Supporting Information

A β-glucuronidase-responsive albumin-binding prodrug programmed for the double release of monomethylauristatin E

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I. Chemistry Section

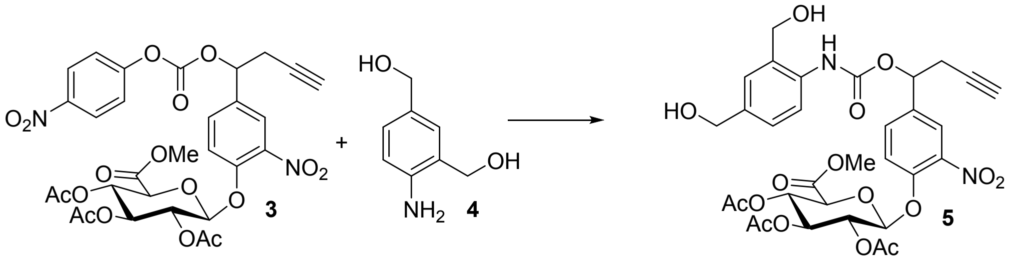
I.1. General experimental methods

All reactions were performed under a nitrogen atmosphere. Unless otherwise stated, solvents used were of HPLC quality. Chemicals were of analytical grade from commercial sources and were used without further purification. The reaction progress was monitored on precoated silica gel TLC plates MACHEREY-NAGEL ALUGRAM® SIL G/UV$_{254}$. (0.2 mm silica gel 60). Spots were visualized under 254 nm UV light and/or by dipping the TLC plate into a solution of phosphomolybdic acid (3 g) in ethanol (100 mL) followed by heating with a heat gun. Automatic chromatographies were performed with a COMBIFLASH RF 200I TELEDYNE ISCO instrument equipped with UV detector and using flash cartridges Interchim silica 15 or 50 µm. ^{1}H and ^{13}C NMR spectra were respectively recorded at 400 MHz and 100 MHz on a Bruker 400 Avance III instrument, equipped with an ultra-shielded magnet and a BBFO 5 mm broadband probe. ^{1}H and ^{13}C NMR of compound **2** were recorded at 500 MHz and 125 MHz on a Bruker spectrometer equipped with a cryoprobe TXI 1H-13C-15N (5 mm) in the Prism platform at University of Rennes. Chemical shifts (δ) are reported in parts per million (ppm) from low to high field and referenced to residual solvent. Coupling constants (*J*) are reported in hertz (Hz). Accurate mass was determined for all derivatives through their infusion on high resolution ESI mass spectrometers in the CBM/ICOA FR2708, at the University of Orléans and in the Organic Analysis Center of IC2MP at University of Poitiers. Analytical RP-HPLC was carried out on a Dionex Ultimate 3000 system equipped with a UV/Visible variable wavelength detector and with a reverse-phase column chromatography Acclaim$^{(R)}$ (120, C18, 250x4.6 mm, 5 µm, 120 Å) at 30 °C and 1 mL.min^{-1}. Method **1** used a linear gradient composed of A (0.2% TFA in water) and B (CH_3CN) beginning with A/B = 80/20 v/v and reaching A/B = 0/100 v/v within 30 min. All chromatograms were recorded at 254 nm. Semi-preparative RP-HPLC was performed with a VWR LaPrep system equipped with a spectrophotometer LaPrep P314 and a preparative pump LaPrep P110. Solvent flow 4 mL.min^{-1} was applied to a semi-preparative column ACE® C18-AR (100x10 cm, 5 µm). Gradient eluent (Method **2**) was composed of A (H_2O + TFA 0,05%) and B (CH_3CN) beginning with A/B = 80/20 v/v and reaching A/B = 0/100 v/v within 30 min.)

HPLC/HRMS experiments were performed on an Accela UHPLC system coupled to a hybrid high resolution mass spectrometer Q-Exactive (Thermo Scientific). An Acclaim® C18 column (250x4.6 mm, 5 µm, 120 Å) at 30°C was used for chromatographic separation at a flow rate of 0.5 mL.min^{-1}. The column effluent was introduced into the electrospray ionisation source (ESI) of the mass spectrometer. Analyses were performed in positive ion mode. The electrospray voltage was set at 4.0 kV. The capillary and heater temperatures were 275°C and 300°C respectively. The sheath, sweep and auxiliary gas (nitrogen) flow rates were set at 35, 10 and 20 (arbitrary units). Analysis of data was performed with Xcalibur software. HPLC/HRMS (Q-Exactive) experiments were performed using a linear gradient composed of A (0.1% formic acid in water) and B (0.1% formic acid in CH_3CN) (Method **3**). After injection of 20 μL of sample, compounds were separated through a linear gradient starting from 20% of B and reaching 100% of B within 30 min.

I.2. Synthetic procedures, characterization details

Preparation of compound 5



To a stirred solution of carbonate **3** (225 mg, 0.326 mmol) in DMF (5 mL) was added aniline **4** (100 mg, 0.653 mmol) and hydroxybenzotriazole (HOBt) (48.5 mg, 0.359 mmol). The mixture was stirred 30 hours at 50°C. The solution was concentrated under reduced pressure and the crude material was purified by column chromatography over silica gel (gradient elution: 10% to 30% ethyl acetate in petroleum ether) to afford **5** (121.8 mg, 0.173 mmol, 53 %).

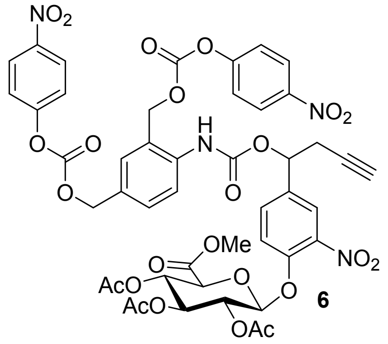
Tr = 16.6 min (Method **1**)

^{1}H NMR (400 MHz, CDCl$_3$) of 5 *as a mixture of two diastereoisomers* : δ 8.21 (s, 1H), 7.88 (s, 1H), 7.73 (d, 1H, *J* = 2.0 Hz), 7.59 (d, 1H, *J* = 8.4 Hz), 7.35 (d, 1H, *J* = 8.7 Hz), 7.18 (d, 1H, 2.0 Hz), 7.09 (s, 1H), 5.81 (t, 1H, *J* = 6.4 Hz), 5.31 – 5.19 (m, 4H), 4.60 (s, 2H), 4.52 (s, 2H), 4.22 (d, 1H, *J* = 8.7 Hz), 3.71 (s, 3H), 2.94 – 2.71 (m, 4H), 2.09 (m, 3H, *J* = 2.0 Hz), 2.04 (s, 7H).

^{13}C NMR (100 MHz, CDCl$_3$) of 5 *as a mixture of two diastereoisomers* : δ 170.15, 169.49, 169.43, 166.89, 166.87, 162.82, 152.72, 149.05, 141.15, 141.06, 136.51, 135.54, 135.49, 132.53, 132.33, 127.74, 127.62, 123.61, 123.37, 119.85, 119.79, 99.73, 78.59, 72.62, 72.07, 71.24, 71.20, 70.29, 68.85, 68.82, 64.63, 64.16, 53.55, 53.21, 36.68, 31.60, 29.80, 26.48, 26.46, 20.69, 20.65, 20.61.

HRESI-MS: m/z 725.1800 (calcd. for $C_{32}H_{34}N_2O_{16}Na$ 725.1806 $[M+Na]^+$).

Preparation of compound 6



6

Anhydrous pyridine (54 µL, 0.669 mmol) was added dropwise to a cooled (0°C) solution of *p*-nitrophenyl chloroformate (135 mg, 0.669 mmol) and compound **5** (117.6 mg, 0.167 mmol) in CH_2Cl_2 (3.9 mL).The mixture was stirred 3 hours at room temperature, hydrolyzed with a saturated solution of $NaHCO_3$ and extracted with ethyl acetate (3*20 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification by column chromatography over silica gel (gradient elution: 0% to 4% MeOH in CH_2Cl_2) afforded **6** (146 mg, 0.141 mmol, 85 %).

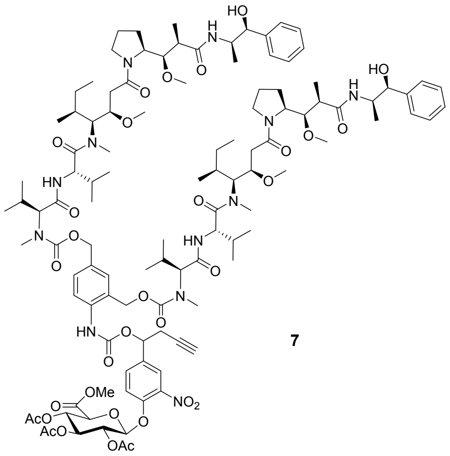
Tr = 25.3 min (Method **1**)

^{1}H NMR (400 MHz, $CDCl_3$) of 6 *as a mixture of two diastereoisomers* : δ 8.28 (m, 4H), 7.91 (s, 1H), 7.86 (d, 1H, *J* = 2.0 Hz), 7.61 (m 2H), 7.53-7.50 (m, 2H), 7.38 (m, 5H), 5.88 (t, 1H, *J* = 6.4 Hz), 5.40 – 5.22 (m, 8H), 4.23 (d, 1H, *J* = 8.6 Hz), 3.74 (s, 3H), 2.86 (m, 2H), 2.18-2.05 (m, 10H).

^{13}C NMR (100 MHz, $CDCl_3$) of 6 *as a mixture of two diastereoisomers* : δ 170.15, 169.43, 169.35, 166.87, 166.83, 155.54, 155.26, 153.09, 152.7, 152.3, 149.21, 145.84, 137.26, 135.15, 135.07, 132.43, 132.0, 131.19, 125.56, 125.48, 123.51, 123.35, 121.89, 120.06, 119.84, 99.74, 99.70, 78.38, 73.49, 72.75, 72.72, 71.18, 71.09, 70.30, 70.10, 68.83, 68.78, 67.68, 53.19, 20.72, 20.68, 20.64.

HRESI-MS: m/z 1055.1925 (calcd. for $C_{46}H_{40}N_4O_{24}Na$ 1055.1930[M+Na]$^+$).

Preparation of compound 7



7

To a stirred solution of biscarbonate **6** (73 mg, 0.0706 mmol) in DMF (3 mL) and pyridine (0.70 mL) was added MMAE (101.5 mg, 0.141 mmol) and hydroxybenzotriazole (HOBt) (19.1 mg, 0.141 mmol). The reaction was stirred 24 hours at room temperature and monitored by analytical RP-HPLC using method 1. The solution was concentrated under reduced pressure. The crude material was purified by column chromatography (gradient elution: 2% to 10% MeOH in CH_2Cl_2) to afford **7** (86.6 mg, 0.0395 mmol, 56 %).

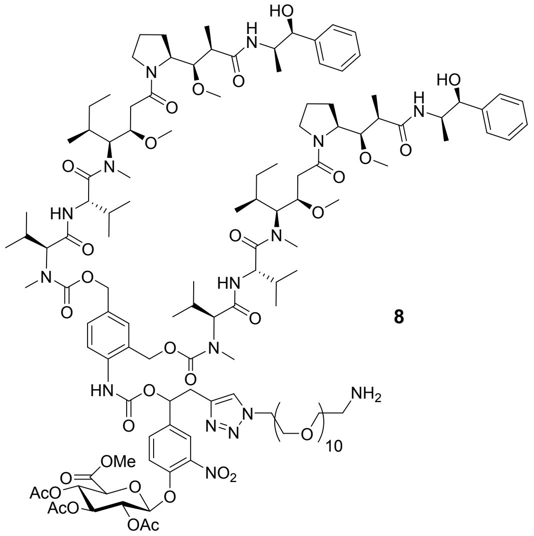
Tr = 27.0 min (Method **1**)

^{1}H NMR (400 MHz, $CDCl_3$) of 7 *as a mixture of two diastereoisomers* : δ 7.95 (s, 1H), 7.79 (s, 1H), 7.63 (s, 1H), 7.39-7.22 (m, 16H), 6.59-6.25 (m, 2H), 5.84 (m, 1H), 5.32-5.20 (m, 4H), 5.17-4.95 (m, 6H), 4.68 (m, 2H), 4.26-4.06 (m, 8H), 3.8-3.68 (m, 4H), 3.51-3.31 (m, 18H), 3.1 (m, 1H), 3.0-2.8 (m, 13H), 2.41 (m, 4H), 2.05 (m, 19H), 1.84-1.71 (m, 5H), 1.26 (m, 10H), 1.06-0.6 (m, 48H).

^{13}C NMR (100 MHz, DMSO-*d6*) of 7 *as a mixture of two diastereoisomers* : δ 173.90, 173.83, 172.36, 172.28, 169.78, 169.73, 169.48, 169.30, 168.71, 166.88, 153.27, 147.78, 143.66, 139.98, 135.08, 132.28, 127.87, 127.77, 127.72, 126.69, 126.63, 126.42, 126.87, 97.82, 97.77, 97.70, 85.42, 81.61, 79.63, 79.30, 78.97, 78.64, 77.67, 76.90, 74.77, 74.75, 73.90, 71.05, 70.69, 69.83, 68.67, 63.32, 63.21, 60.90, 60.26, 58.64, 58.17, 57.12, 57.09, 55.00, 54.96, 54.91, 54.23, 54.19, 54.12, 52.61, 49.74, 49.16, 47.19, 46.22, 43.74, 43.19, 31.99, 31.94, 31.80, 31.76, 31.54, 31.48, 29.92, 29.89, 29.79, 29.71, 29.69, 29.04, 26.86, 26.81, 26.75, 25.50, 25.49, 25.34, 25.28, 25.26, 25.17, 24.33, 23.10, 20.28, 20.22, 20.16, 18.87, 18.86, 18.73, 18.71, 18.51, 18.48, 18.28, 15.74, 15.60, 15.44, 15.31, 15.26, 14.99, 10.37, 10.27.

HRESI-MS: m/z 2190.16473 (calcd. for $C_{112}H_{165}N_{12}O_{32}$ 2190.1653 $[M+H]^+$).

Preparation of compound 8



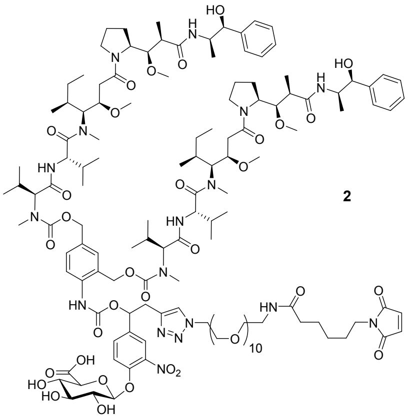
8

To a solution of **7** (55.4 mg, 0.0252 mmol) and O-(2-aminoethyl)-O'-(2-azidoethyl) nonaethyleneglycol (14.6 mg, 0.0278 mmol) in CH_2Cl_2 (2.1 mL) was added $Cu(MeCN)_4PF_6$ (14.1 mg, 0.0379 mmol). The mixture was stirred at room temperature for 15 hours. A solution of ethylenediaminetetraacetic acid disodium salt dihydrate ($EDTA.2Na.2H_2O$) (160 mg) in water (2.4 mL) was added. The resulting mixture was stirred for 30 min. and extracted with CH_2Cl_2. The combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. The crude product was purified by column chromatography over silica gel (gradient elution: 2% to 10% MeOH in CH_2Cl_2) to give instable amine **8** (22.4 mg, 0.0086 mmol, 34 %).

Tr = 22.2 min (Method **1**)

HRESI-MS: m/z 2716.4861 (calcd. for $C_{134}H_{211}N_{16}O_{42}$ 2716.4867 $[M+H]^+$).

Preparation of compound 2


2

8 (20.9 mg, 0.0081 mmol) was dissolved in MeOH (0.63 mL). The mixture was cooled at 0°C and a solution of lithium hydroxide monohydrate (3.95 mg, 0.0941 mmol) in water (0.83 mL) was added dropwise. The mixture was stirred for 1h30, hydrolyzed with acidic resin (Amberlite Weakly Acidic Cation Exchanger Hydrogen Form) for 20 min., filtrated and concentrated in vacuo. The crude product was dissolved in DMSO (0.4 mL). 3mg (0.00975 mmol) of **9** was added. The mixture was stirred at room temperature for 14 hours and the solvent was removed under reduced pressure. High degree of purity for **2** was obtained using preparative-reverse phase HPLC (Method **2**, 7.4 mg, 33% over 2 steps).

Tr = 20.1 min (Method **1**)

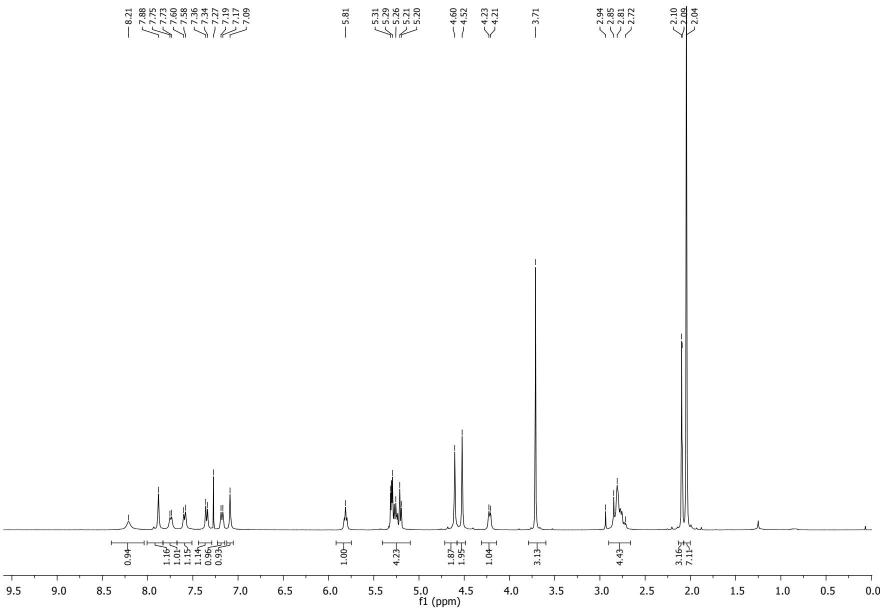
^{1}H NMR (500 MHz, DMSO-*d6*) of 2 *as a mixture of two diastereoisomers* δ 9.3 (m, 1H), 8.3 (m, 1H), 8.05 (m, 1H), 7.9-7.8 (m, 3H), 7.7-7.6 (m, 2H), 7.4-7.2 (m, 15H), 7.0 (s, 2H), 6 (m, 1H), 5.5-5.4 (m, 3H), 5.1-4.9 (m, 9H), 4.7-4.6 (m, 2H), 4.5-4.4 (m, 9H), 4.3 (m, 2H), 4.0 (m, 7H), 3.7 (m, 8H), 3.5-3.2 (m, 42H masked by residual H_2O), 3.1-2.8 (m, 14H), 2.6 (m, 1H), 2.4 (m, 1H), 2.3 (m, 3H), 2.1-1.9 (m, 8H), 1.8-1.7 (m, 6H), 1.5-1.4 (m, 14H), 1.3-1.1 (m, 6H), 1.1-0.8 (m, 52H).

^{13}C NMR (125 MHz, DMSO-*d6*) of 2 *as a mixture of two diastereoisomers* δ 172.37, 172.33, 172.07, 172.05, 171.10, 168.76, 143.61, 134.51, 127.75, 127.71, 126.67, 126.64, 126.61, 126.40, 126.37, 81.61, 74.75, 74.72, 71.88, 69.74, 69.70, 69.60, 69.57, 69.12, 68.81, 63.42, 63.34, 60.89, 60.25, 58.63, 58.18, 57.12, 57.08, 49.73, 49.31, 49.15, 47.20, 46.22, 43.72, 43.20, 38.42, 36.97, 35.23, 35.09, 35.04, 32.01, 31.99, 31.79, 31.77, 31.49, 31.46, 29.91, 29.86, 27.74, 25.75, 25.32, 25.24, 24.74, 24.32, 23.08, 18.89, 18.85, 18.80, 15.75, 15.41, 15.23, 14.96, 12.91, 10.36, 10.35, 10.30, 10.25.

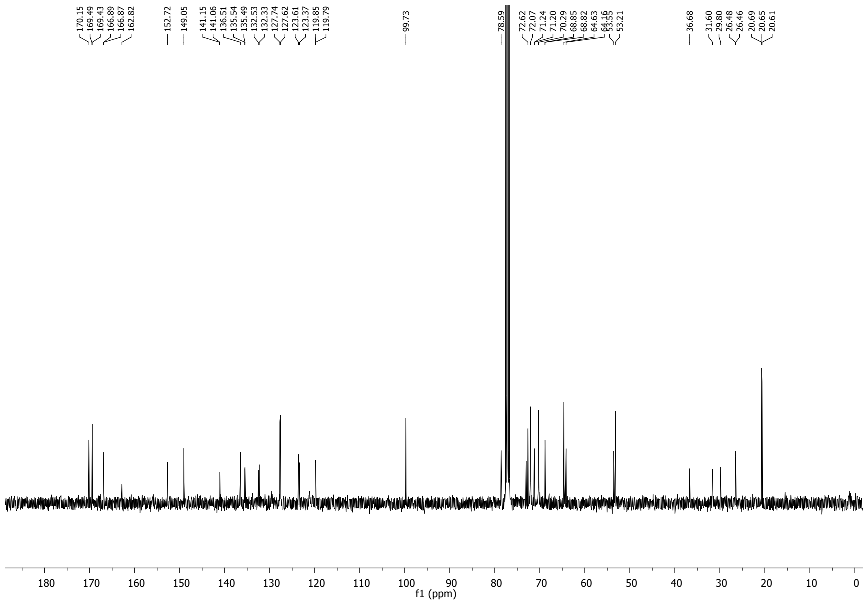
HRESI-MS: m/z 2767.4983 (calcd. for $C_{137}H_{212}N_{17}O_{42}$ 2767.49813 $[M-H]^-$)

I.3. ^{1}H NMR and ^{13}C NMR plots

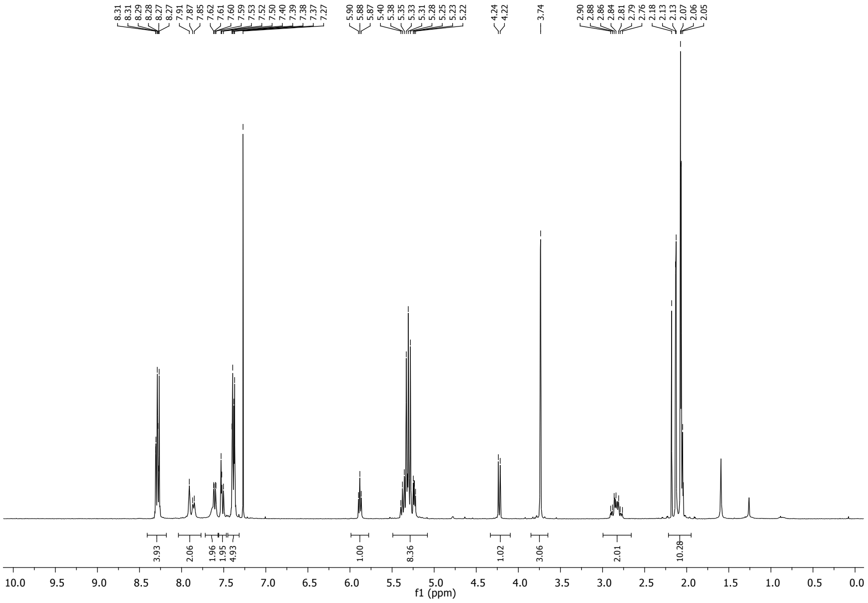
^{1}H NMR spectrum (400 MHz, 298 K, CDCl$_3$) of **5**



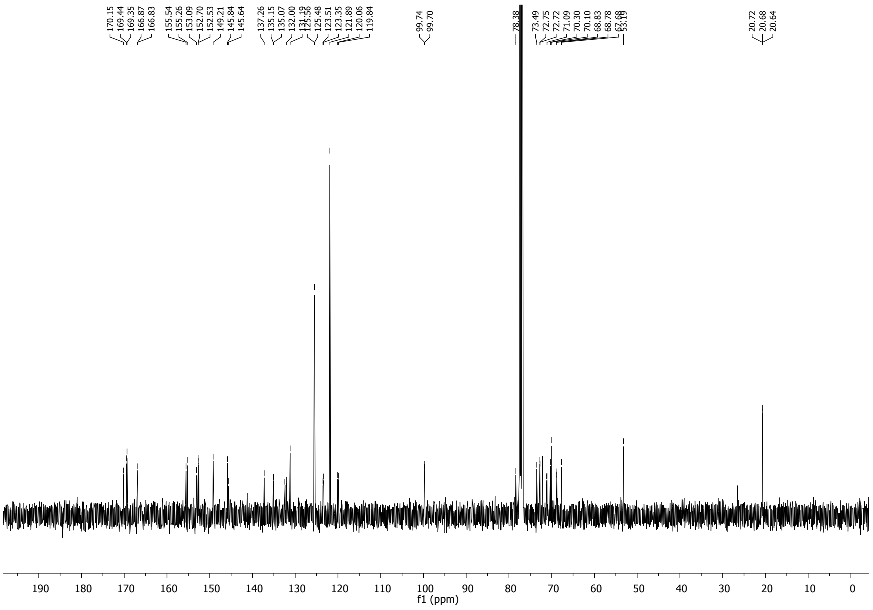
^{13} C NMR spectrum (100 MHz, 298 K, CDCl$_3$) of **5**



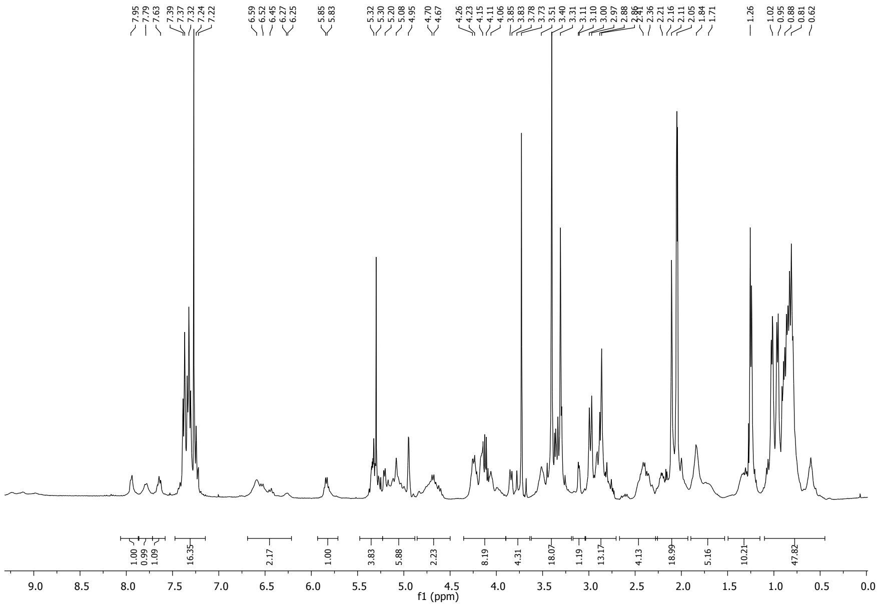
^{1}H *NMR spectrum (400 MHz, 298 K,* $CDCl_3$*) of* **6**



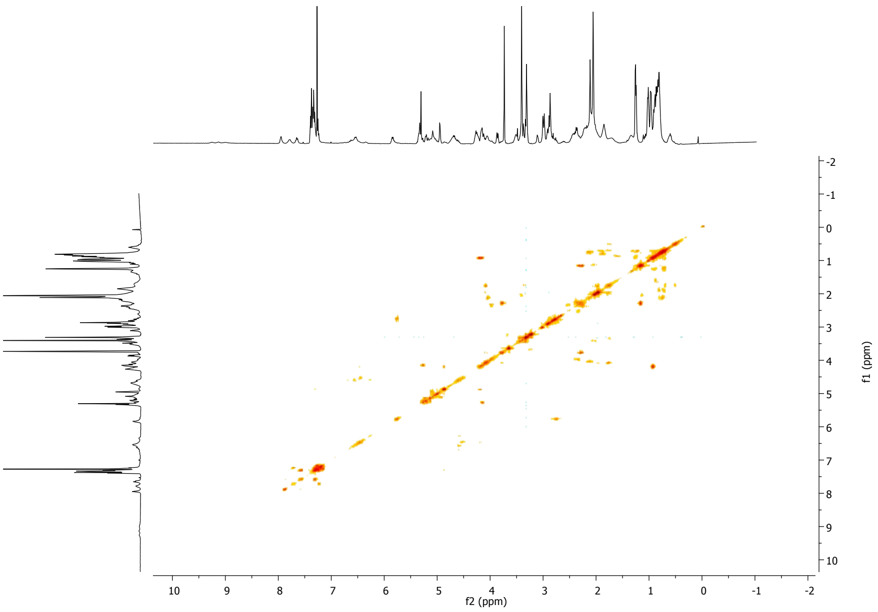
^{13} *C NMR spectrum (100 MHz, 298 K,* $CDCl_3$*) of* **6**



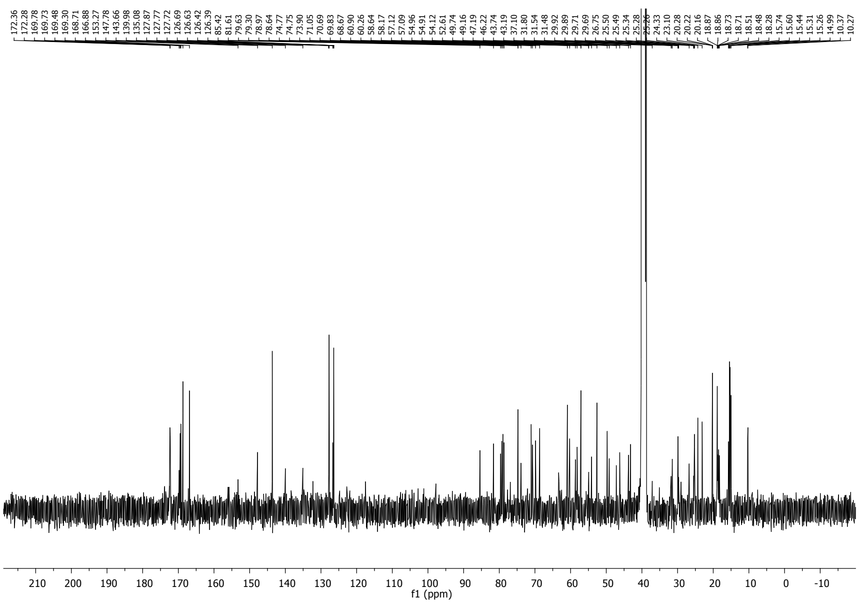
^{1}H NMR spectrum (400 MHz, 298 K, CDCl$_3$) of **7**



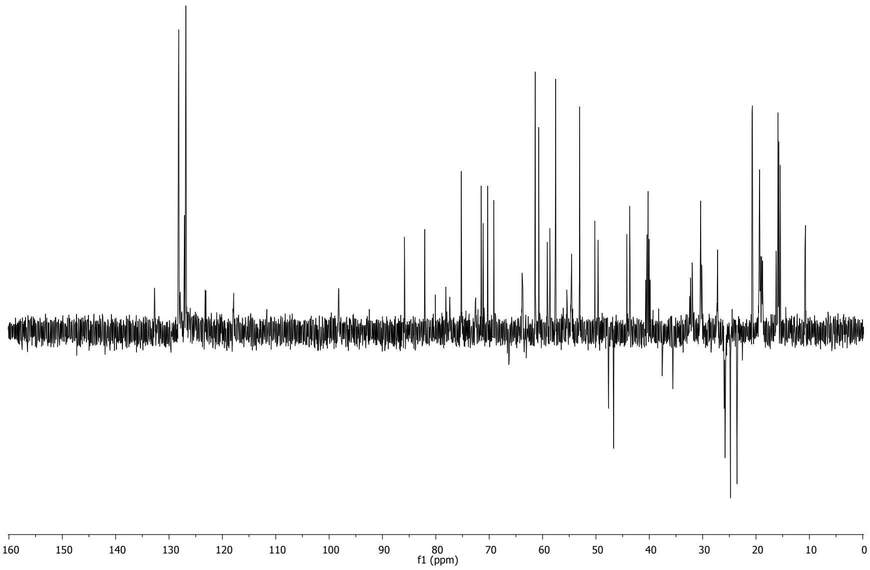
COSY spectrum (400 MHz, CDCl$_3$) of **7**



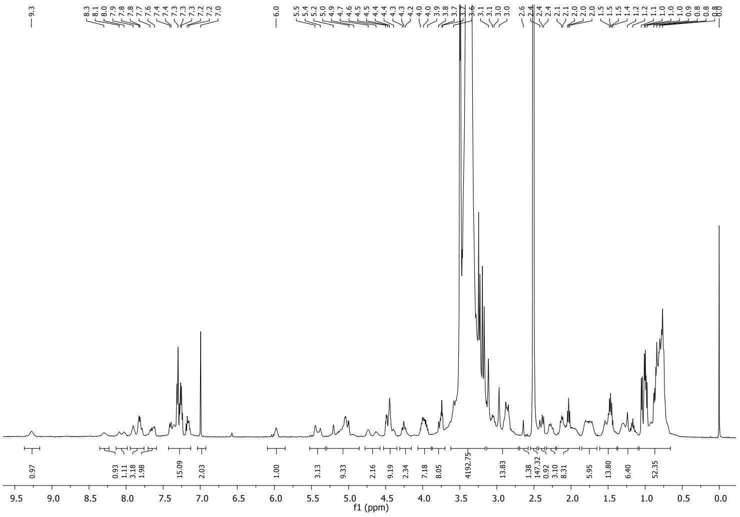
^{13} C NMR spectrum (100 MHz, 298 K, DMSO-d6) of ***7***



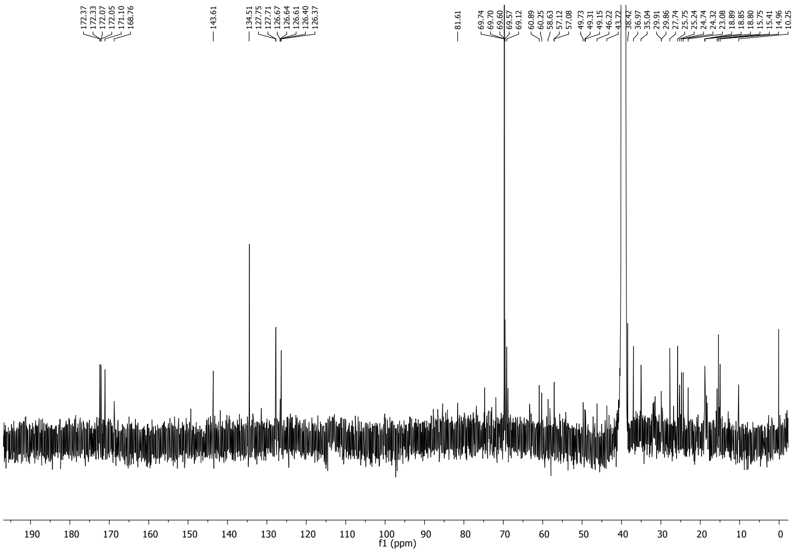
DEPT 135 NMR spectrum (100 MHz, DMSO-d6) of 7



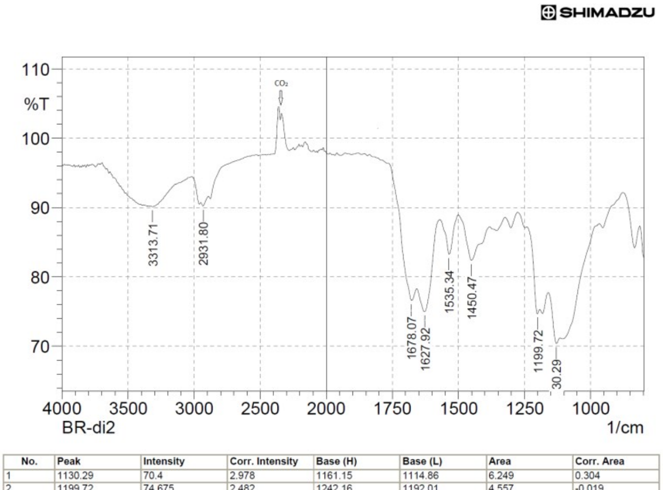
^{1}H NMR spectrum (500 MHz, DMSO-d6) of **2**



^{13}C NMR spectrum (125 MHz, DMSO-d6) of **2**



IR spectrum of **2**



No.	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	1130.29	70.4	2.978	1161.15	1114.86	6.249	0.304
2	1199.72	74.675	2.482	1242.16	1192.01	4.557	-0.019
3	1450.47	82.377	5.856	1500.62	1381.03	8.202	1.617
4	1535.34	83.24	5.363	1570.06	1500.62	4.44	0.79
5	1627.92	74.998	6.766	1658.78	1570.06	8.664	1.545
6	1678.07	76.607	4.927	1766.8	1658.78	7.485	0.891
7	2931.8	90.22	0.782	2951.09	2893.22	2.429	0.1
8	3313.71	90.109	0.116	3329.14	3298.28	1.388	0.01

I.4. Mechanism of drug release

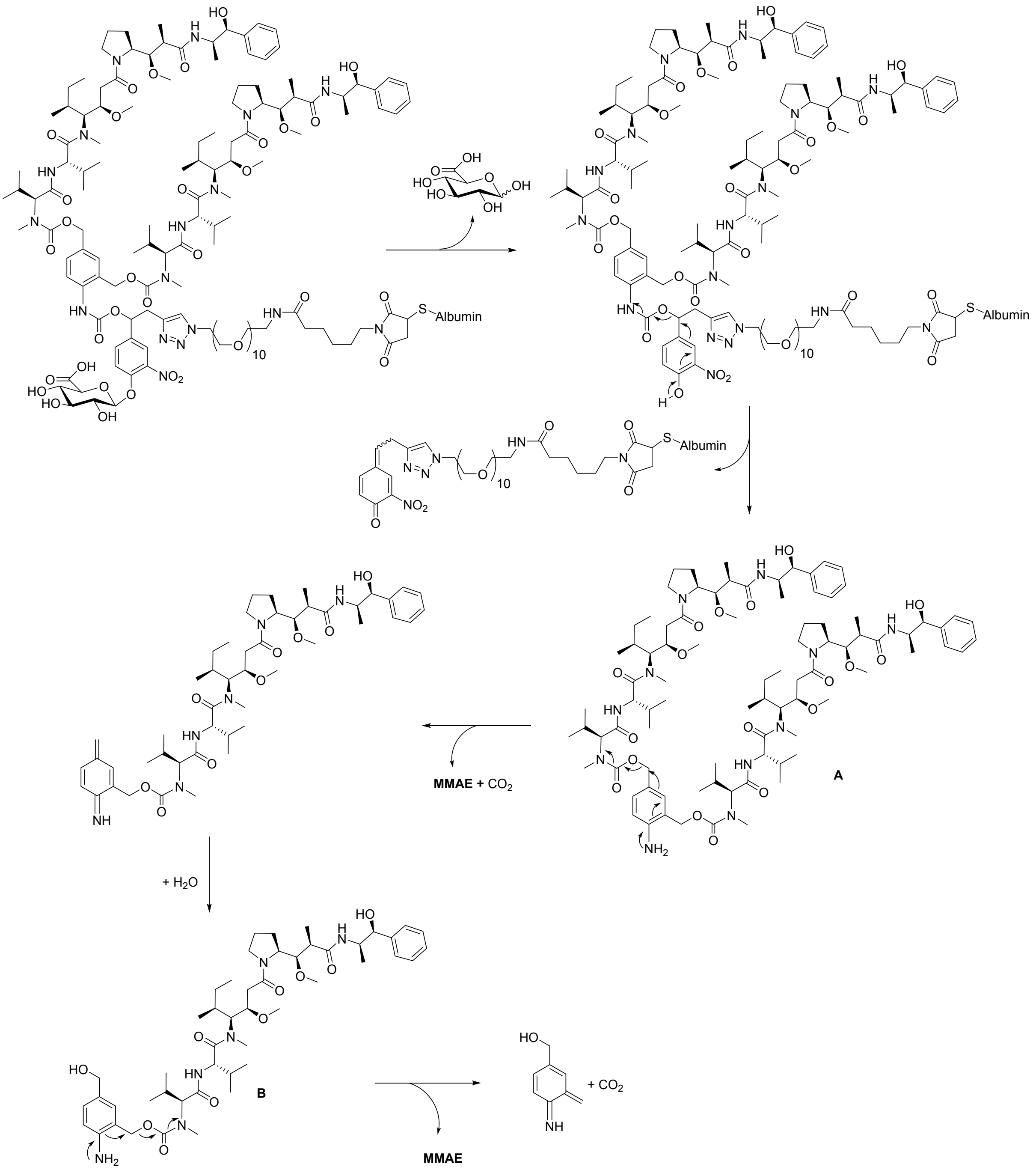


Fig. S1 Mechanism of drug release from prodrug **2** when activated by β-glucuronidase

Along the process of drug release, intermediates **A** and **B** have been detected by HPLC-HRMS with:

- **A**: m/z = 821.0402 $[M + 2H]^{2+}$
- **B**: m/z = 897.5739 $[M + H]^{+}$

I.5. Binding assays with HAS and β-glucuronidase cleavage

Prodrug **2** (0.49 mg.mL^{-1}) was incubated at 37°C in Human Serum Albumin (HSA) 5 % solution (purchased from Octapharma) for 4 hours to form the corresponding albumin adduct. The mixture was diluted 1:3 using 0.02 M phosphate buffer (pH 7) and *Escherichia coli* β-glucuronidase (133 U/mL; purchased from Sigma Adrich) was added at 37°C. The release of MMAE along the time was followed by HPLC/HRMS/MS (screened MMAE m/z: 718.5117 $[M+H]^+$ for the precursor ion; 443,29099 $[M+H]^+$ and 686,48523 $[M+H]^+$ for the fragment ions).

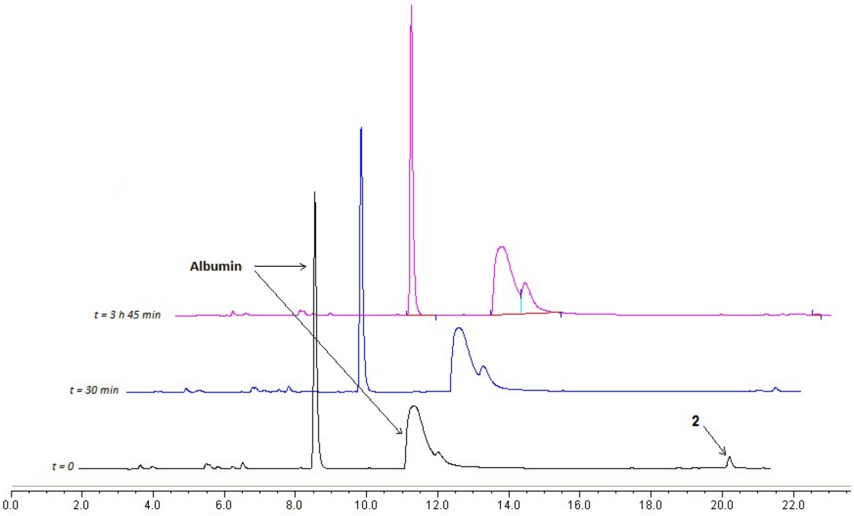


Fig. S2 Chromatograms of HSA incubated with prodrug **2**

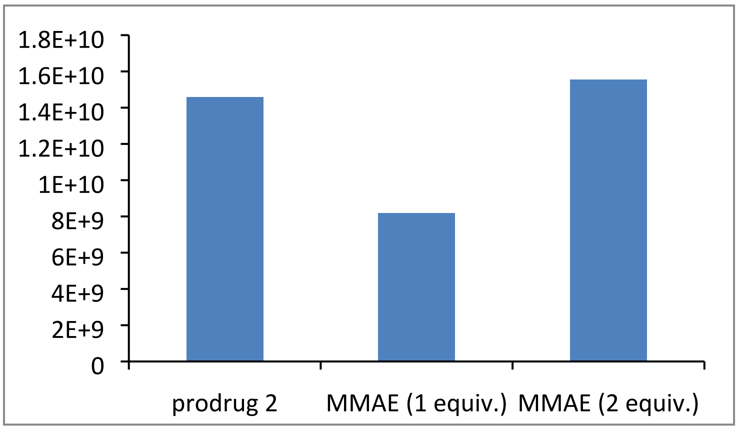


Fig. S3 Relative quantity of MMAE released from prodrug **2** in the presence of β-glucuronidase determined by HPLC-HRMS and compared to standard solutions containing 1 or 2 equivalents of MMAE.

II. Biological Section

II.1. Cell Culture

LS174T (human colon adenocarcinoma – ECACC 87060401) cells were grown in complete medium composed of EMEM-GlutaMax (Life Technologies) supplemented by 1 % Non-Essential Amino-Acid, 10 % fetal bovine serum (Lonza), and 100 U/ml Penicillin/Streptomycin (Life Technologies). Cells were purchased from European Collection of Cell Cultures (ECACC), used at early passages (<10) and cultured as recommended by the manufacturers.

II.2. Soft agar colony formation assay

Soft agar colony formation assay is a relevant method to monitor anchorage-independent growth of transformed cells. Briefly, wells of a 6-well plate were coated with complete culture medium containing 0.6 % melted agarose. After solidification of this bottom layer, 20×10^3 LS174T isolated cells were seeded in complete culture medium containing 0.35 % melted agarose. Cell colonies were allowed to develop in this semi-solid medium for 10 days with adding of fresh complete medium every 3 days. Cells were then treated for 3 additional days with 5 nM of prodrugs **1** or **2** in presence or in absence of β-glucuronidase (≈ 3700 U/well). Colony size was assessed using Olympus MVX10 macroscope and ImageJ software. Scale bar: 250 µm. Results are presented as mean values ± s.e.m from 5 independent experiments.

II.3. *In vivo* experiments

Male BALB/c nu/nu mice (Janvier Labs), purchased from Janvier Labs, were the only animals used in this study. All experimental procedures (2015072111521773) involving animals were validated by the regional ethical committee and carried out in accordance with the guidelines of the French Agriculture and Forestry Ministry (decree 2013-118) and of the European Communities Council Directive (2010/63/UE). Eight-week-old mice were anesthetized with 2 % vaporized isoflurane before 1×10^6 LS174T cells were transplanted subcutaneously in PBS containing 50 % of Matrigel® (Corning) into the dorsal flank (day 0). Animals were then randomly distributed in 4 experimental groups of 6 mice. Mice received on day 17 and 24 intravenous injection of MMAE (0.5 mg/kg), prodrugs **1** (2 mg/kg) or **2** (3 mg/kg) in PBS – 2 % DMSO or intravenous injection of the vehicle solution. Tumor sizes were measured three times a week in two dimensions using caliper. Individual tumor volumes (V) were calculated by the formula $V=[\text{length} \times (\text{width})^2]/2$. Mice weights were also evaluated three times a week. Mice were euthanized when tumor exceeded a volume of 2 cm^3.