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Biotin-decorated anti-cancer nucleotide theranostic conjugate of human serum albumin: Where the seed meets the soil?

Tatyana V. Popova [a,b], Hamda Khan [c,d], Alexey S. Chubarov [a,b], Vladimir A. Lisitskiy [a], Natalya M. Antonova [a,b], Andrey E. Akulov [e], Oleg B. Shevelev [e], Evgenii L. Zavjalov [e], Vladimir N. Silnikov [a], Saheem Ahmad [c,d], Tatyana S. Godovikova [a,b,*]

[a] Institute of Chemical Biology and Fundamental Medicine, SB RAS, Lavrentiev Ave. 8, Novosibirsk 630090, Russia
[b] Novosibirsk State University, Pirogova St. 2, Novosibirsk 630090, Russia
[c] Integral Research Centre-I, Integral University, Dasauli, P.O. Bas-ha Kursi Road, Lucknow 226026, U.P., India
[d] Department of Biosciences, Integral University, Dasauli, P.O. Bas-ha Kursi Road, Lucknow 226026, U.P., India
[e] Institute of Cytology and Genetics, SB RAS, Lavrentiev Ave. 10, Novosibirsk 630090, Russia

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ABSTRACT

Human serum albumin is playing an increasing role as a drug carrier in clinical settings. Biotin molecules are often used as suitable tags in targeted anti-tumor drug delivery systems. We report on the synthesis and properties of a new multimodal theranostic conjugate based on an anti-cancer fluorinated nucleotide conjugated with a biotinylated dual-labeled albumin. Interestingly, *in vitro* and *in vivo* study revealed stronger anti-tumor activity of the non-tagged theranostic conjugate than that of the biotin-tagged conjugate, which can be explained by decreased binding of the biotin-tagged conjugate to cellular receptors. Our study sheds light on the importance of site-specific albumin modification for the design of albumin-based drugs with desirable pharmaceutical properties.

The development of state-of-the-art theranostic strategies, which can help simultaneously diagnose early stage cancer, deliver therapeutic drugs to tumors, monitor drug release kinetics, and visualize cancer stages, offers techniques for effective and personalized medicine for cancer patients.[1] Combining these modalities for cancer treatment into one has the potential to prolong, localize, and minimize various toxic side effects and improve the therapeutic indexes of existing drugs.[2] Generally, an effective diagnostic and therapeutic delivery system is composed of three parts: (1) a cancer targeting moiety that can selectively guide the whole system into the tumor region; (2) an effector unit such as a reporter (e.g. a fluorophore or MRI contrast agent), and a pharmaceutical agent (e.g. a chemical drug, peptide, small interfering RNA, or gene); (3) a cleavable linker that is sufficiently stable to reduce cytotoxicity prior to its delivery to the target cells and cleaved efficiently enough to release its effector molecule upon arrival at the target and triggering by bio-species, pH, light, or other factors.[3–9] Recently[10], we have reported on a human serum albumin-therapeutic nucleotide conjugate that meets these design criteria.

The long circulatory half-life of approximately 19 days for human serum albumin (HSA), facilitated by engagement with the recycling cellular neonatal Fc receptor (FcRn),[11,12] has contributed to its application in drug half-life extension technology,[13] as well as other clinical fields.[14] Though the mechanism of HSA uptake in tumors is still unclear, it has been known that albumin targets tumors based on the leaky blood vessel-related enhanced permeability and retention (EPR) effect,[15,16] which means that albumin accumulates in tumor by simple infiltration, not by specific uptake. However, it has been suggested that secreted protein acidic and rich in cysteine (SPARC) could sequester albumin in tumor stroma, which would partially cause the tumor specific uptake of albumin.[17] It has been reported that SPARC is overexpressed in brain tumors, serving as a promoter to glioma progression and invasion, suggesting its potential therapeutic value.[17,18] To our interest, we conceived an idea of using albumin as a SPARC-ligand and

Abbreviations: Bio, biotin; HSA, human serum albumin; Hcy, homocysteine; Hcy-HSA, *N*-homocysteinylated human serum albumin; HTL, homocysteine thiolactone; MI, maleimide; MRI, magnetic resonance imaging; PEG, poly(ethylene glycol); PEGBio, biotin functionalized poly(ethylene glycol); PFT, perfluorotoluene; PFT-HTL, *N*-(2,3,5,6-tetrafluoro-4-(trifluoromethyl)phenyl)homocysteine thiolactone; TFT, 5-trifluoromethyl-2′-deoxyuridine (trifluorothymidine).

* Corresponding author at: Institute of Chemical Biology and Fundamental Medicine, SB RAS, Lavrentiev Ave. 8, Novosibirsk 630090, Russia.
E-mail address: godov@niboch.nsc.ru (T.S. Godovikova).

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transporter for an effector unit containing a fluorophore and MRI contrast agent as a reporter and, a chemotherapeutic agent 5-trifluoromethyl-2′-deoxyuridine 5′-monophosphate. We have developed a novel anti-cancer albumin-trifluorothymidine theranostic conjugate PFT-Hcy-HSA-Cy7-pTFT with disulfide and phosphamide bonds for on-demand delivery of the chemotherapeutic agent 5-trifluoromethyl-2′-deoxyuridine 5′-monophosphate (pTFT) in response to redox and pH dual trigger.[10] Our initial results demonstrate the potential of PFT-Hcy-HSA-Cy7-pTFT conjugate to serve as an optical and ^{19}F MR imaging agent. Based on their detection sensitivity, the combination of ^{19}F MRI and fluorescence imaging is probably the most valuable one in multimodal molecular imaging.

In addition to albumin, biotin (vitamin H) has been classified as a key micronutrient for cellular function and cell growth.[19] Humans and other mammals cannot synthesize biotin and thus must obtain it from exogenous sources via intestinal absorption. Rapidly dividing cells such as cancer cells have a voracious appetite to vitamins including biotin, and biotin levels have been found to be significantly higher in some cancer cells compared to normal tissue.[20,21] Therefore, biotin transporters have also attracted great attention for their promising application in biomimetic delivery.[22–24] Accordingly, several research groups have tried different biotinylated chemotherapeutic agents for cancer cell-specific drug delivery.[24] It was shown that biotin-conjugates are favorably integrated into tumors because of overexpression of biotin-specific uptake systems on the cell surface in rapidly proliferating malignant cells.[24]

Biotin is transported also into the brain by a sodium-dependent multivitamin transporter (SMVT).[25] Therefore, biotin transporters have also attracted our great attention for their promising application in biomimetic delivery. Motivated by this idea, we developed a method for ligand-directed albumin conjugate synthesis, based on an anti-cancer fluorinated nucleotide conjugated with a biotinylated dual-labeled albumin.

The synthetic scheme for preparation of the theranostics conjugates PFT-Hcy-HSA-Cy7-pTFT and PFT-Hcy-HSA-PEGBio-Cy7-pTFT is given in Fig. 1. To synthesize PFT-Hcy-HSA-PEGBio-Cy7-pTFT (Fig. 1, route A) we first obtained a biotinylated dual-labeled human serum albumin PFT-Hcy-HSA-PEGBio-Cy7. PFT-Hcy-HSA-PEGBio-Cy7 conjugate was prepared by acylation of PFT-Hcy-HSA-Cy7 using biotin functionalized polyethylene glycol *N*-hydroxysuccinimide ester (PEGBio). Detailed description of the synthetic procedure and characterization of PFT-Hcy-HSA-PEGBio-Cy7 conjugate can be found in the Supporting Information. The extent of biotinylation of the albumin was quantified using the HABA/avidin Thermo Fisher Scientific method (supplementary data). On average, 1 biotin residue was conjugated to an albumin molecule. According to MALDI-ToF MS data (Table S2), the PEGBio residue was attached to Lys-536/Lys-560 residues of HSA.

Finally, we coupled the biotinylated dual-labeled albumin PFT-Hcy-HSA-PEGBio-Cy7 with the maleimide derivative of pTFT (MI-pTFT) (Fig. 1, route A). This conjugation scheme was inspired by the work of Lisitskiy and co-authors,[10] who employed the same strategy to couple pTFT to non-targeted PFT-Hcy-HSA-Cy7 (Fig. 1, route B). Detailed description of the synthetic procedure and characterization of biotin-tagged PFT-Hcy-HSA-PEGBio-Cy7-pTFT theranostic can be found in the Supporting Information. The chemical structure of the obtained conjugate was confirmed by ^{19}F NMR, ^{31}P NMR and UV–vis spectroscopy (Figs. S1–S3, ESI†).

We investigated whether PFT-Hcy-HSA-Cy7-pTFT and PFT-Hcy-HSA-PEGBio-Cy7-pTFT could affect the survival of cancer cells. A549 cells treated with either of the pTFT-HSA conjugates survived at decreased rates relative to matched cells treated with PFT-Hcy-HSA-Cy7 (Fig. 2).

As expected, the decreased survival of the cells treated with the theranostic conjugates was associated with the increased rates for apoptosis, as measured by the Annexin V assay. Compared with pTFT, PFT-Hcy-HSA-Cy7-pTFT and PFT-Hcy-HSA-PEGBio-Cy7-pTFT conjugates induced higher apoptosis rate in A549 cells (Fig. 2A and B). Interestingly, A549 cells treated with PFT-Hcy-HSA-Cy7-pTFT conjugate survived at decreased rates relative to the matched cells treated with the biotinylated conjugate. The apoptosis rates induced by non-tagged PFT-Hcy-HSA-Cy7-pTFT and biotin-tagged PFT-Hcy-HSA-PEGBio-Cy7-pTFT were about 2 and 1.5 times, more than pTFT, respectively.

Next, we evaluated the anti-tumor effect of biotin-tagged PFT-Hcy-HSA-PEGBio-Cy7-pTFT and compared it with that for non-tagged PFT-Hcy-HSA-Cy7-pTFT on tumor-bearing mice *in vivo*. The anti-cancer efficacy of the conjugates was evaluated in mice bearing A549 lung adenocarcinoma. A single dose of PFT-Hcy-HSA-Cy7-pTFT showed stronger *in vivo* anti-tumor activity than free pTFT. Fifteen days post treatment, an average tumor volume in the PFT-Hcy-HSA-Cy7-pTFT-treated group was decreased to 28.2% of the initial tumor volume. The inhibitory tumor growth effect of the PFT-Hcy-HSA-Cy7-pTFT conjugate was found to be 1.25-fold more than that of the free pTFT under similar conditions. At the same time, the biotin-tagged PFT-Hcy-HSA-PEGBio-Cy7-pTFT exhibited a very low inhibitory tumor growth effect. Fifteen days post treatment, an average tumor volume in the PFT-Hcy-HSA-PEGBio-Cy7-pTFT-treated group was decreased to 9.1% of the initial tumor volume.

The anti-cancer efficacy of the conjugates was also evaluated in SCID mice with the brain tumor caused by the intracranial injection of a human glioblastoma cell line U87.[27] Changes in tumor volume were measured using a horizontal 11.7 T magnet interfaced with a digital spectrometer (BioSpec117/16USR, Bruker, Germany). A single dose of PFT-Hcy-HSA-Cy7-pTFT showed stronger *in vivo* anti-tumor activity (Table 1). Thirty days post treatment, an average tumor volume in the PFT-Hcy-HSA-Cy7-pTFT-treated group was decreased to 42% of the initial tumor volume (Table 1). At the same time, the average tumor volume in the PFT-Hcy-HSA-PEGBio-Cy7-pTFT-treated group grew rapidly and reached 342% of the initial tumor volume (Table 1).

Then, we studied accumulation of the HSA conjugates in the target tissue (Fig. 3). For this purpose, we used fluorescence-based molecular imaging in combination with computed tomography (CT) to generate anatomical details of an animal subject studied. The trials were performed on the SCID SPF mice with the brain tumor caused by the intracranial injection of the human glioblastoma cell line U87.[27] The xenograft-bearing SCID mice was intravenously injected with PFT-Hcy-HSA-Cy7-pTFT or the biotin-tagged PFT-Hcy-HSA-PEGBio-Cy7-pTFT conjugates at the concentration of 10^{-4} M. The fluorescence images of the whole body were taken at 1 h and 72 h post-injection by using InSyTe FLECT/CT system (TriFoil Imaging, Chatsworth, USA) (Fig. 3). FLECT/CT system allows one to capture true 3D tomographic images by acquiring projection images 360° around the animal subject and sequent accurate image reconstruction.

These initial animal experiments in a glioma model demonstrated a marked accumulation of the fluorescent signal of PFT-Hcy-HSA-Cy7-pTFT in the tumor lesion (Fig. 3B) At the same time, the biotin-tagged PFT-Hcy-HSA-PEGBio-Cy7-pTFT construct did not accumulated in target tissue (Fig. 3D). It could be explained by rapid elimination of the modified albumin by the reticuloendothelial system of the liver. Further investigations of tumor pharmacokinetics and its correlation with the tumor histology and pharmacodynamics endpoints are on-going, the results of which will be reported elsewhere.

Interestingly, our *in vivo* study revealed stronger anti-tumor activity of the non-tagged theranostic conjugate than that of the