasminogen activator (uPA) [7], was inserted at its N-terminus between the cysteine residue and the DTox module. To study the kinetics of accumulation in the tissues of laboratory animals, the obtained MNT variants were labeled with the isotope ^{111}In as described in [6]. For this purpose, the chelator S-2-(4-isothiocyanatobenzyl)-1,4,7-triazocyclononane-1,4,7-triacetate (Macrocyclics, United States) was attached to MNT, MNT-PEG_n, and MNT-PEG. The degree of labeling for all MNT variants was 2–3 chelator molecules per MNT molecule. Male DBA/2J mice weighing 22–24 g (Pushchino Laboratory Animal Breeding Facility) were inoculated with Cloudman's melanoma S91, clone M3 (5000 subtype 1 melanocortin receptors per cell [8]). Mice (three animals in each group) with tumors larger than 0.2 mL were injected into the tail vein under anesthesia (inhalation of 0.8–1.5% isoflurane in air) with 300 μL of ^{111}In-MNT, ^{111}In-MNT-PEG_n, or ^{111}In-MNT-PEG in an amount of 1 μg per mouse and a total activity of approximately 400 kBq. The kinetics of radioactivity distribution in the mouse body was studied under isoflurane anesthesia using the SPECT procedure on a U-SPECT-II/CT scanner (MILabs, Netherlands) coupled to an X-ray computed tomograph and equipped with a collimator with a hole diameter of 1 mm. Each image was recorded for 15 min. Image reconstruction was performed using the U-SPECT-Rec2.34b software, and subsequent quantitative analysis was performed using the PMOD 3.4 software. Anatomical reference of the obtained biodistribution patterns was performed using X-ray computed tomography. The radioactivity measured in the heart was taken as the radioactivity in the blood. To determine the radioactivity in the entire volume of the heart or tumor, they were circumscribed with one or several ellipsoids, in which the total radioactivity was then measured. The dose of radioactivity in tissue per gram was obtained by dividing this radioactivity by the volume of the ellipsoids and assuming that the tissue density is close to 1 g/mL. The total radioactivity of the entire mouse organism immediately after administration of ^{111}In-MNT, ^{111}In-MNT-PEG_n, or ^{111}In-MNT-PEG was considered as the injected dose (ID). The ratio of the radioactivity dose in tissue per gram to the injected dose gave the percentage of the injected dose per gram of tissue ((%)ID/g). When calculating (%)ID/g, we took into account the fact that ^{111}In has a half-life of 2.8 days.

The accumulation of MNT in the tumor was described using a compartmental mathematical model (Fig. 1), similar to that proposed by Thurber et al. [9] but adapted for the analysis of SPECT data and taking into account the biexponential decline in the concentration of MNT in the blood. In this model, which describes the standard exchange processes between the blood and tumor, the blood and tumor are considered as separate compartments into which molecules enter and exit with certain rate constants. The concentration of MNT, MNT-PEG, or MNT-PEG_n in the blood was designated as MNT_{bl} (Fig. 1).

Usually, the concentration of protein in the blood is well described by the sum of two exponents that are presumably responsible for its absorption by the liver and kidneys [9]. Therefore, we assumed that

$$MNT_{bl} = MNT_{bl01} \cdot e^{-k_1 \cdot t} + MNT_{bl02} \cdot e^{-k_2 \cdot t}, \quad (1)$$

where k_1 and k_2 are the rate constants of MNT departure from the blood, and MNT_{bl01} and MNT_{bl02} are the MNT concentrations in the blood corresponding to the contributions of each of these components. The concentration of MNT, MNT-PEG, or MNT-PEG_n in the tumor was designated as MNT_{tum}, and the rate constants of MNT entry into the tumor and exit from the tumor were designated as k_{in} and k_{out}, respectively (Fig. 1).

For this model, the change in the MNT concentration in the tumor is described by the equation

$$\frac{dMNT_{tum}}{dt} = k_{in} \cdot MNT_{bl} - k_{out} \cdot MNT_{tum}, \quad (2)$$

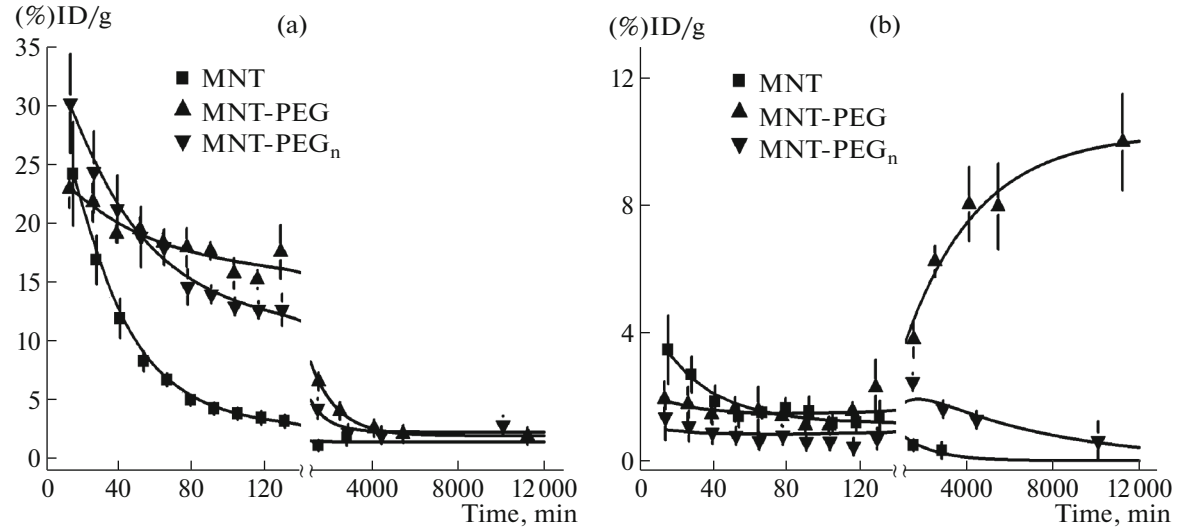

Fig. 2. Changes in the accumulation of MNT, MNT-PEG, and MNT-PEG$_n$ labeled with ^{111}In in the blood (a) and tumor (b) of mice after intravenous administration of MNT. Accumulation is shown as % of the injected dose per gram of tissue ((%)ID/g). Mean values ± standard error (n = 2–3) are shown. Lines show the results of interpolation according to dependencies (1), (3), and (4).

where the concentration of MNT in the blood is given by expression (1). Taking into account the initial conditions, we obtained from expressions (1) and (2) that the concentration of MNT in the tumor, MNT_{tum}, is

$$MNT_{tum} = \frac{MNT_{bl01} \cdot k_{in}}{k_{out} - k_1}(e^{-k_1 t} - e^{-k_{out} t}) + \frac{MNT_{bl02} \cdot k_{in}}{k_{out} - k_2}(e^{-k_2 t} - e^{-k_{out} t}). \quad (3)$$

The signal in the tumor obtained using SPECT reflects the accumulation of MNT labeled with ^{111}In not only in the tumor tissue but also in the tumor vasculature. To take into account the contribution of blood, we introduced parameter α – the volume fraction of blood in the tumor. Then, the accumulation in the tumor obtained by the SPECT procedure, $MNT_{tum\ SPECT}$, is

$$MNT_{tum\ SPECT} = (1 - \alpha)MNT_{tum} + \alpha MNT_{bl}, \quad (4)$$

where MNT_{tum} and MNT_{bl} are given by expressions (3) and (1), respectively. Interpolation by expressions (4), (3), and (1) of the obtained kinetic dependences of MNT accumulation in the blood and tumors in the studied animals showed that, on average, α = 8.8 ± 1.7%. The contribution of blood to the tumor signal is especially large in the first hour after MNT administration.

The obtained kinetics of MNT, MNT-PEG, or MNT-PEG$_n$ accumulation in the blood and tumor of mice show that PEGylation leads to both an increase in the MNT lifetime in the blood and a marked increase in the MNT accumulation in the tumor (Fig. 2). The presence of a PEG cleavage site by tumor-specific proteases has a positive effect on the MNT-PEG accumulation in the tumor (Fig. 2b). Quantitative characteristics of MNT, MNT-PEG, or MNT-PEG$_n$ accumulation were obtained using the proposed mathematical model (Fig. 1). The change in the MNT concentration in the blood (Fig. 2a) was well described by dependence (1) (r^2 = 0.999, 0.996, and 0.993 for MNT, MNT-PEGn, and MNT-PEG, respectively). In turn, the accumulation of MNT in the tumor (Fig. 2b) was interpolated quite well by dependence (4), where dependences (1) and (3) were used as components (r^2 = 0.955, 0.761, and 0.985 for MNT, MNT-PEG$_n$ and MNT-PEG, respectively), and the parameters of dependence (1) were fixed according to the values determined from the kinetics of MNT accumulation in the blood. The parameters obtained as a result of such interpolation are summarized in Table 1. As can be seen in Fig. 2a and Table 1, the increase in the MNT lifetime due to the attachment of PEG is largely associated with a decrease in the contribution of the component with the highest excretion rate, MNT_{bl01}, presumably corresponding to the proportion of MNT absorbed by the liver, rather than with a change in the excretion rate constants.

It is known that, if a molecule is not retained in the tissue, the k_{in}/k_{out} ratio reflects the fraction of intercellular space in the tissue, which is 0.05–0.5 for different tissues [9]. For MNT and MNT-PEG$_n$, this ratio is approximately 1.5 (Table 1), which is at least 3 times greater than the fraction of intercellular space in the tumor. This indicates a significant retention of these MNTs in the tumor, which can be explained by the interaction of MNTs with tumor cells. Moreover, a similar k_{in}/k_{out} ratio for MNT and MNT-PEG$_n$ indi-

Table 1. Parameters obtained as a result of interpolation of experimental data in accordance with dependences (1), (3), and (4). The description of parameters is given in the text. Data are presented as values of estimated parameters ± standard error

	Blood				Tumor	
	MNT_{bl01}, (%) ID/g	MNT_{bl02}, (%) ID/g	$k_1 \times 10^{-3}$, min^{-1}	$k_2 \times 10^{-3}$, min^{-1}	$k_{in} \times 10^{-4}$, min^{-1}	$k_{out} \times 10^{-4}$, min^{-1}
MNT	35.1 ± 1.9	1.9 ± 2.5	35 ± 4	3 ± 10	10.1 ± 0.9	6.7 ± 2.2
MNT-PEG	8.0 ± 1.3	15.3 ± 1.2	24 ± 12	0.80 ± 0.14	2.09 ± 0.19	≤0.15
$MNT\text{-}PEG_n$	24.1 ± 1.1	10.6 ± 1.0	25 ± 3	1.2 ± 0.3	2.8 ± 0.4	1.6 ± 0.6

cates that the attachment of PEG does not hamper the interaction of the ligand module (α-MSH) with its receptor in the tumor. It should be noted that PEGylation resulted in a more than threefold decrease in k_{in} (Table 1), which may be due to an increase in the size of the molecule.

For MNT-PEG, which has a site for PEG cleavage by tumor-specific proteases, k_{in} is the same as for $MNT\text{-}PEG_n$ (Table 1). When interpolating the data using the proposed model (Fig. 1), all constants were considered greater than or equal to zero. When interpolating the data for MNT-PEG in the tumor (Fig. 2b), k_{out} was indistinguishable from zero (Table 1). In other words, only the presence of the site for tumor-specific proteases led to a significant retention of MNT-PEG in the tumor. Presumably, this retention is associated with the interaction of this hydrolysis site with tumor-specific proteases in an inactive state, which can form fairly large aggregates [10, 11].

Thus, the analysis of the obtained kinetics of changes in the MNT concentration in the blood and accumulation in the tumor allowed us to establish that PEGylation not only increases the lifetime of MNT in the blood but also significantly increases its accumulation in the tumor. It was found that the insertion of a site for PEG cleavage from MNT by tumor-specific proteases leads to the retention of this MNT in the tumor, which increases its maximum accumulation in the tumor at least 3 times. The data obtained make it possible to optimize the structure of MNT for its systemic use by PEGylation of MNT with the possibility of PEG cleavage in the tumor.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (http://oacu.od.nih.gov/regs/index.htm). Animal protocols were approved by the Bioethics Committee of the Institute of Gene Biology of the Russian Academy of Sciences, Moscow, Russia (Approval ID no. 11, March 15, 2021).

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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