SUPPORTING INFORMATION

AuNPs pyrazolo[3,4-*d*]pyrimidine nanosystem in combination with radiotherapy against glioblastoma

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CHEMISTRY

1. REAGENTS AND INSTRUMENTS

All commercially available chemicals were used as purchased from Sigma Aldrich. CH_2Cl_2, was dried over sodium hydride. Anhydrous reactions were run under a positive pressure of dry N_2. TLC was carried out using Merck TLC silica gel 60 F254. Chromatographic purifications were performed on columns packed with Merck silica gel 60, 23-400 mesh, for flash technique. ^{1}H NMR and ^{13}C NMR spectra were recorded at 400 MHz on a Bruker Advance DPX400. Chemical shifts are reported relative to tetramethylsilane at 0.00 ppm. Mass spectra (MS) data were obtained using an Agilent 1100 LC/MSD VL system (G1946C) with a 0.4 mL/min flow rate using a binary solvent system of MeOH:H_2O 95:5. UV detection was monitored at 254 nm. MS were acquired in positive and negative mode scanning over the mass range 100-1500. The following ion source parameters were used: drying gas flow, 9 mL/min; nebulizer pressure, 40 psi; drying gas temperature, 350 °C. All target compounds possessed a purity of ≥ 95% verified by LC-UV/MS.
LC analyses were performed by Agilent 1100 LC/MSD VL system (G1946C) (Agilent Technologies, Palo Alto, CA) constituted by a vacuum solvent degassing unit, a binary high-pressure gradient pump, a 1100 series UV detector and a 1100 MSD model VL benchtop mass spectrometer. The Agilent 1100 series mass spectra detection (MSD) single-quadrupole instrument was equipped with the orthogonal spray API-ES (Agilent Technologies, Palo Alto, CA). Nitrogen was used as nebulizing and drying gas. The pressure of the nebulizing gas, the flow of the drying gas, the capillary voltage, the fragmentor voltage and the vaporization temperature were set at 40 psi, 9 L/min, 3000 V, 70 V and 350 °C, respectively. UV detection was monitored at 254 nm. The HPLC-ESI-MS determination was performed by operating the MSD in the positive ion mode. Spectra were acquired over the scan range *m/z* 105-1500 using a step size of 0.1 u. Chromatographic analysis were performed using a Phenomenex Kinetex C18-100A column (150 x 4.6 mm, 5 μm particle size) at room temperature. Analysis were carried out using gradient elution of a binary solution; (eluent A: ACN, eluent B: H_2O, both eluents were acidified with formic acid 0.1% v/v). The analysis started with 5% of A (from t = 0 to t = 3 min), then A was increased to 95% (from t = 3 to t = 12 min), then kept at 95% (from t =

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12 to t = 20 min) and finally return to 5% of eluent A in 1.0 min. The analyses were performed at a flow rate of 0.6 mL/min with 20 µL as injection volume.

2. SYNTHETIC PROCEDURES, NMR AND LC-MS CHARACTERIZATION

Synthesis of 5-(1,2-dithiolan-3-yl)-1-(4-(2-hydroxyethyl)piperazin-1-yl) pentan-1-one (2)

To a solution of 1,2-dithiolane-3-pentanoic acid (100.00 mg, 0.4850 mmol, 1.0 eq) in 10.00 mL of CH_2Cl_2, 1-(2-hydroxyethyl)piperazine (0.017 mL, 0.5820 mmol, 1.2 eq), HOBt (65.53 mg, 0.4850 mmol, 1.0 eq) and EDC (75.29 mg, 0.4850 mmol, 1.0 eq) were added. After 3 hours, 0.6 eq of 1-(2-hydroxyethyl)piperazine were added. The reaction mixture was stirred at room temperature for 9 h. The obtained solution was evaporated under reduced pressure to obtain a solid which was solubilized in CH_2Cl_2, then washed with water, dried (Na_2SO_4), filtered, and concentrated under reduced pressure to afford a yellow oil. After, the crude was purified by flash chromatography (CH_2Cl_2:MeOH 95:5) affording **2** as a yellow oil (479.00 mg 1.5040 mmol, Yield: 62%).

MW: 318,49 $C_{14}H_{26}N_2O_2S_2$

MS (ESI): *m/z* calcd: 341.13 ; found: 341.10 $[M+Na]^+$

^{1}H NMR: ($CDCl_3$, 400MHz) δ, 3.54 (m, 5H), 3.46 (m, 2H), 3.12 (m, 2H), 2.54 (t, 2H, J=5.2Hz), 2.45(m, 5H), 2.30(t, 2H, *J*=7.6Hz), 1.90(m, 1H), 1.66(m, 4H), 1.46(m, 2H).

^{13}C NMR: ($CDCl_3$, 400MHz) δ, 171.17, 77.37, 77.05, 76.74, 59.35, 57.84, 56.42, 53.17, 52.62, 45.54, 41.54, 40.23, 38.48, 34.73, 32.92, 29.06, 24.95.

Synthesis of *SI306-liker/hydrolyzable function system* (5)

To a solution of SI306 (19.40 mg, 0.0338 mmol, 1.0 eq) in 8.00 mL of dry CH_2Cl_2, $NaHCO_3$ (14.20 mg, 0.1690 mmol, 5.0 eq) was added and the mixture was stirred for 5 minutes. A solution of triphosgene (15.04 mg, 0.0507 mmol, 1.5 eq) in 2.00 mL of dry CH_2Cl_2 was prepared and stirred until solubilization. Then, this solution was added to the solution of SI306, refrigerating with an ice bath. After 30 min the ice bath was removed, and the reaction mixture was stirred at room temperature for 5 hours. After, a solution of the linker **2** (16.14 mg, 0.0507 mmol, 1.5 eq) in dry CH_2Cl_2 was added. The reaction mixture was stirred at room temperature for 64 hours. Then the solvent was removed under reduced pressure and the crude residue was purified by flash chromatography (CH_2Cl_2:MeOH 95:5) affording **5** as a yellow oil (11.64 mg, 0.0127 mmol, Yield: 60%).

MW: 918.43 $C_{40}H_{50}BrClN_8O_4S_3$

MS (ESI): *m/z* calcd: 939.19-941.19 $[M+Na]^+$; found: 939.16-941.17 $[M+Na]^+$

^{1}H NMR: ($CDCl_3$, 400MHz) δ, 7.56 (s, 1H), 7.54 (s, 1H), 7.46 (m, 2H), 7.34 (m, 4H), 7.19 (m, 2H), 5.54 (m, 1H), 5.01b(m, 1H), 4.76 (m, 1H), 4.40 (m 2H), 4.12 (m, 1H), 3.75 (m, 4H), 3.60 (m, 3H), 3.44 (m, 3H), 3.15 (m, 4H), 2.61 (m, 4H), 2.49 (m, 4H), 2.35 (m, 5H), 1.92 (m, 1H), 1.68 (m, 4H), 1.50 (m, 2H).

^{13}C NMR: ($CDCl_3$, 400MHz) δ, 171.13, 156.06, 145.15, 140.97, 139.13, 137.94, 137.83, 135.32, 132.00, 131.21, 130.58, 130.38, 129.07, 129.01, 128.79, 127.53, 127.36, 122.22, 103.43, 96.47, 64.55, 60.35, 57.53, 56.47, 56.39, 53.85, 53.27, 52.98, 45.51, 41.49, 40.23, 38.48, 34.75, 32.92, 29.66, 29.10, 24.96.

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PREPARATION AND CHARACTERIZATION OF AuNPs AND AuNPs-SI306

1. MATERIALS

All solvents and reagents were purchased from Sigma-Aldrich Srl (Milan, Italy).
The human plasma for plasma stability was obtained by volunteers' donors. Milli-Q quality water (Millipore, Milford, MA, USA) was used.

2. SYNTHESIS OF AuNPs

To a solution of $HAuCl_4 \cdot 3H_2O$ (7.88 mg, 0.02 mmol) in 20.00 mL of H_2O Milli-Q heated upon boiling, was added a solution of sodium citrate dehydrate 1% prepared with 2.00 mL of H_2O Milli-Q (19.97 mg, 0.0679 mmol). The reaction mixture was stirred until the appearance of a red color.
The resulting solution was cooled at room temperature and stored in the dark.

3. FUNCTIONALIZATION OF AuNPs

2.79 mL of AuNPs was diluted with 8.00 mL of H_2O Milli-Q. A solution of **5** (3.00 mg, 0.0033 mol) solubilized in 0.20 mL of DMSO and diluted with 2.00 mL of H_2O Milli-Q was added dropwise to obtain a dark pink solution. The solution was stirred at room temperature for 8 hours. After, the solution was stored in the dark.

4. DRUG FUNCTIONALIZATION EFFICACY % AND PURIFICATION

In order to determine the loading efficacy (LE%) of SI306 for AuNPs and to remove the unreacted compound, the AuNPs-SI306 suspension was ultracentrifugated by three repeated cycles at 13.000 rpm for 90 min at 24 °C each (BeckMan Coulter Optima L-90 K). Washing waters (H_2O:DMSO 1.5:2) were collected at the end of each cycle for the LC-UV/MS determination of free SI306. Finally, after the third ultracentrifugation cycle, the AuNPs-SI306 pellet was resuspended in water.
LE% was calculated with the following Equation:

$$\text{LE\%} = \frac{\text{Amount of total drug} - \text{Amount of drug in the supernatant}}{\text{Amount of total drug}} \cdot 100$$

The experiments were repeated in triplicate and results compared with the standard curve.

With the aim to further confirm the successful functionalization of AuNPs by Pro-SI306, UV-vis absorption measurements have been performed for three samples and are reported in the figures below (Figure S1, S2 and S3):

- the free prodrug spectrum which shows a maximum of absorption around 250 nm (**Figure S1**);
- the AuNPs-SI306 nanosystem spectrum which shows an absorption peak attributable to the prodrug (350-250 nm) and an absorption peak attributable to gold nanoparticles (530 nm) (**Figure S2**);
- The AuNPs and ProSI306 mixed together and immediately measured (**Figure S3**).

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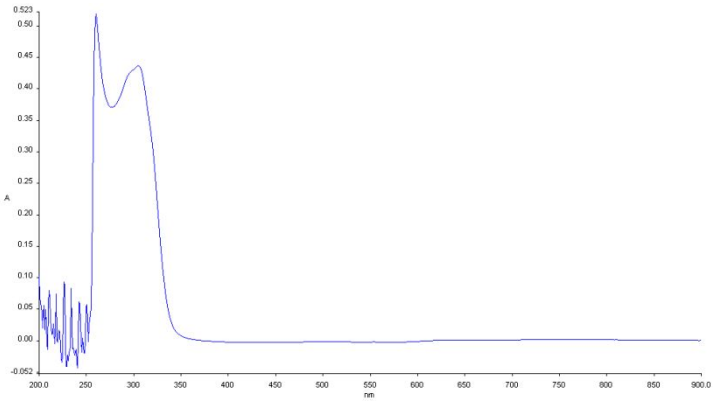

Figure S1. Free prodrug UV-vis spectrum (concentration 0.012 µg/mL)

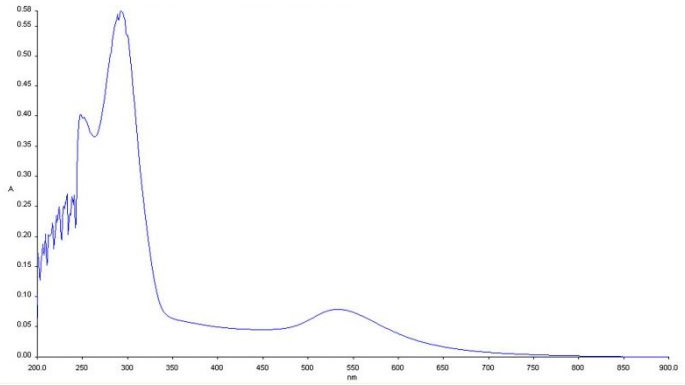

Figure S2. AuNPs-SI306 nanosystem UV-vis spectrum

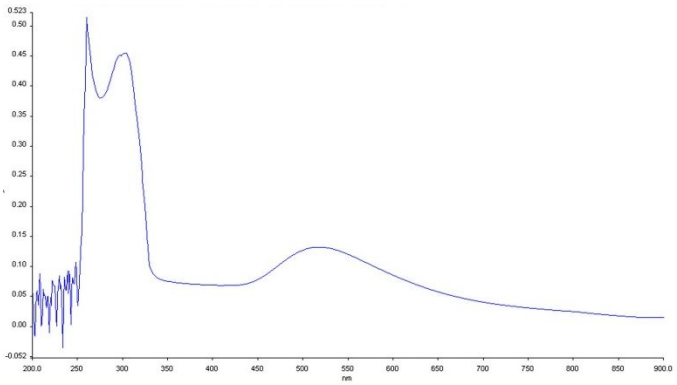

Figure S3. AuNPs and ProSI306 mixed together and immediately measured UV-vis spectrum

When measured as free or immediately after the mix with AuNPs, Pro-SI306 showed a maximum of absorption around 250 nm. After the functionalization, the maximum of absorption changed to around 300 nm. This is a further confirmation of the successful functionalization of gold nanoparticles by ProSI306.

5. UV-VIS SPECTRA OF AuNPs UPON INTERACTION WITH SALTS AND PROTEIN HSA

The UV-VIS analyses of AuNPs and AuNPs-SI306 nanosystem were performer by UV-VISIBLE spectrophotometer Lambda 2 (Perkin Elmer).

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The interaction of AuNPs versus salts (NaCl and KCl) and protein (HSA, human serum albumin) and the state of nanoparticles aggregation have been investigated by UV-vis absorption measurements. AuNPs suspension (200 µL) was added with:

- Salt solution 0.02 M, 200 µL (final concentration 0.01 M)
- Salt solution 0.1 M, 200 µL (final concentration 0.05 M)
- Salt solution 0.14 M, 200 µL (final concentration 0.07 M)
- Salt solution 0.2 M, 200 µL (final concentration 0.1 M)

For each mixture, the UV-vis absorption was measured at 1, 5, 10 minutes (for NaCl + AuNPs mixture spectra, see table S1 and for KCl + AuNPs mixture spectra, see table S2).

Table S1. Time-resolved UV-vis spectra of NaCl upon interaction with AuNPs and picture of the NaCl-AuNPs mixture immediately after salt addition.

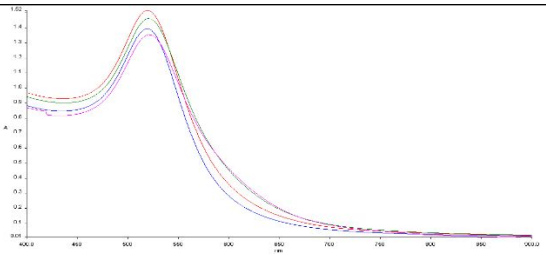
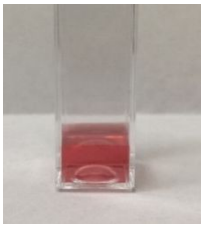
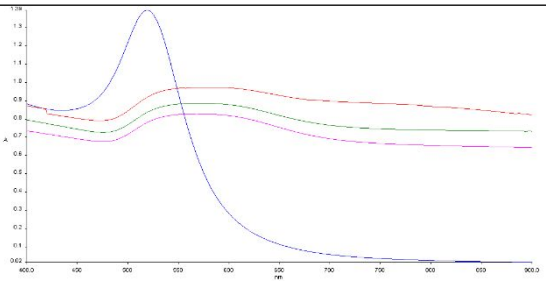
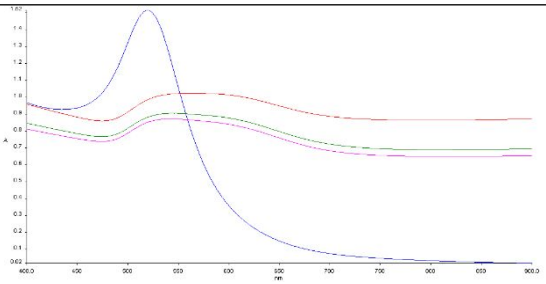
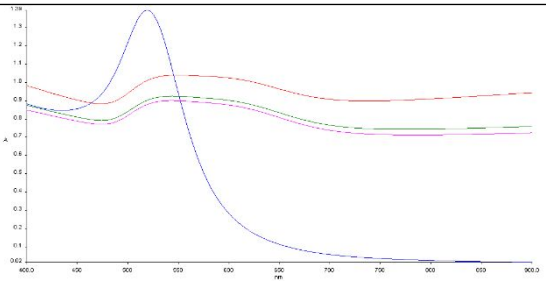
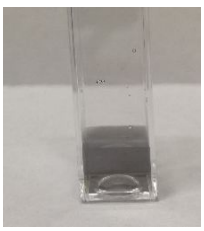
NaCl final concentration	**AuNPs + NaCl spectra** AuNPs (Blue); AuNPs + NaCl after 1 min (Red); AuNPs/NaCl after 5 min (Green); AuNPs/NaCl after 10 min (Pink)	Aspect immediately after salt addition
NaCl 0.01 M		
NaCl 0.05 M		
NaCl 0.07 M		
NaCl 0.1 M		

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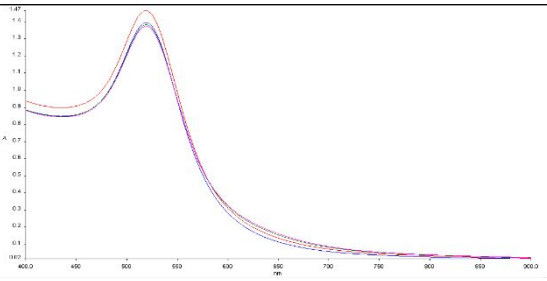
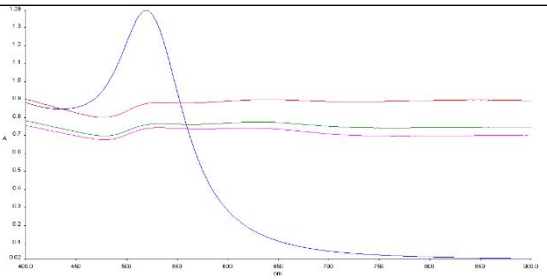
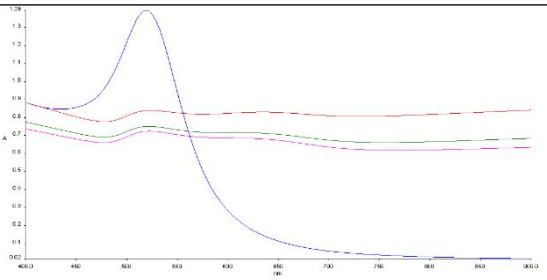
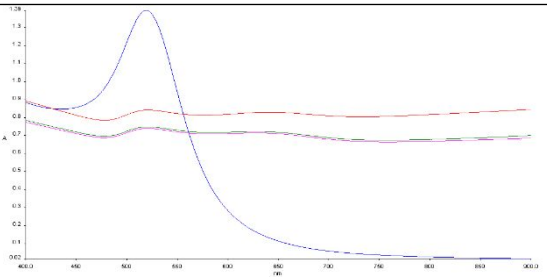
Table S2. Time-resolved UV-vis spectra of KCl upon interaction with AuNPs and picture of the KCl-AuNPs mixture immediately after salt addition.

KCl final concentration	**AuNPs + KCl spectra** AuNPs (Blue); AuNPs + KCl after 1 min (Red); AuNPs + KCl after 5 min (Green); AuNPs + KCl after 10 min (Pink)	Aspect immediately after salt addition
KCl 0.01 M		
KCl 0.05 M		
KCl 0.07 M		
KCl 0.1 M		

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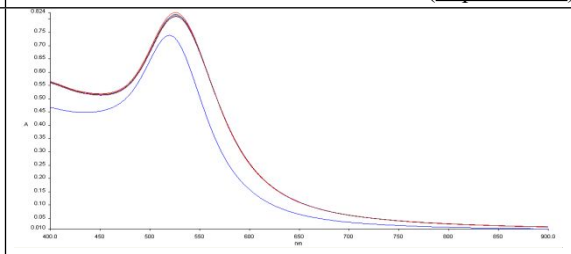
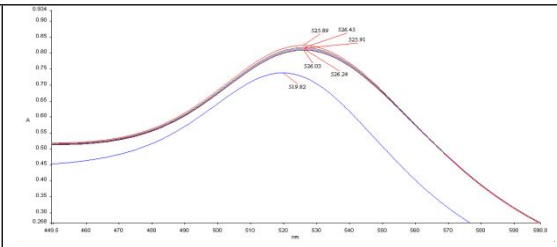
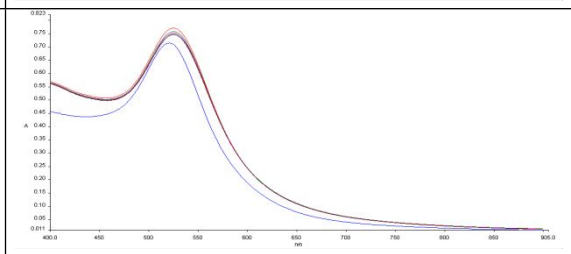
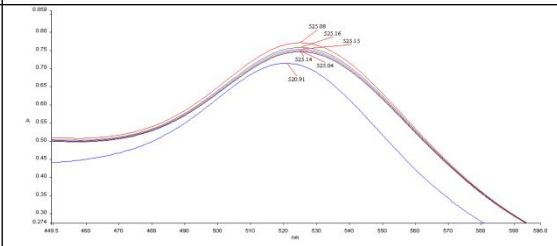
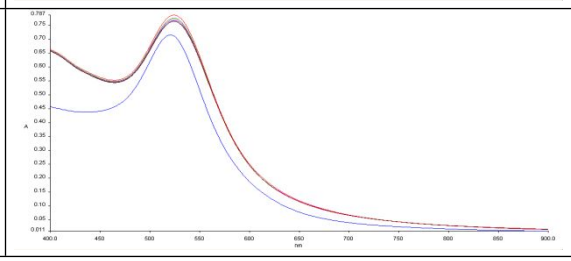
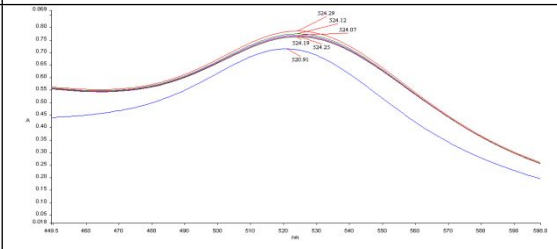
AuNPs suspension (200 µL) was added with:

- HSA solution 25 mg/mL, 200 µL (final concentration 12.5 mg/mL)
- HSA solution 50 mg/mL, 200 µL (final concentration 25 mg/mL)
- HSA solution 100 mg/mL, 200 µL (final concentration 50 mg/mL)

For each mixture, the UV-vis absorption was measured immediately, at 10, 20, 40, 60 minutes.
For HSA + AuNPs mixture spectra, see table S3.

Table S3. Time-resolved UV-vis spectra of HSA upon interaction with AuNPs and picture of the KCl-AuNPs mixture immediately after salt addition.

	AuNPs + HSA Spectra	**Zoom maximum absorption**
HSA final conc.	AuNPs (Blue); AuNPs + HSA immediantly (Red); AuNPs + HSA after: 10 min (Green), AuNPs + HSA 20 min (Pink), AuNPs + HSA 40 min (Aquamarine) AuNPs + HSA 60 min (Bordeaux).	
12.5 mg/mL		
25 mg/mL		
50 mg/mL		

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6. DLS MEASUREMENTS

Mean particle size, polydispersity index (PDI) and ζ-potential were determined by Dynamic Light Scattering (DLS) (Zeta Sizer Nano ZS90, Malvern Instruments Ltd, Malvern, UK). AuNPs-SI306 nanosystem was diluted in water in order to give an optical density (OD) of 0.1 unit of absorbance at 420 nm and then measured at 24° C with a scattering angle of 90°. Z-potential measurements were carried out using folded capillary cells (Malvern). Results are expressed as mean values ± S.D. calculated from three independent experiments (n=3).

7. STABILITY IN POLAR MEDIA

AuNPs-SI306 nanosystem was dissolved at room temperature in H_2O, ACN, methanol, DMSO or phosphate buffer (25 mM, pH 7.4) up to a final concentration of SI306 equal to 100 μM. Aliquot samples (20 μL) were taken at fixed time points (0.25, 0.50, 0.75, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 24.0 and 48 h), ultracentrifugated (BeckMan Coulter Optima L-90 K, 13000 rpm for 90 min at 25 °C) and the supernatant was analyzed by LC-UV/MS (for method details see Chemistry section, paragraph 1. Reagents and Instruments). The experiments were repeated in triplicate and results compared with the standard curve.

8. STABILITY IN HUMAN PLASMA

Pooled human plasma (1.50 mL, 55.70 μg protein/mL), phosphate buffer (1.40 mL, pH 7.4, 25 mM) and a solution of AuNPs-SI306 nanosystem (100 μL, SI306 3.0 mM) were mixed in a test tube that was incubated at 37 °C. At set time points (0.25, 0.50, 1.0, 3.0, 7.0 and 24.0 h), samples of 150 μL were taken, mixed with 600 μL of cold acetonitrile and ultracentrifuged (BeckMan Coulter Optima L-90 K, 13000 rpm for 90 min at 25 °C). The supernatant was removed and analyzed by LC-UV/MS to monitor the hydrolysis process (for method details see Chemistry section, paragraph 1. Reagents and Instruments). The experiments were repeated in triplicate and results compared with the standard curve.

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BIOLOGICAL ASSAY

1. LOW DENSITY GROWTH ASSAY

U87 cells capacity for growth at clonal density, was evaluated by plating cells at density of 10 cells/cm^2 in 10% FBS supplemented DMEM. Treated cells received one 3 Gy irradiation (RT) three days after plating. Treatment with SI306, AuNPs and AuNPs-SI306 nanosystem was performed two hours prior RT. After 2 weeks of culture, adherent cells were fixed with cold methanol, washed with PBS/BSA and air-dried. Adherent cells were detached with trypsin/EDTA solution and viable cells were counted after Trypan blue exclusion staining. Each experimental point was performed in 3 replicates and results were expressed as mean ± S.D.

2. STATISTICAL ANALYSIS

Bonferroni's Multiple Comparison Test (One-way ANOVA) was performed by Software GraphPad Prism 6.0.

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