

SUPPLEMENTARY DATA

Remote Loading of Liposomes with a ^{124}I -Radioiodinated Compound and their *in vivo* Evaluation by PET/CT in a Murine Tumor Model

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S1 - Detailed synthetic protocol for Amino Diatrizoic Acid (ADA)

General experimental conditions

Starting materials, reagents and solvents were purchased from commercial suppliers and used without further purification. All solvents beside 2-methoxy-ethanol were HPLC grade and dry solvents were obtained via an Innovative Technology PS-MD-7 puresolv system.

All reactions were stirred in a flame-dried, one-necked, round-bottomed flask equipped with a Teflon-coated stirring bar and a rubber-septum. The reaction flask was purged and sealed with argon prior to use. All solid reagents were weighed out in ambient atmosphere and subsequently added to the reaction flask. All liquid reagents and solvents were added to the reaction flask by means of polypropylene/polyethylene disposable syringes equipped with needles, by penetrating the rubber septum. All stated reactions were heated in an oil-bath for the specified time. Thin Layer Chromatography (TLC) analysis was conducted using TLC silica gel 60 F₂₅₄ aluminum sheets (Merck KGaA, 64271 Darmstadt, Germany). Ultra Performance Liquid Chromatography Mass Spectrometry analysis (LC-MS) was conducted on a Waters AQUITY UPLC system equipped with PDA and SQD electrospray MS detector. The column used was a Thermo Accucore C18, 2.6 μm , 2.1 x 50 mm from Thermo Scientific, operating at a column temperature of 50 °C. The flow rate was set to 0.6 mL/min. The solvent used for elution was Solvent A: 0.1% formic acid in Milli-Q H₂O, Solvent B: 0.1% formic acid in CH₃CN. The gradient applied for analysis was 5-100% Solvent B over 3 min followed by 1 min at 100% of Solvent B. Dry column vacuum chromatography purification was conducted according to literature using silica gel 60 (0.015-0.040 mm, Merck KGaA, 64271 Darmstadt, Germany) [1].

Nuclear Magnetic Resonance Spectroscopy (NMR) was recorded on a Bruker Ascend[™] 400 MHz - operating at 401.3 MHz for ¹H and 100.62 MHz for ¹³C – with a 5 mm H - Broadband Dual Channel z-gradient Prodigy cryoprobe instrumentation. All spectra were recorded at 298 K and calibrated according to solvent residues [2]. Structural assignment of synthesized compounds was conducted from standard 1D and 2D NMR-experiments (¹H, ¹³C, COSY, HSQC, HMBC).

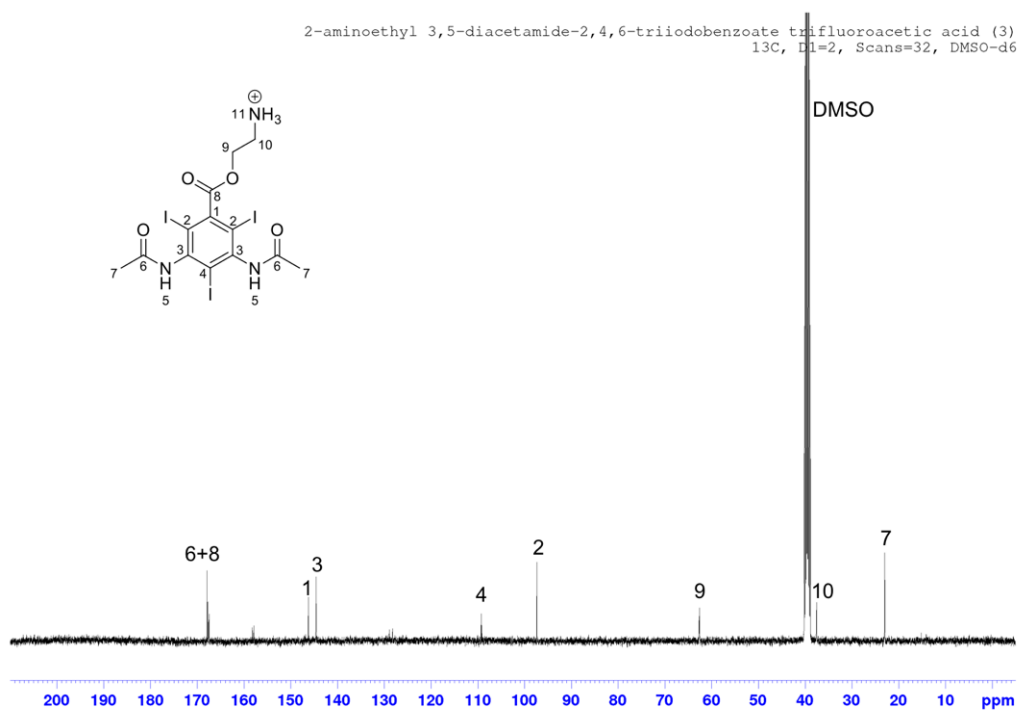
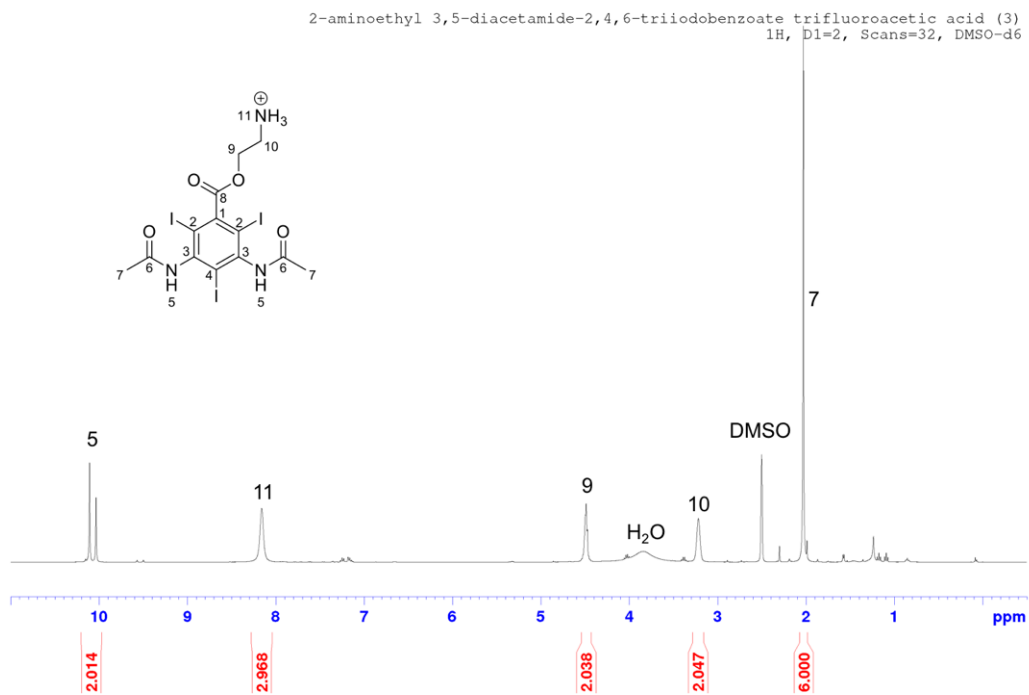
High Resolution Mass Spectroscopy (HR-MS) was performed on a Bruker Daltonics MaXis 3G QTOF-MS fitted with a Dionex Ultimate 3000 UHPLC instrumentation. Attenuated Total reflection Fourier Transformation Infrared Spectroscopy (ATR-FTIR) was conducted on a Bruker Alpha platinum ATR instrumentation with a single reflection diamond ATR module. Melting point (m.p.) was measured using a Stuart SMP 30 instrumentation and reported uncorrected in degrees Celsius.

2-(Boc-amino)ethyl 3,5-diacetamide-2,4,6-triiodobenzoate (2). Diatrizoic acid **1** (1.00 g, 1.59 mmol) was added to a suspension of K₂CO₃ (270 mg, 1.93 mmol) in dry DMF (20 mL). 2-(Boc-amino)ethyl bromide (556 mg, 2.39 mmol) was subsequently added and the reaction mixture was stirred for 30 min at 20 °C and then heated at 50 °C overnight. The reaction was monitored by LC-MS analysis. The reaction was quenched by cooling the reaction mixture to ambient temperature and pouring the reaction mixture into H₂O (50 mL) leading to precipitation. The suspension was extracted using EtOAc (3x50 mL). Upon extraction, the alkylated product **2** occasionally precipitated which in this case was collected by filtration. In case no precipitate was observed, the EtOAc-phases

were combined, dried (Na_2SO_4) and purified by dry column vacuum chromatography using a 0.5% (vol%) $\text{CH}_3\text{OH}/\text{CH}_2\text{Cl}_2$ increment until elution of **2**. The alkylated product **2** was upon concentration *in vacuo* afforded as a white solid. **Yield:** 0.578 g (48%). **m.p.:** Decomposition > 265 °C. **¹H-NMR** (400 MHz, $\text{DMSO}-d_6$): δ (ppm) = 10.1-9.96 (2 s, 2H), 7.01 (t, J = 5.9 Hz, 1H), 4.25 (t, J = 6.1 Hz, 2H), 3.30 (m (overlap with H_2O residue), 2H), 2.02 (s, 6H), 1.38 (s, 9H). **¹³C-NMR** (100 MHz, $\text{DMSO}-d_6$): δ (ppm) = 167.9 (2C), 167.8, 155.5, 146.8, 144.5 (2C), 108.9, 97.1 (2C), 78.0, 64.3, 38.4, 28.2 (3C), 22.9 (2C). **HR-MS:** Calculated adducts $[\text{M}-\text{boc}+\text{H}]^+ = 657.8191$, $[\text{M}+\text{Na}]^+ = 779.8535$. Observed adducts $[\text{M}-\text{boc}+\text{H}]^+ = 657.8202$, $[\text{M}+\text{Na}]^+ = 779.8535$. **ATR-FTIR** (neat, cm^{-1}): 3319, 3225, 2978, 2940, 1729, 1681, 1667, 1500, 1366, 1330, 1255, 1199, 1160, 1025, 995, 864, 638, 592, 565.

2-aminoethyl 3,5-diacetamide-2,4,6-triiodobenzoate trifluoroacetic acid (3). The boc-protected amine **2** (553 mg, 0.72 mmol) was dissolved in CH_2Cl_2 and the mixture was cooled to 0 °C in an ice-water bath. Trifluoroacetic acid (0.95 mL, 12.3 mmol) was added and the reaction was left in the ice bath overnight, allowing to reach 20 °C. The deprotection was monitored by LC-MS analysis. Upon full deprotection, the reaction mixture was concentrated *in vacuo*. In order to remove any excess of trifluoroacetic acid, the residue was azeotropically concentrated *in vacuo* with toluene to afford the amine **3** as the trifluoroacetate salt. **Yield:** 0.546 g (98%). **m.p.:** Decomposition > 260 °C. **¹H-NMR** (400 MHz, $\text{DMSO}-d_6$): 10.1-10.0 (2 s, 2H), 8.16 (s, 3H), 4.49 (m, 2H), 3.22 (s, 2H), 2.03 (s, 6H). **¹³C-NMR** (100 MHz, $\text{DMSO}-d_6$): δ (ppm) = 167.9 (2C), 167.7, 146.2, 144.6 (2C), 109.3, 97.4 (2C), 62.6, 37.5, 22.9 (2C). **HR-MS:** Calculated adducts $[\text{M}+\text{H}]^+ = 657.8191$, Observed adducts $[\text{M}+\text{H}]^+ = 657.8210$. **ATR-FTIR** (neat, cm^{-1}): 3212, 2991, 1709, 1654, 1503, 1366, 1327, 1207, 992, 833, 779, 703, 573.

^1H and ^{13}C NMR spectra of ADA



S2 – Estimation of the LogP and LogD value of ADA

The partition coefficient (LogP and LogD) values are the physicochemical parameters that describe the lipophilicity of ADA by its distribution between two immiscible phases. These parameters are used to estimate the partitioning of ADA into the liposomal membrane, and the solubility in the aqueous core upon protonation of the ADA compound.

The partition coefficients were calculated using ChemAxon software. The calculation method defined by ChemAxon is based on the modified algorithm described by Viswanadhan et al. [3, 4].

logP and logD

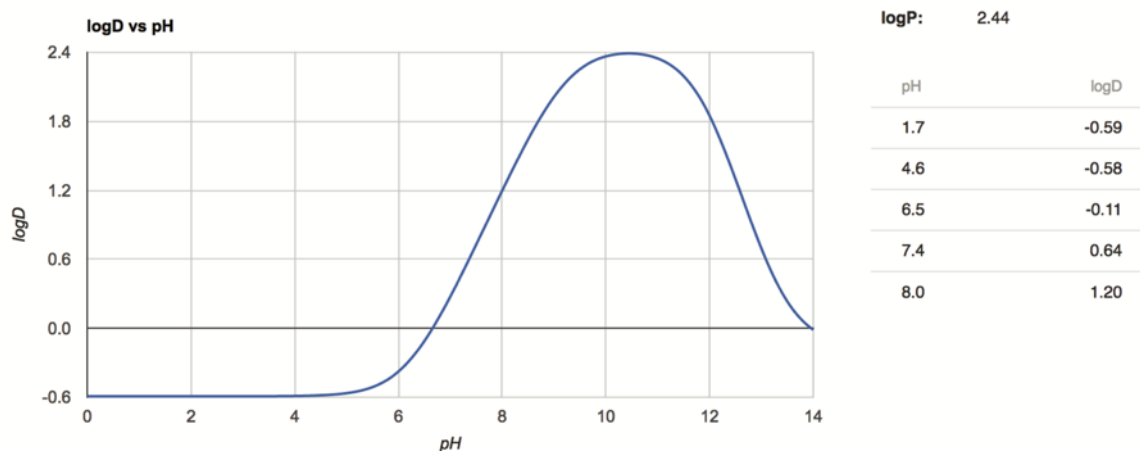


Figure S1. Estimates of LogP and LogD for ADA were obtained by the ChemAxon software (www.chemicalize.org). LogP and logD were used to predict the ability of ADA to load into liposomes by a pH gradient method.

S3-Determination of the pKa value of ADA by acid-base titration

The pKa of ADA was determined by acid-base titration. Briefly, ADA (2.4 mg) was dissolved in 150 mM NaCl (10.00 mL) and titrated with aq. NaOH (10.0 mM) using an automated burette (Metrohm 888). For validation of the result, ADA in basic solution was also titrated with acid. In this titration, ADA (2.7 mg) was dissolved in 150 mM NaCl (10.00 mL) and the pH was adjusted to 10-11 using aq. NaOH (100 mM). The ADA solution was subsequently titrated with aq. HCl (10.0 mM) using an automated burette. The pKa of ADA was found to be 8.56 ± 0.04 (HP₂). The pH of ADA in NaCl solution versus the volume of added NaOH or HCl are shown in Fig. S2. The autotitration was performed by the halfway-point titration mode (HP), and equivalence points (EP) values were calculated based on the first derivative of the titration curve. HP, the halfway-point value, is equal to the pKa of the analyte.

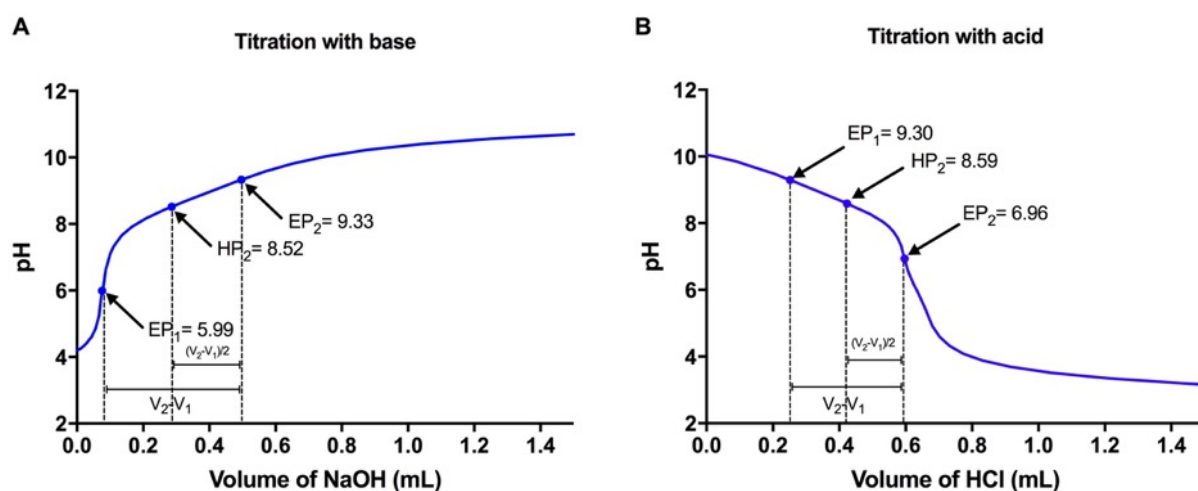


Figure S2. pH-titration curves of ADA. (A) ADA dissolved in NaCl (pH=4.2) was titrated with 10 mM NaOH. (B) ADA dissolved in NaCl (starting pH > 10.0) was titrated with 10 mM HCl. On the titration curves, EP₁ and EP₂ denote the first and second equivalence point of the titration, respectively, whereas V₁ and V₂ are the volume of added NaOH or HCl (10 mM) to complete the titration. HP₂ denotes the halfway-point between EP₁ and EP₂.

S4 - Quantification of the ADA loading efficiency by UV-VIS Spectroscopy

Briefly, liposome samples collected before and after spin column separation were diluted in absolute ethanol with 0.6 v/v% TFA prior to UV-VIS measurement as described in the manuscript. Proper levels of dilution were chosen to keep the absorption above 0.25 and below 1.5. Some samples were measured at two different levels of dilution to determine both the ADA and lipid absorbance concentration accurately. The concentration of ADA was determined via the absorbance at 243 nm and the lipid concentration via absorption of Rho-DPPE at 560 nm. In the UV-VIS spectra, the full and dotted lines show the spectrum of ADA loaded liposomes before and after removal of non-loaded ADA via spin column separation respectively (Fig. S3). Changes in lipid concentration upon spin column separation were corrected for, by either normalizing the absorbance of Rho-DPPE at 560 nm to unity (Fig. S3) or by multiplication of the Rho-DPPE absorption ratio (before and after spin column separation) as described by Eq. 1 in the manuscript. The UV-VIS spectra of ADA loaded ammonium sulfate or citrate gradient liposomes are shown in Fig. S3 and S4 respectively.

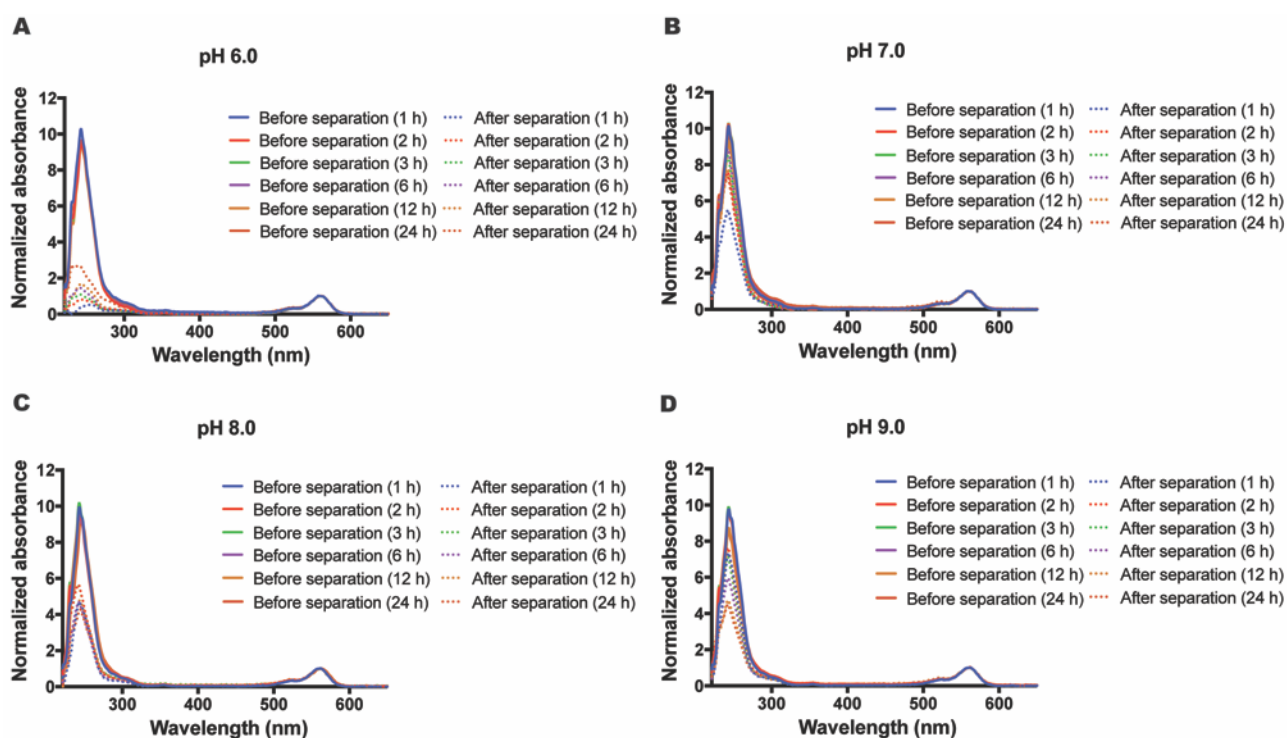


Figure S3. Normalized UV-VIS spectra of ADA loaded ammonium sulfate gradient liposomes. The spectra are shown before and after spin column separation. The normalization of the UV-VIS spectra was achieved by setting the absorbance of the lipid rhodamine dye to unity, i.e. each spectrum was divided by the absorbance of Rho-DPPE at 560 nm. Ammonium sulfate gradients were established at different external pH values and loading efficiencies were determined as a function of incubation time and external pH. The ADA-to-lipid ratio was 0.1 and the samples were diluted 150-fold. External pH values were pH 6.0 (A), pH 7.0 (B), pH 8.0 (C), and pH 9.0 (D).

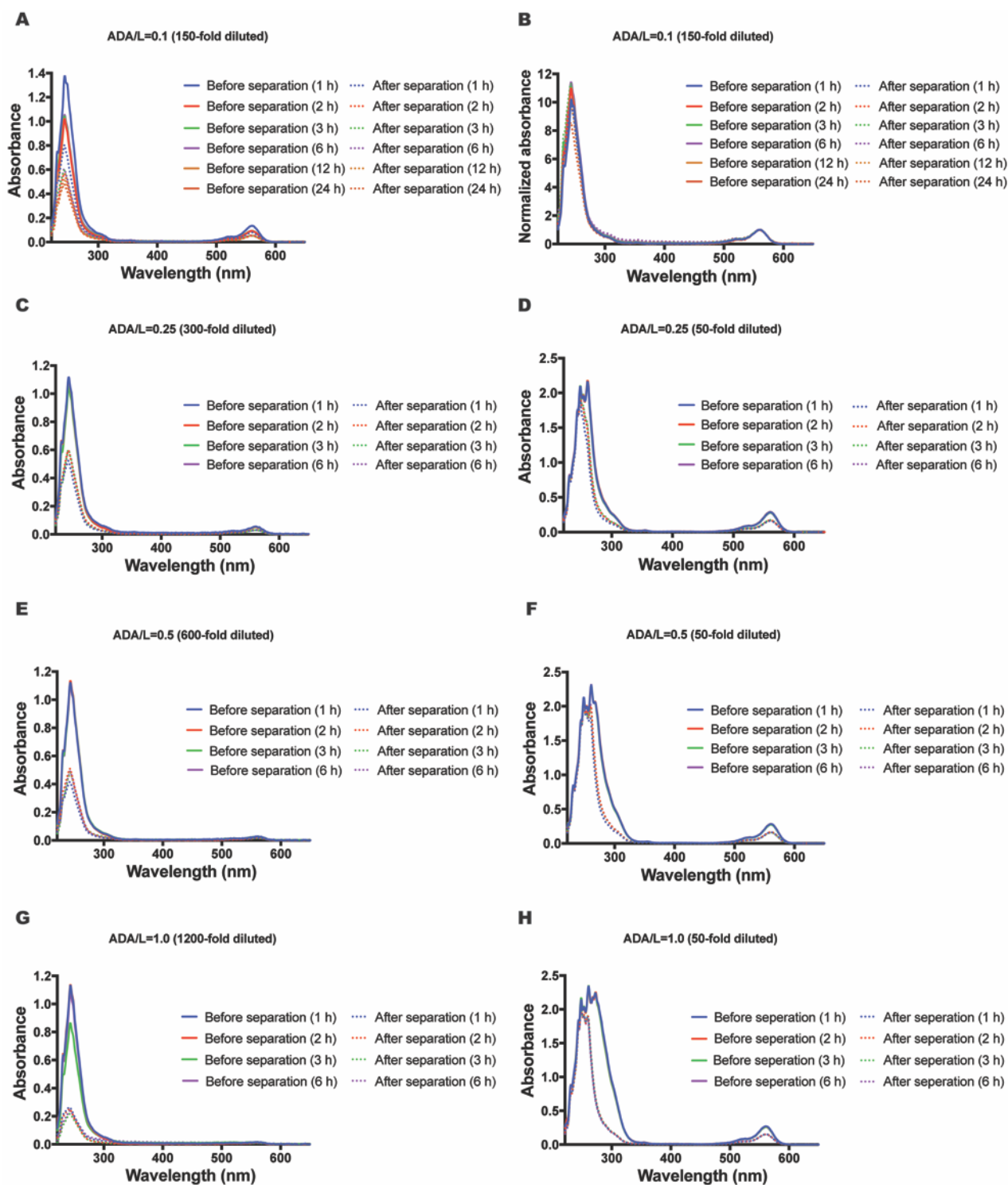


Figure S4. UV-VIS spectra of **3** citrate gradient loaded liposomes. The spectra are shown before and after spin column separation. Loading of citrate gradient liposomes with **3** were performed at varying **3**-to-lipid ratio and incubation time. The normalized UV-VIS spectrum was obtained as described above for the loading of ammonium sulfate gradient liposomes (Fig. S3). The absorbance of **3** and liposomes were determined from separate UV-VIS spectra of **3** and liposomes for the higher ratio of ADA to lipid, e.g., 0.25, 0.5 and 1.0. The UV-VIS spectra of the 50-fold diluted liposomes samples were used to determine the absorbance peak of Rho-DPPE at 560 nm, whereas the absorption of ADA

at 243 nm was determined at higher dilution level. The ADA-to-lipid ratio is 0.1 (A-B), 0.25 (C-D), 0.5 (E-F), and 1.0 (G-H).

S5 - Quantification of the ADA loading efficiency by ICP-MS

Iodine and phosphor-lipid concentrations of liposomes were in addition quantified by ICP-MS (Thermo Scientific, iCAP Q), which confirms the UV-VIS spectroscopy based loading efficiencies presented in the manuscript. The loading efficiencies were quantified by determining the concentration ratio of iodine to phosphor (I/P) before and after removal of the non-loaded ADA by spin column separation. The liposome samples were diluted with either 0.5% tetramethylammonium hydroxide (prepared from 25% TMAH in H₂O, TraceSelect[®]) (50 ppb Te as internal standard) or with 2% nitric acid (100 ppb Ga as internal standard) for iodine or phosphor quantification respectively.

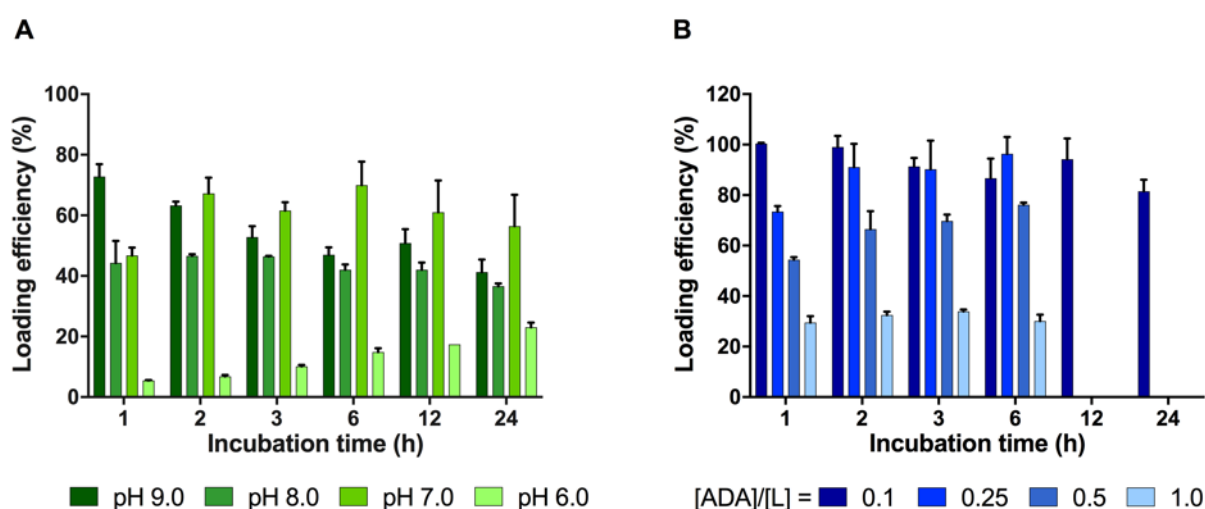


Figure S5. Remote loading of ADA into liposomes via transmembrane ammonium sulfate (A) or citrate gradients (B). (A) The loading efficiency of ADA into ammonium sulfate gradient liposomes is shown as a function of time and external pH. These loadings were conducted at an ADA-to-lipid ratio of 0.1 and at 55°C. (B) The loading efficiency of ADA into citrate gradient liposomes is shown as function of the ADA-to lipid ratio and time. These loadings were conducted at 55 °C with an external pH of 7.0. The data are presented as mean ± SEM.

S6- Determination of radiochemical purity of [¹²⁴I]ADA by radio-TLC

[¹²⁴I]ADA was synthesized as described in Materials & Methods. After synthesis, the product was isolated by a sequential gradient elution of 10 fractions on a Sep-Pak Plus cartridge. Fractions 5-10 were pooled and radio-TLC was performed on the combined sample. The radiochemical purity was determined by analyzing the radio-TLC on a phosphor imager (Cyclone Plus, PerkinElmer). The obtained radio-TLC chromatogram of [¹²⁴I]ADA is shown in Fig. S6.

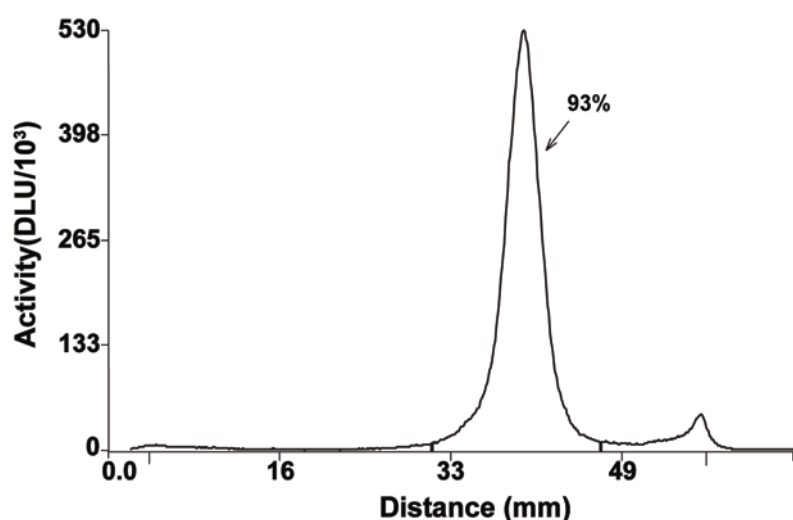


Figure S6. Representative radio-TLC peak of [¹²⁴I]ADA and impurities. [¹²⁴I]ADA was purified on a Sep-Pak Plus Silica cartridge and the product with a radiochemical purity of 93% was remote-loaded into citrate gradient liposomes.

S7- Stability of [¹²⁵I]ADA in serum, liver homogenate and PBS by radio-TLC

Liver homogenate was prepared by homogenization of three livers freshly dissected from 12 week old female Balb/C mice (about 2 g in total) in PBS (20 mL). The resulting homogenate was used without further modification. Mouse serum was prepared from freshly drawn mouse blood that was allowed to stand at room temperature for about 10 minutes, then centrifuged. Liver homogenate, serum or PBS were then added in aliquots of 100 μ L to solutions of [¹²⁵I]ADA in PBS (100 μ L, 0.65 MBq, 0.1 mg ADA, RCP: 93%). The resulting mixtures were incubated at 37 $^{\circ}$ C and monitored by radio-TLC for 3 days. The experiments were conducted in triplicate.

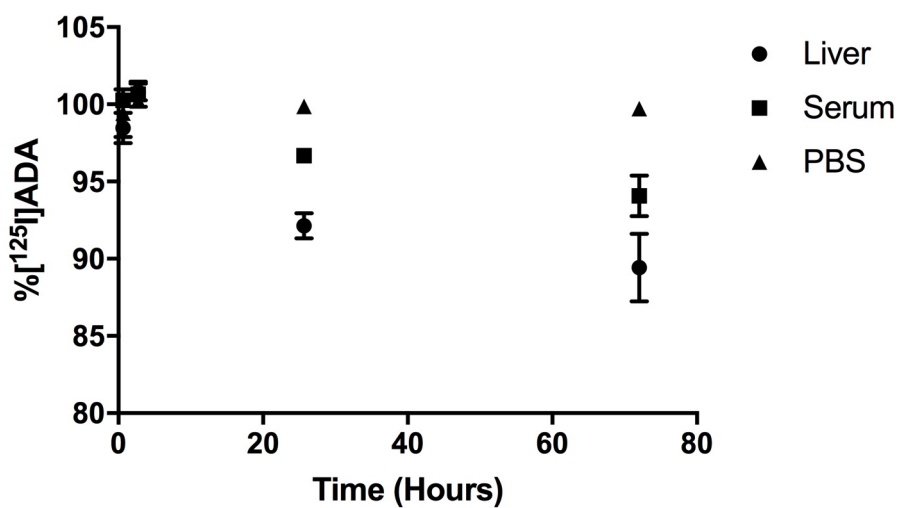


Figure S7: In vitro stability of $[^{125}\text{I}]\text{ADA}$ in liver homogenate, serum (50%) and PBS. Data was recorded by radio-TLC and is plotted as remaining $[^{125}\text{I}]\text{ADA}$ as a percentage of added $[^{125}\text{I}]\text{ADA}$, corrected for radiochemical purity (93%). n = 3 in all cases.

S8- Coronal and Sagittal PET/CT images

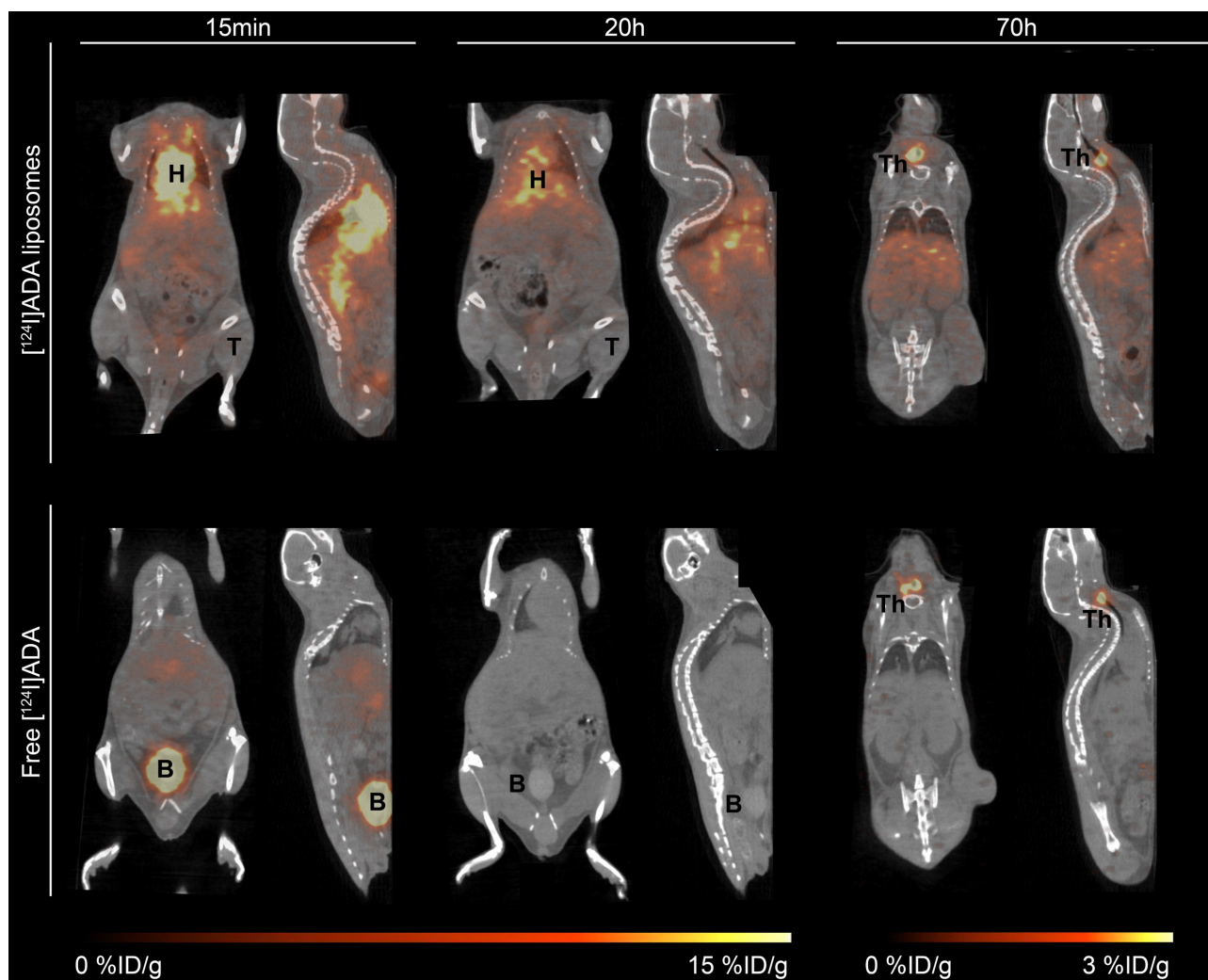


Figure S8. Coronal and sagittal PET/CT images of systemically administered free $[^{124}\text{I}]\text{ADA}$ and $[^{124}\text{I}]\text{ADA}$ loaded liposomes in CT26 tumor-bearing mice at 15 min, 20 h and 70 h pi.. The color bar spans the range 0-15 %ID/g for the 15 min and 20h images, and 0-3 %ID/g for the 70 h images. The thyroid, heart and bladder are indicated by Th, H and B in the images, respectively.

References

- [1] D. S. Pedersen and C. Rosenbohm, Dry Column Vacuum Chromatography, *Synthesis*. 2001, 2431-2434.
- [2] H. E. Gootlieb, V. Kotlyar and A. Nudelman, NMR Chemical Shifts of Common Laboratory Solvents as Trace Impurities", *Journal of Organic Chemistry*. 62, 1997, 7512-7515.
- [3] V. N. Viswanadhan, A. K. Ghose, G. R. Revankar and R. K. Robins, Atomic physicochemical parameters for three dimensional structure directed quantitative structure-activity relationships. 4. Additional parameters for hydrophobic and dispersive interactions and their application for an automated superposition of certain naturally occurring nucleoside antibiotics, *J. Chem. Inf. Comput. Sci.* 29, 1989, 163-172.
- [4] <https://docs.chemaxon.com/display/docs/LogP+and+logD+calculations>