Electronic Supplementary Information

Protein modification by thiolactone homocysteine chemistry: a multifunctionalized human serum albumin theranostic

Tatyana V. Popova, [a,b] Olesya A. Krumkacheva, [b,c] Anna S. Burmakova, [a,b] Anna S. Spitsyna, [b,d] Olga D. Zakharova, [a] Vladimir A. Lisitskiy, [a] Igor A. Kirilyuk, [d] Vladimir N. Silnikov, [a] Michael K. Bowman, [d,e] Elena G. Bagryanskaya, [b,d] Tatyana S. Godovikova. [a,*]

[a]Institute of Chemical Biology and Fundamental Medicine, SB RAS, 630090 Novosibirsk, Russia;
[b]Novosibirsk State University, 630090 Novosibirsk, Russia;
[c]International Tomography Center, SB RAS, 630090 Novosibirsk, Russia;
[d]Novosibirsk Institute of Organic Chemistry, SB RAS, 630090 Novosibirsk, Russia;
[e]University of Alabama, Tuscaloosa, Alabama 35487-0336, United States.

*Corresponding author

Dr. Tatyana S. Godovikova

E-mail godov@niboch.nsc.ru

Table of contents

I. Synthesis general remarks

All chemical reagents and solvents were purchased from Sigma-Aldrich Chem. Co. (St. Louis, MO, USA), TCI (Japan), Merck (Germany) or Invitrogen and used as received, without any further purification. All reactions were carried out at atmospheric pressure. Room temperature is defined as between 19–21^{o}C. The term *in vacuo* refers to solvent removal using Büchi rotary evaporation between 15–60°C, at approximately 10 mm Hg. Reactions were monitored by thin layer chromatography (TLC), using TLC plates pre-coated with Kieselgel 60 F_{254} on aluminium (Merck, Darmstand, Germany) in CH_2Cl_2. Detection was by UV (254 nm) or chemical stain (ninhydrin, iodine).

^{1}H NMR spectra were recorded at ambient temperature on a Bruker Advance AV-400 instrument, operating at 400 MHz in the stated solvent, using $CDCl_3$ (δ = 7.30), CD_3CN (δ = 2.00) or D_2O (δ = 4.80) as the internal standard. Chemical shifts (δ) are reported in parts per million (ppm) and coupling constants (J) in Hertz (Hz). The multiplicity of each signal is indicated as s-singlet, d-doublet, t-triplet, m-multiplet (i.e. complex peak obtained due to overlap) or a combination of these. Broad peaks are indicated by the addition of br.

Mass spectra of the low molecular weight compounds were recorded on Ion trap XTC ultra (Agilent Technologies, USA) mass spectrometer with an electrospray ionization (ESI) interface.

Electronic absorption spectra were acquired on a UV-1800 spectrometer (Shimadzu, Japan).

II. Synthesis and characterization of compounds (Scheme 1)

(*Z*)-6-((2-(4-(1,2-diphenylbut-1-en-1-yl)phenoxy)ethyl)dimethylammonio)-hexanoic acid (TMXhex). To a solution of TMX (74.2 mg, 0.2 mmol) in DMF (0.5 mL) was added 6-bromohexanoic acid (156 mg, 0.8 mmol) in DMF (0.5 mL). The resultant mixture was stirred at 77 ^{o}C for 3 days. Reaction was monitored by TLC. After the starting material was consumed, the product TMXhex was isolated by a HPLC system with a C-18 column (Lichrosorb, 4.6 mm × 250 mm, 10–25 µm) (Figure S1A). The mobile phase was composed of solvent A (50 % acetonitrile in 0.1 M TEAA pH 7.0) and solvent B (80 % acetonitrile in 0.1 M TEAA pH 7.0) and eluted at 2.5 mL/min. The fraction at 48 min was collected. The solvent was removed *in vacuo* to afford the target compound as a solid.

The yield: 70%. R_f = 0.15. ^{1}H NMR (CD_3CN, D_2O, δ, ppm, J, Hz): 7.34-7.05 (m, 10H aromatic systems A and B); 6.81 (d, 2H, 9-H, 12-H, $J_{9,10} = J_{12,11}$ = 8.7); 6.58 (d, 2H, 10-H, 11-H, $J_{10,9} = J_{11,12}$ =

8.7); 4.18 (m, 2H, 7-H); 3.55 (m, 2H, 8-H); 3.22 (m, 2H, 5-H); 2.98 (s, 6H, 6-H); 2.31 (q, 2H, 13-H, $J_{13,14}$ = 7.5); 2.08 (m, 2H, 1-H); 1.67 (m, 2H, 4-H), 1.53 (m, 2H, 2-H), 1.23 (m, 2H, 3-H); 0.80 (t, 2H, 14-H, $J_{14,13}$ = 7.5). ESI MS (*m/z*) calculated for $[C_{32}H_{40}NO_3]^+$ $[M]^+$: 486.30, observed 486.29.

(*Z*)-*N*-(2-(4-(1,2-diphenylbut-1-enyl)phenoxy)ethyl)-*N,N*-dimethyl-6-oxo-6-(2-oxotetrahydrothiophen-3-ylamino)hexan-1-aminium chloride (HTL-TMX). TMXhex bromide (31.7 mg, 0.056 mmol) was dissolved in 56 µL toluene. To a solution of TMXhex was added 2 µL DMF and 10 µL $SOCl_2$ (0.14 mmol,). The resultant mixture was stirred for 1 h at RT. The solvent was then removed *in vacuo* and the acyl chloride of TMXhex was used for the subsequent condensation without additional purification. The crude residue was dissolved in toluene (56 µL) and then allowed to cool under ice. Homocystein thiolactone hydrochloride (17.8 mg, 0.112 mmol) was dissolved in CH_2Cl_2 (3.3 mL) and DIPEA (68 µL, 0.39 mmol) was added. To a solution of HTL was dropwise added acyl chloride of TMXhex. The mixture was stirred at room temperature for 12 h. The organic phase was washed with saturated aq. NaCl (20 mL), 0.23 M HCl (20 mL) and dried (Na_2SO_4).

The yield 52%. R_f = 0.9. ^{1}H NMR (CD_3CN, D_2O δ, м. д. и J, Гц): 7.37-7.06 (m, 10H aromatic systems A and B); 6.80 (d, 2H, 9-H, 12-H, $J_{9,10} = J_{12,11}$ = 8.7); 6.60 (d, 2H, 10-H, 11-H, $J_{10,9} = J_{11,12}$ = 8.7); 4.54 (m, 1H, 15-H); 4.20 (m, 2H, 7-H); 3.56 (m, 2H, 8-H); 3.31 (m, 1H, 17α-H); 3.23 (m, 3H, 5-H, 17β-H); 3.00 (s, 6H, 6-H); 2.48 (m, 1H, 16α-H); 2.34 (q, 2H, 13-H, $J_{13,14}$ = 7.5); 2.26 (m, 2H, 1-H); 2.09 (m, 1H, 16β-H); 1.69 (m, 2H, 4-H), 1.55 (m, 2H, 2-H), 1.25 (m, 2H, 3-H); 0.82 (t, 2H, 14-H, $J_{14,13}$ = 7.5). ESI MS (*m/z*) calc. $[C_{36}H_{45}N_2O_3S]^+$ $[M]^+$: 585.31, observed 585.3.

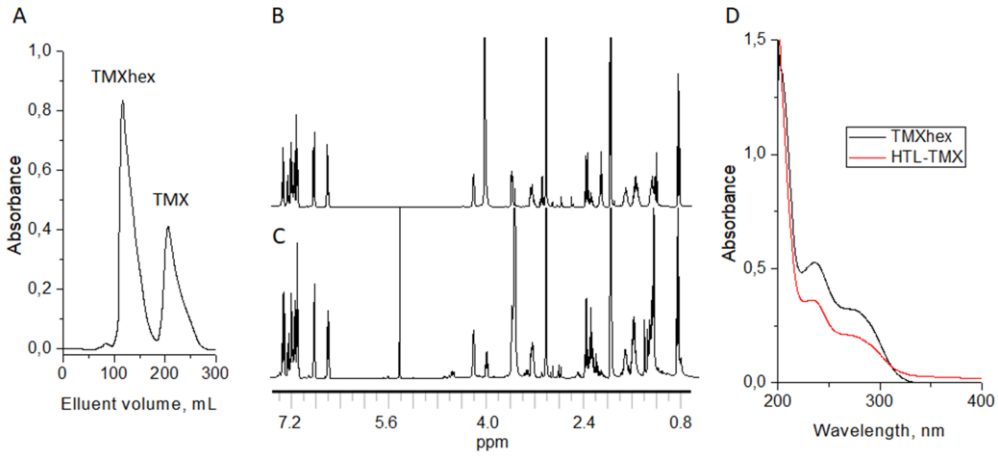

Figure S1. Purification and characterization of tamoxifen derivatives. A: HPLC of the reaction mixture after TMXhex synthesis (column 4.6 × 250 mm with Lichrosorb C18 10-25 µm, gradient CH_3CN 50 – 80 % in 0.1 M TEAA, pH 7.0, 2.5 mL/min). B: ^{1}H NMR spectrum (at 400 MHz) of TMXhex in CD_3CN/D_2O (1/1) at 25°C. The chemical shifts are referred to the resonance of CD_3CN at 2.0 ppm; C: ^{1}H NMR spectrum (at 400 MHz) of HTL-TMX in CD_3CN/D_2O (1/1) at 25°C. The chemical shifts are referred to the resonance of CD_3CN at 2.0 ppm. D: Electronic absorption spectra of the TMXhex and HTL-TMX compounds in CH_3CN.

III. Bioconjugation general remarks

Conjugation experiments were carried out in standard polypropylene Eppendorf® safe-lock tubes (1.5 mL) at atmospheric pressure with mixing at 37 °C unless otherwise stated. Reagents and solvents were purchased from commercial sources and used as supplied. All buffer solutions were prepared with doubly deionized water. Conjugation buffer was phosphate-buffered saline (PBS) (12 mM phosphates, 140 mM NaCl at pH 7.4). Low molecular weight materials (M_r < 3 000 Da) were removed from solution of protein conjugate by ultrafiltration at 9000 rpm twenty four times using a Millipore ultrafiltration tube (Amicon Centriprep YM30, Millipore, Bedford, MA). Purification by size exclusion chromatography (SEC) was carried out on a 300 × 12 mm column of Sephadex-G 150 superfine at a flow rate of 0.18 mL/min. The sample was applied to the top of the column by gravity; elution was carried out with the 0.1 M sodium chloride. Detection was by absorption at 280 nm and 646 nm. HSA was purchased from Sigma-Aldrich Chem. Co. (St. Louis, MO, USA). The concentrations of protein solutions were determined by absorption at 294 nm, pH 13, using the molar extinction coefficient $\varepsilon = 4.44 \times 10^4$ $M^{-1}cm^{-1}$ with a UV-1800 spectrometer (Shimadzu, Japan). Determination of the amount of thiol groups per albumin molecule was done using the Ellman's method as described in the literature[1] and employed 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB) spectrophotometrically at pH 8 at 412 nm ($\varepsilon = 1.36 \times 10^4$ $M^{-1}cm^{-1}$) with a UV-1800 spectrometer using unmodified albumin as control. DTNB produces a measurable yellow-colored product when it reacts with free sulfhydryl group.

Fluorescence spectra were recorded in PBS (pH 7.4, 1.7 mM KH_2PO_4, 5.2 mM Na_2HPO_4, 150 mM NaCl) at 37 °C ClarioStar spectrofluorometer (BMG LabTech, USA) using 96-well plate. Emission spectra were obtained in the range of 660–710 nm using the excitation wavelength λ_{ex} = 630 nm.

All HSA conjugates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 7% PAAG under Laemmli condition with subsequent Coomassie Brilliant Blue (Bio-Rad) staining (see Fig. 3B, Fig. 4C and S2C). Quantitative data were obtained by digitizing the gel using GelPro Analyzer software (Media Cybernetics) (Table S1). Intensities of fluorescence of sulfo-Cy5 residue (λ_{ex} = 650 nm, λ_{em} = 670 nm) in HSA-Cy5-Hcy-TMX-MTSL, HSA-Cy5-Hcy-TMX, and HSA-Cy5 conjugates were analyzed in gel by «Molecular Imager FX» («Bio-Rad», USA) after electrophoresis (see Fig. 3A and 4C), and then the gel was stained with Coomassie Brilliant Blue.

Table S1. Quantitative data of the SDS–PAGE analysis of HSA conjugates presented in Figure 3[a]

HSA type	Higher aggre-gates	Dimer ~130kDa	Monomer ~66.5 kDa	Lower bands	Total oligomers
HSA	14	24	62	0	38
HSA-Cy5	11	22	55	12	33
HSA-Cy5-Hcy-TAM	5	18	46	31	23
HSA-Cy5-Hcy-TAM-MTSL	8	21	45	26	29

[a]Values are given as percentage of intensity to total intensity in the lane (%). Quantitative data were obtained by digitizing the gel using GelPro Analyzer software (Media Cybernetics).

Mass spectra of proteins were recorded on Bruker Autoflex Speed (Bruker Daltonics, Germany) MALDI-ToF mass spectrometer in a positive linear mode. A smart beam-II laser was used. Protein samples were desalted by ZipTip C18 pipette tips. A 2 µL of the protein sample solution was mixed with 2 µL of a 2% TFA (trifluoroacetic acid). To the latter solution 2 µL of the matrix (2,5-dihydroxyacetophenone) was added. The mixture was pipetted up and down until the crystallization starts. Mass spectra were obtained by averaging 3,000 laser shots. External calibration was provided by $[M + H]^+$ HSA at *m/z* 66.5 kDa.

IV. Preparation of HSA Modified by Sulfo-Cy5 dye (HSA-Cy5)

The HSA-Cy5 conjugate is a known HSA conjugate. The synthesis of HSA-Cy5 was adapted from Chubarov et al.[2] Briefly, HSA (33.3 mg, 0.5 µmol) was dissolved in PBS buffer (0.5 mL, 1 mM) and mixed with threefold excess of sulfo-Cy5 maleimide derivative (1.5 µmol, 0.96 mg) dissolved in 0.025 mL DMSO. The reaction was carried out overnight while protected from light. Ellman's test showed that no free sulfhydryl groups were left. Subsequently, low molecular weight materials were removed from solutions of protein conjugates by ultrafiltration at 9000 rpm twenty four times using a Millipore ultrafiltration tube (Amicon Centriprep YM30, Millipore, Bedford, MA). The yield of HSA-Cy5 was ~ 90%. UV-vis (PBS buffer, pH 7.4): λ_{max} 278 nm (ε = (4.7 ± 0.1) × 104), λ_{max} 646 nm (ε = (2.7 ± 0.1) × 105). MS (MALDI ToF) *m/z*. Calcd. for HSA-Cy5, 67.302 kDa, found 67.303 kDa (*MW* for HSA-Cy5). Note: the calculated value is obtained as 66.537 (measured *MW* of reference HSA) + 766.4 (Calculated *MW* of sulfo-Cy5).

V. Purification of HSA Theranostic Conjugates by Size Exclusion Chromatography (SEC)

Serum albumin samples were chromatographed on a 300 × 12 mm column of Sephadex-G 150 superfine at a flow rate of 0.18 ml/min. The sample was applied to the top of the column by gravity; elution was carried out with the 0.1 M sodium chloride. Tubes were read at 280 nm and 646 nm. The appropriate peaks were pooled. Low molecular weight materials were removed from solution of protein conjugate by ultrafiltration at 9000 rpm twenty four times using a Millipore ultrafiltration tube (Amicon Centriprep YM30, Millipore, Bedford, MA). The protein conjugates were then freeze-dried and stored at 4 °C.

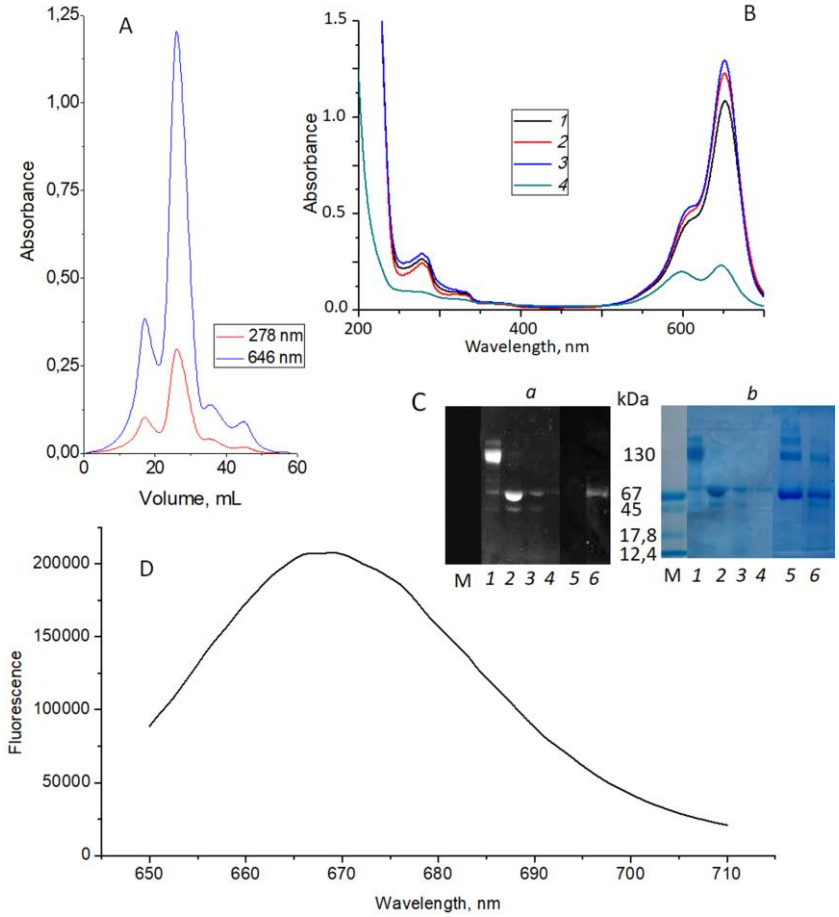

Figure S2. Purification and characterization of HSA theranostic conjugate HSA-Cy5-Hcy-TMX. Panel A. Representative chromatogram of HSA-Cy5-Hcy-TMX products obtained by SEC utilizing a Sephadex-G 150 superfine column (300 × 12 mm column) at a flow rate of 0.18 mL/min (0.1 M sodium chloride). Protein elution was monitored at 278 and 646 nm. Panel B. UV-vis spectra of albumin homocystamides in PBS buffer, pH 7.4. Products from SEC: Peak *1* – black; Peak *2* – red; Peak *3* – blue; Peak *4* – green. Panel C. SDS-PAGE of homocystamide conjugates of the albumin under Laemmle condition[2] with subsequent fluorescence detection (*a*) and Coomassie blue staining (*b*). SDS-PAGE had been performed with a molecular weight marker (*M*), Lane 1 – products from SEC Peak *1*, Lane 2 – products from SEC Peak *2*, Lane 3 – products from SEC Peak *3*, Lane 4 – products from SEC Peak *4*, Lane 5 –HSA, Lane 6 – HSA-Cy5-Hcy-TMX after remove of low molecular weight homocysteine derivatives from solution of protein conjugate by centrifugal filtration using Centricon concentrators. Panel D. fluorescence spectra of HSA-Cy5-Hcy-TMX.

The SEC showed the main product, Peak 2, eluting at approximately 60–150 minutes post-injection for all samples (Fig. 4A and Fig. S2A). Higher molecular weight products, Peak 1,

eluting between 12–60 minutes were observed for all HSA samples. The HSA sample generally showed a higher percentage of these dimers/oligomers (Table S1). Products from SEC Peak 3 eluting later than Peak 2 were observed for HSA-Cy5-Hcy-TMX-MTSL and another HSA conjugate HSA-Cy5-Hcy-TMX had elution times between 180–270 minutes potentially indicative of degradation fragments (Fig. 4C and Fig. S2C). The UV-vis spectra from Peaks 1–3 are almost identical (Fig. 4B and Fig. S2B).

VI. Identification of specific Hcy-TMX sites in HSA-Cy5-Hcy-TMX conjugate

In order to identify sites of modification in HSA-Cy5-Hcy-TMX conjugate, the modified albumin and HSA were digested with trypsin prior to MS analysis according standard procedure. For the digest of HSA-Cy5-Hcy-TMX were used SDS-PAGE bands with MW ~ 66 kDa. The trypsin digest was subjected to MALDI-ToF mass spectrometric analysis. Prior to analysis, samples were purified and fractionated on ZipTip C18 pipette tips. Purified peptides were eluted directly on MALDI plate, mixed with α-cyano-4-hydroxy-cinnamic acid (HCCA) or 2,5-dihydroxyacetophenone (DHB) (saturated matrix solution dissolved in 50% acetonitrile 0.1% TFA) and allowed to air dry. Samples were analyzed in reflectron positive ions mode, mass calibration was performed using peptide calibration standards (Bruker, Germany). The mass spectra were recorded in positive ion mode in the m/z range from 500 to 4000 Da. The masses of the peptides detected in the HSA, HSA-Cy5 and HSA-Cy5-Hcy-TMX digests were compared to the masses predicted for a theoretical digest of HSA by using PEPTIDEMASS software (http://web.expasy.org/peptide_mass/). MALDI ToF data was deconvoluted using the software package mMass (http://www.mmass.org).[3-5] All peptides that could be matched in theoretical and experimental digest of HSA were removed from the analysis of the HSA-Cy5-Hcy-TMX digests data. Peptides sequence and *MW* calculation presented in the Table S2.

Table S2 Identification of specific *N*-homocysteinylation site in HSA-Cy5-Hcy-TMX conjugate

Hcy-TMX site	Sequence	peptide MW calc.	MW calc., peptide MW + …[a]			*m/z* measured	missed cleavages
			+ IAA	+ Hcy-TMX+IAA	Other modifications		
Lys-174	163AAFTECCQAAD***K****AACLLPK181	1953.9	2181.9 [b]	2768.2	+2 Li^+	2781.2	1
Lys-199	198L***K****CASLQK205	890.5	947.5	1533.8	+Cl^-	1568.8	1
Lys-524	522QI***K****K525	516.3	573.3[c]	1159.6	-	1157,3	1

K* lysine residues carrying *N*-linked Hcy;

[a]MW: Hcy-TMX 586.3 Da, IAA 57 Da.

[b] + 4 IAA residue, because of the presence of three cysteine residue in the peptide and one SH group in Hcy.

[c]One IAA residue change, because of the presence of two SH groups in Hcy

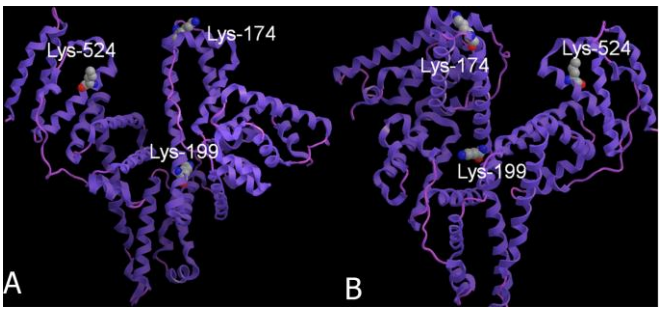

Figure S3 Model of human albumin structure based on 1AO6 PDB entry. *N*-Hcy-TMX-lysine residues are marked with white color. A – front view, B – back view.

VII. Cellular uptake and *in vitro* imaging of multifunctionalized HSA

T98G cells were seeded on 12 mm round coverslips (1×10^6 cells per plate) and incubated for 24 h at 37 °C. The medium was then replaced with HSA-Cy5/HSA-Cy5-Hcy-TMX/HSA-Cy5-Hcy-TMX conjugates with 12 µM of the final albumin conjugate concentration in fresh culture. After being incubated for 1 h, the medium was removed and the coverslips were washed with cold PBS three times. The cells were fixed with 2% paraformaldehyde in PBS for 20 min at 25 °C and the cell nuclei were dyed with DAPI for 20 min. The fluorescence images of HSA conjugates in cells were observed by Carl Zeiss LSM 510 Laser Scanning Microscopy System (Carl Zeiss, Germany) at the Center for Microscopic Analysis of Biological Objects of the Institute of Cytology and Genetics SB RAS. Standard filters were used for detection of Cy5 labeled conjugates (645 nm for excitation and 670 nm for emission). The results were processed using Zen (Carl Zeiss) software.

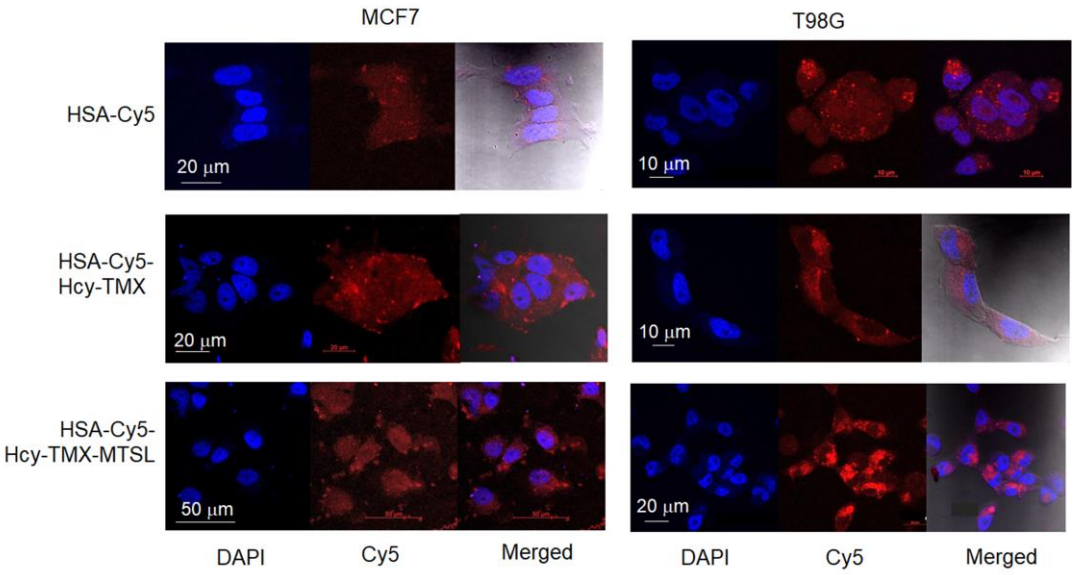

Figure S4. Representative fluorescence micrographs showing HSA conjugates in cells (DAPI nuclear stain, blue; Cy5-fluor 510 HSA labeled conjugates, red). Scale bar: 10/50 µm.

VIII. Flow cytometry

Cellular uptake of Cy5-labeled human serum albumin conjugates was investigated by flow cytometry. Here, 1×10^6 T98G cells per well, were seeded into 6-well plates in complete medium and treated with the albumin conjugates. After 1 h of incubation, the cells were washed twice with PBS, trypsinized by using 0.25 % Trypsin-EDTA, the cells were centrifuged (1000 rpm × 5 min, LMC-3000 centrifuge, Biosan, Latvia). The cells were fixed with 2% formaldehyde in PBS (20 min, 25 ^{o}C). The samples were assayed by flow cytometry using a NovoCyte 3000 (ACEA Biosciences, USA) and data were processed with NovoExpress software (ACEA Biosciences, USA). The fluorescence intensity of individual cells was measured in relative fluorescence units (RFU). All experimental points were run in triplicate for statistical analysis; the standard deviation did not exceed 5%. Cells incubated in the absence of HSA conjugates were used as a negative control: in these samples the percentage of fluorescence-positive cells and fluorescence intensity did not exceed the experimental error (1–3%).

IX. *In vitro* cytotoxicity assay

The cytotoxicity of HSA conjugates was evaluated in vitro by MTT assay.[6] Briefly, exponentially growing cells were plated in 96-well plates (~ 2000 cells per well) and were incubated (24 h) at 37 °C in RPMI-1640 (5% CO2). The solutions of HSA conjugates were applied in medium with TMX equivalent concentrations ranging from 0.06–60 µM. Cell viability was assessed at 72 h after the start of the treatments by MTT. An aliquot (10 µL) of MTT solution (25 mg/mL in PBS) was added to each well, and the plates were incubated at 37 °C for 3 h. The medium was removed, and the dark blue crystals of formazan that had formed were dissolved in 100 µL of isopropanol. The absorbance at 570 nm (peak) and 620 nm (baseline) was read using a microplate reader Multiscan FC (Thermo Fisher Scientific). Results were expressed as a percentage to the control values. All values in the Figure 6 of the present study are given as mean ± standard deviation (S.D.) values, and all measurements were repeated three times.

Reference

1. J. Janatova, J. K. Fuller and M. J. Hunter, *J. Biol. Chem.,* 1968, **243***,* 3612–3622.
2. A. S. Chubarov, O. D. Zakharova, O. A. Koval, A. V. Romaschenko, A. E. Akulov, E. L. Zavjalov, I. A. Razumov, I. V. Koptyug, D. G. Knorre and T. S. Godovikova, *Bioorg. Med. Chem*., 2015, **23**, 6943–6954.

3. D. W. Cleveland, S. C. Fischer, M. W. Kirschner and U. K. Laemmli, *J. Biol. Chem.,* 1977, **252**, 1102–1106.
4. M. Strohalm, D. Kavan, P. Novák, M. Volný and V. Havlíček, *Anal. Chem.,* 2010, **82**, 4648–4651.
5. T. H. J. Niedermeyer and M. Strohalm, *PLoS One,* 2012, **7**, e44913.
6. P. Jackson and M. I. Attalla, *Rapid Commun. Mass Spectrom.,* 2010, **24**, 3567–3577.
7. T. Mosmann, *J. Immunol. Methods,* 1983, **65**, 55–63.