Supporting Information

Signature Effects of Vector-Guided Systemic Nano Bioconjugate Delivery Across Blood-Brain Barrier of Normal, Alzheimer's and Tumor Mouse Models

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Figures:

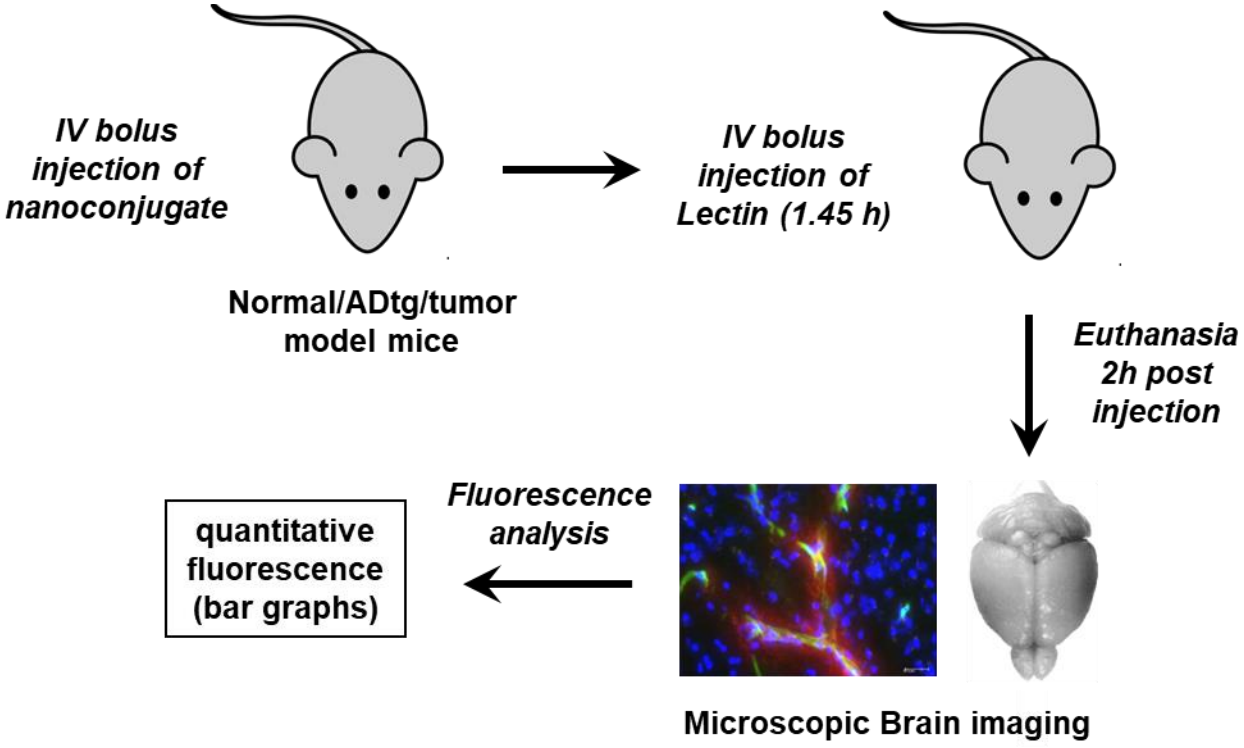


Figure S1. Measurement of in vivo BBB permeability through transcytosis of brain vascular endothelial cells by using ex-vivo microscopic quantitative fluorescence after *in vivo* IV injection into the mouse tail.

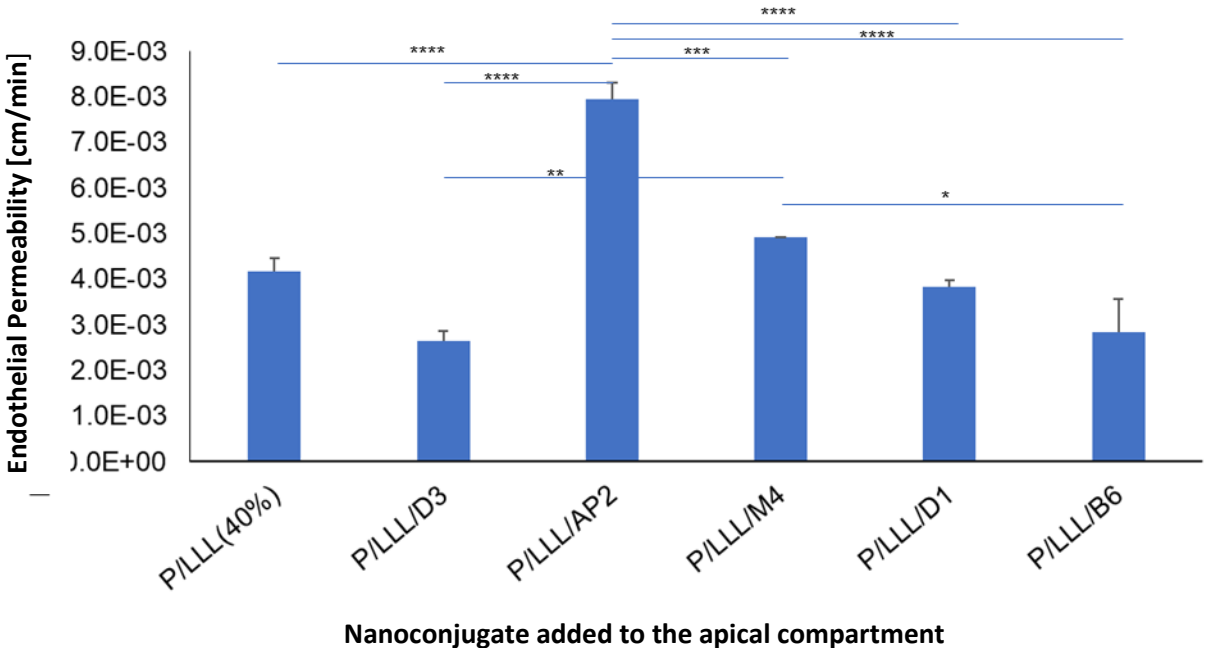


Figure S2. *In vitro* Transwell model of the blood-brain barrier (BBB, hCMEC/D3 immortalized human cerebral microvascular endothelial cells). The concentration of nanoconjugates in the apical chamber was 9.1 µM (µmol/L), compared to blood concentration of 1.4 µM after injection of nanoconjugate dose 0.25X (0.068 µmol/Kg administered in vivo (see dose conversion to vascular concentration in mice, Table S2). Endothelial permeability (EP; cm/min) at 37^{o}C was measured by sampling the medium in the basolateral compartment 3 x every 30 min for 3 h following the addition of nanoconjugate. Fluorescence intensity of the rhodamine labelled nanoconjugates was measured using a plate reader. Error bars represent SEM. Note short names given for nanoconjugates.

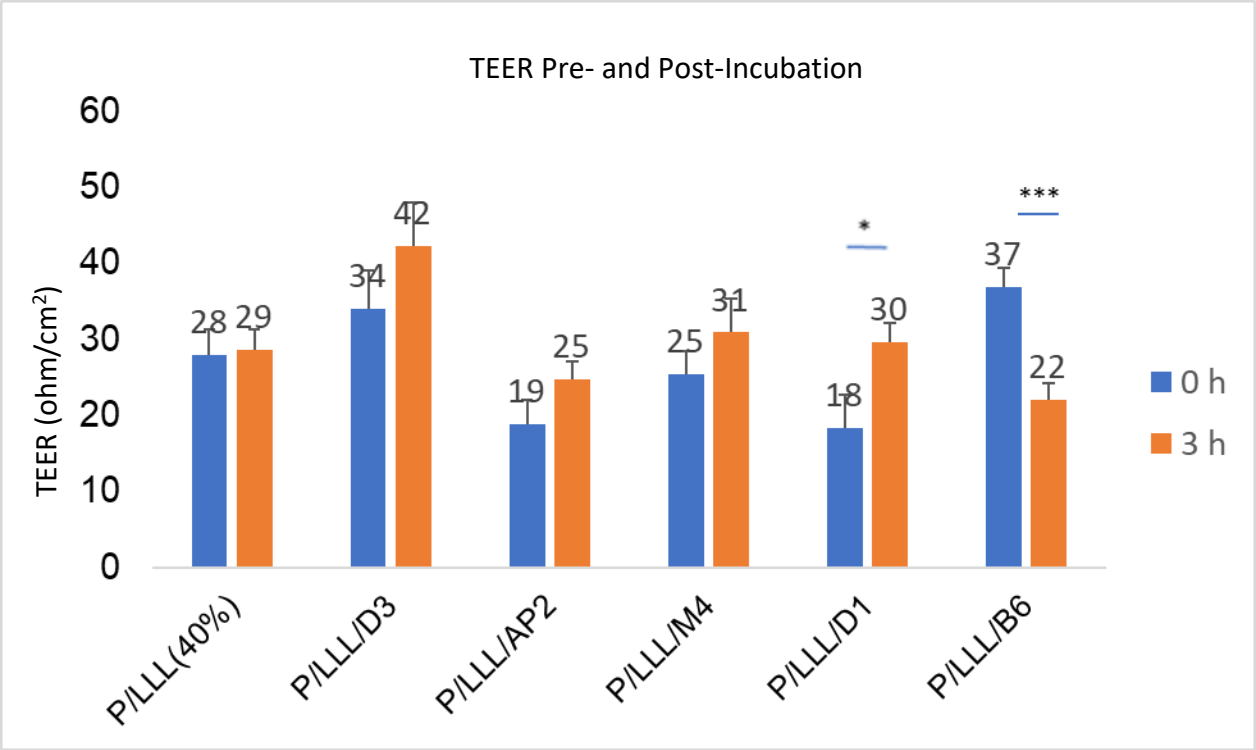


Figure S3. Trans-endothelial electrical resistance (TEER; Ω/cm2) of hCMEC/D3 cells seeded in Transwell plates at 0 h and 3 h after incubation of nanoconjugates at 37 ^{o}C. Experiments carried out in triplicate. Student's two-tailed t-test. Statistical significance is indicated as * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$.

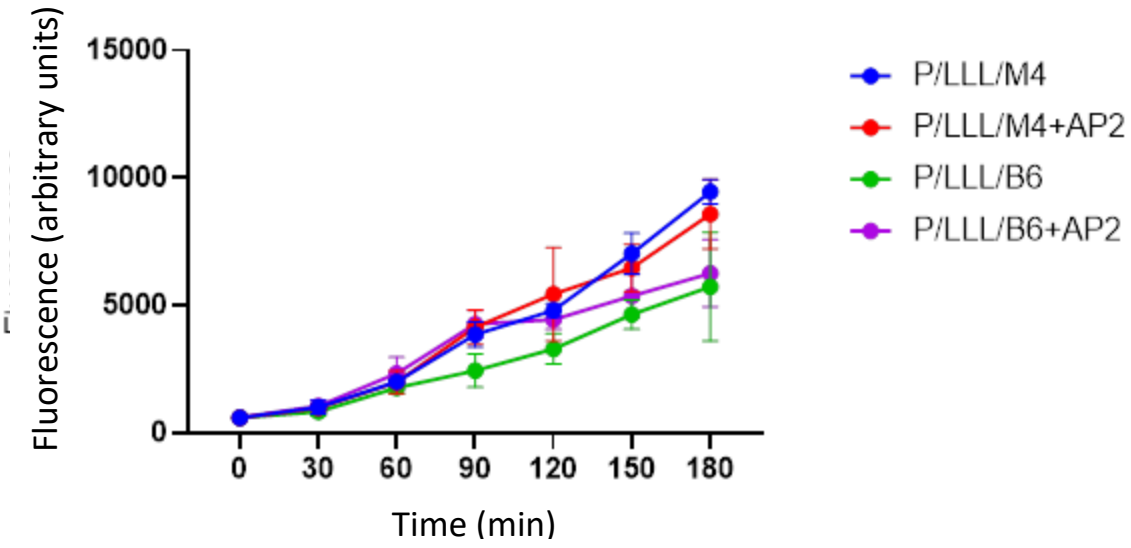


Figure S4. Transwell competition assays of M4 and B6 conjugates with AP2.

Experiments were done as describes in the materials and method section, with P/LLL/M4 (blue) as the control for competition P/LLL/M4+AP2 (red) and P/LLL/B6 (green) as the control for competition P/LLL/B6+AP2 (purple). AP2 was not labeled. Experiments (Filter without cell layer) were also used as a control. No significant deviation was observed between control and competition groups during a period of 3 hours (Prism, one-way ANOVA with Tukey t-tests (t)).

Competition of P/LLL/AP2 and P/LLL/B6 with P/LLL (20X) for Brain permeation in normal mice

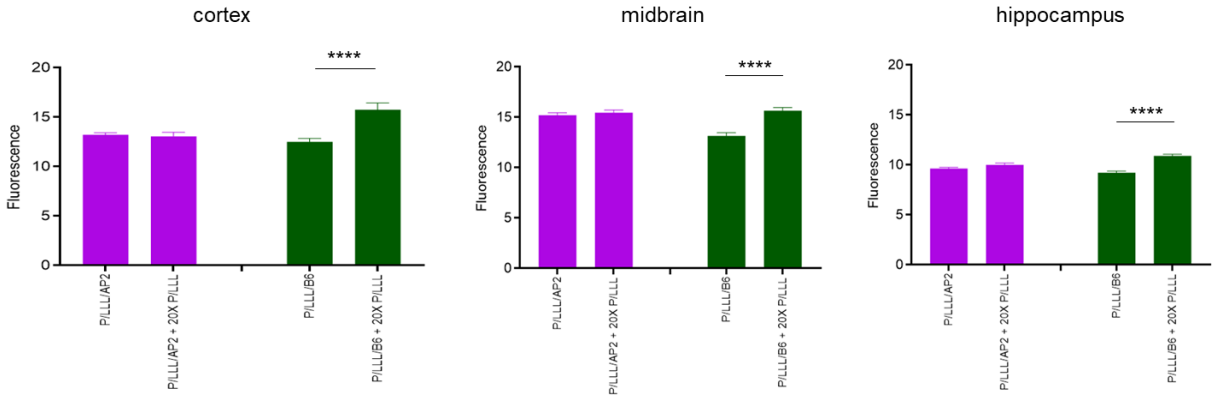


Figure S5: Normal brain enhancement of BBB-permeation for P/LLL/AP2 and P/LLL/B6 in the presence of P/LLL(40%).

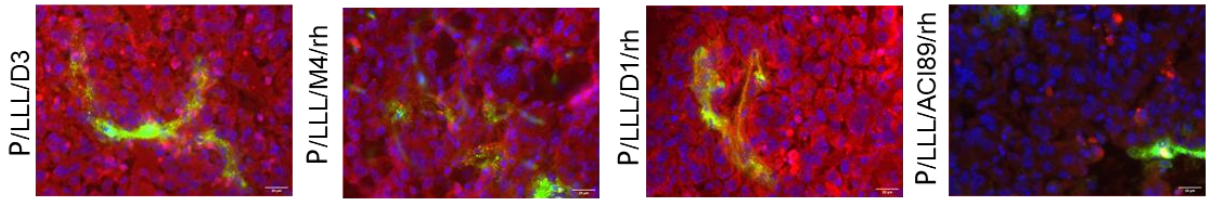
We performed an *in vivo* experiment following the protocol for competition of P/LLL/AP2 and P/LLL/B6 (both at 1X) by the addition of 20X P/LLL(40%) to see whether we could enforce a competition at high concentration of competitor which was ineffective at low concentration. However, again no competition expected as a fluorescence intensity decrease was indicated for P/LLL/AP2, whereas a significant increase was noted for P/LLL/B (Fig. S5). The difference is tentatively attributed to an allosteric boosting mechanism with maintained binding of P/LLL(40%) only in the case of P/LLL/B6. All statistical tests were conducted as a one-way ANOVA with Tukey t-tests (t) comparing nanoconjugate permeability in each brain region. Statistical significance (p) is indicated as * = $p<0.01$, ** = $p<0.001$, *** = $p<0.0001$ and **** = $p<0.00001$. Error bars represent SEM.

Figure S6.

A) Optical Imaging of Glioblastoma. *Ex-vivo* imaging after

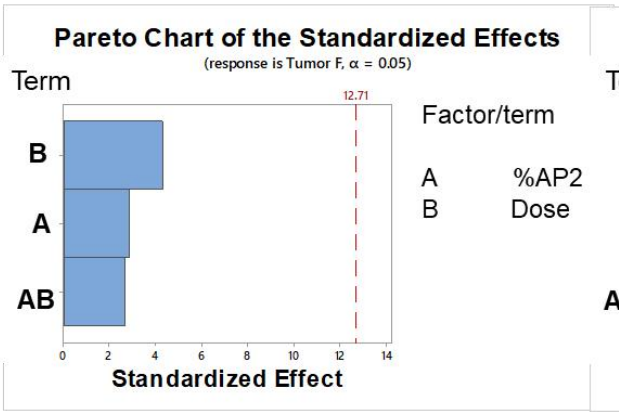
***In vivo* injection of nanoconjugates D3, D1, AC189, and M4.**

Red: Nanoconjugates (Rhodamine labeled), Green: Lectin, Blue: Nuclei

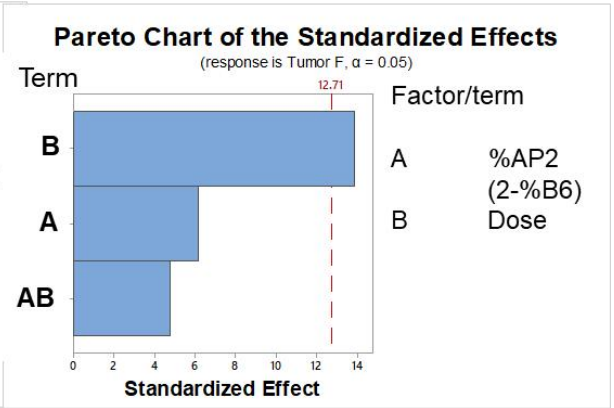


B) Factorial study pareto charts

B1 Only AP2 matrix

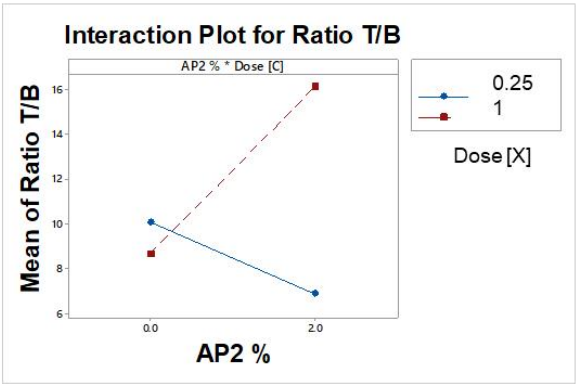


B2 AP2/B6 Combined matrix



C) Factorial study pareto charts

C1 Only AP2 matrix



C2 AP2/B6 Combined matrix

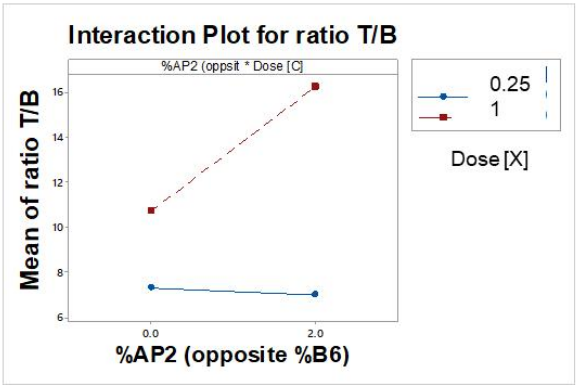
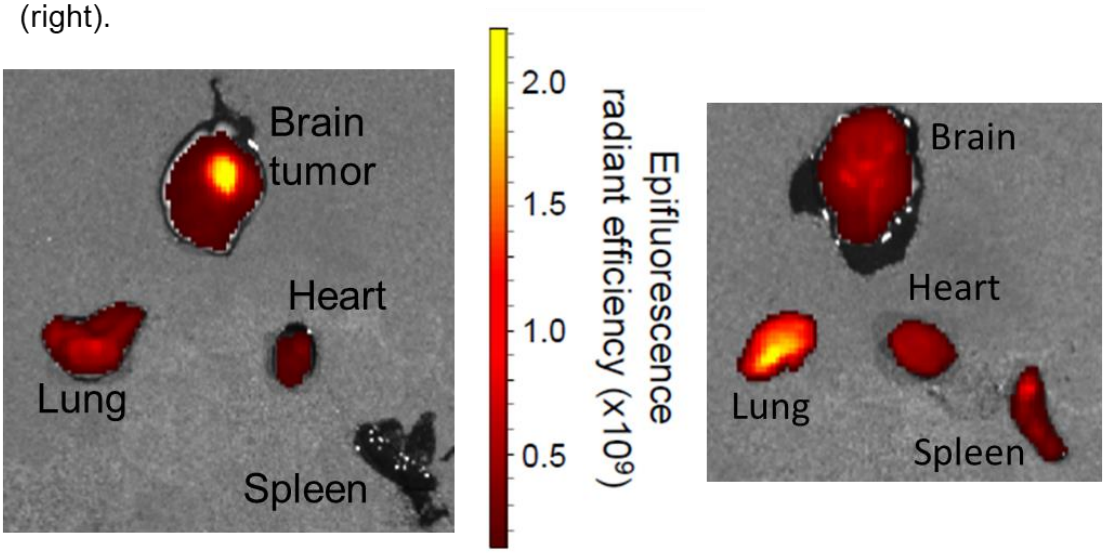


Figure S6A. Nanoconjugate tumor uptake Fluorescence microscope imaging of the cortex 120 min after intravenous injection into mouse tail vein at a dose of 1X (0.274 μmol/Kg) for P/LLL/D3,P/LLL/M4, P/LLL/D1, P/LLLAC189 (A). Note the shortened names of nanoconjugates.

Figure 6 B, C. Puerto Charts and Interaction Plots for Multi Factorial Assay

B). Pareto Charts (calculated significance of factors or combination of factors for an indicated response). Panel B1, the single peptide matrix (P/LLL/AP2); B2, the combined matrix (P/LLL/AP2/B6)C). Interaction Plots for the T/NB-ratio (tumor selectivity) response. C1, data matrix for P/LLL/AP2; C2, data matrix for P/LLL/AP2/B6.

A) Biodistribution of nanoconjugates in tumor and AD mice models – representative images of P/LLL/AP2 in tumor mice (left) and P/LLL/D1 conjugate in 2XTg-AD mice (right).



B) Biodistribution of nanoconjugates in tumor and AD mice models – summary of results for P/LLL/AP2, P/LLL/D3 and P/LLL/D1

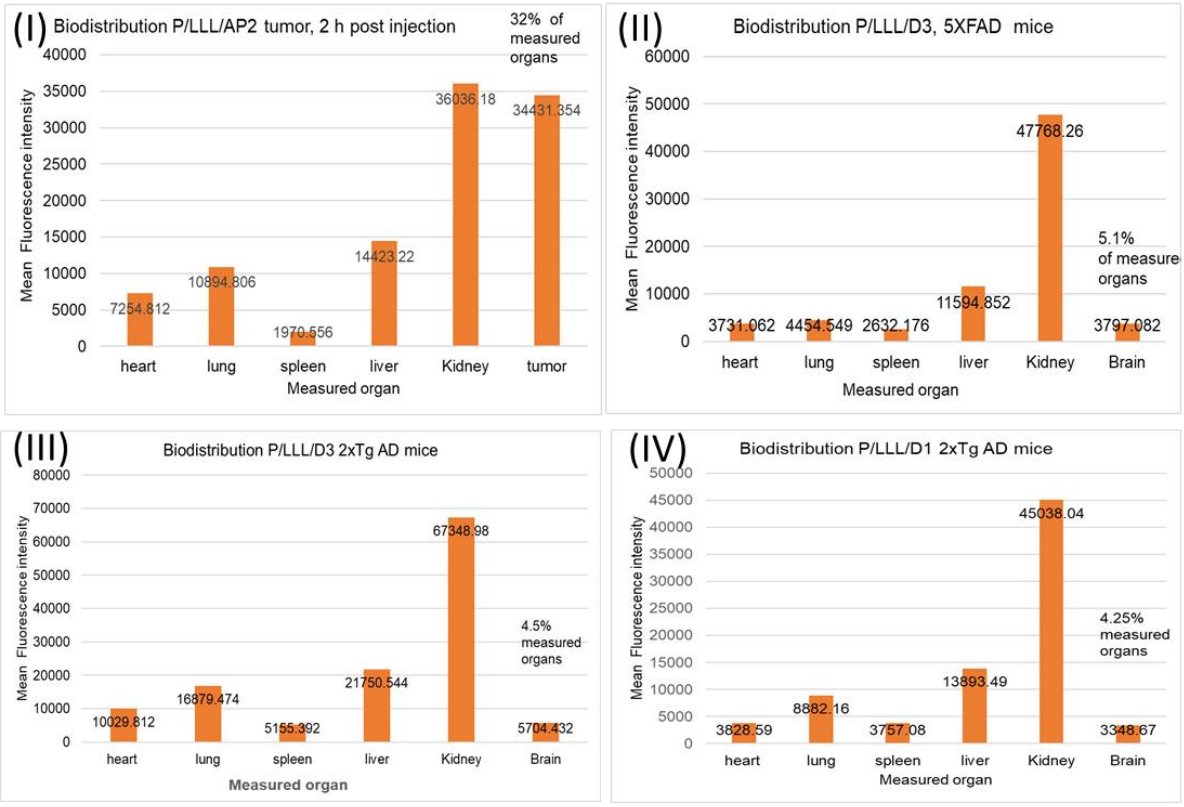


Figure S7. Biodistribution of nanoconjugates in tumor and AD mice models shows increased uptake in the tumor. A) Biodistribution of nanoconjugates 120 min after tail vein injection of P/LLL(40%)/vector/rhodamine in mice. Fluorescence intensity of whole organs brain, lungs, kidneys, liver, spleen, and heart P/LLL/AP2 in tumor mice (left) and P/LLL/D1 in 2XTg-AD mice (right). B) Fluorescence intensity of extracts for P/LLL/AP2 in tumor mouse (I), P/LLL/D3 in 5XFAD mouse (II) and 2XTg-AD mouse (III) and P/LLL/D1 in 2XTg-AD mouse (IV). (IVIS Lumina XR Optical Imaging System (PerkinElmer, Richmond, CA, USA) applying DsRed filters sets.)

Table S1. Nomenclature, structure, and analytical properties of nanoconjugates. [a]AP2 (TFFYGGSRGKRNNFKTEEY), [b]D1 (qshyrhispaqv), [c]D3 (rprtrlhthrnr), [d]ACI89 (pshyrhispaqk), [e]B6 (CGHKAKGPRK) and [f]M4 (H-[Dap]KAPETALD-NH_2) lactam-bridged exocycle. [g]Rhodamine. [h]rt = Retention time (SEC-HPLC). [i]Note, that percent (%) loading and "/rh" indicated under "structure" is omitted for simplicity throughout the publication and is only repeated when under special consideration.

Nano conjugates	Structure	Calculated MW [g/mol]	Zeta-Potential [mV]	SEC HPLC rt[h] [min]
P/LLL/AP2[a]	PMLA/LLL(40%)/AP2(2%)/rh(1%)	164,000	-11.6	7.22
P/LLL/AP2-1	PMLA/LLL(40%)/AP2(1%)/rh(1%)	139,000	-2.5	7.52
P/LLL/AP2/B6	PMLA/LLL(40%)/AP2(1%)/B6(1%)/rh(1%)	159,000	-6.2	7.36
P/LLL/D1[b]	PMLA/LLL(40%)/D1(2%)/rh(1%)	154,000	-3.1	7.20
P/LLL/D3[c]	PMLA/LLL(40%)/D3(2%)/rh(1%)	155,000	-7.3	7.20
P/LLL/ACI89[d]	PMLA/LLL(40%)/ACI89(2%)/rh(1%)	154,000	-13.8	7.15

P/LLL/B6[e]	PMLA/LLL(40%)/B6(2%)/rh(1%)	153,000	-6.1	7.50
P/LLL/B6-1	PMLA/LLL(40%)/B6(1%)/rh(1%)	160,000	-10.8	7.52
P/LLL/M4[f]	PMLA/LLL(40%)/M4(2%)/rh(1%)	138,000	-10.4	7.20

Table S2. Definition of doses used for *in Vivo* intravenous injections. In the molar concentration, μmol/Kg refers to μmol as the molar quantity of the entire nanoconjugate, Kg = kilograms to mouse mass. Average mouse mass was 0.020 Kg at the time of injection.

Dose [X]	**μmol/Kg mouse**
0.125X	0.034
0.25X	0.068
0.5X	0. 137
0.625X	0.17
1X	0.274
1.5X	0.408
2X	0.548

Table S3. Fluorescence intensities measured by optical imaging of tumor-bearing brain and brain tissue in the contralateral hemisphere of BL/6-mice after intravenous injection of nanoconjugates at doses 0.25X and 1X. Brain sections were not fixed, and 2 sec

exposure was used for fluorescence measurements. Fluorescence background measured in mice injected with phosphate buffered saline (PBS) only is subtracted.

		Corrected fluorescence intensity ±SEM		
Nanoconjugate	**Dose**	**Tumor tissue**	**Brain tissue from contralateral hemisphere**	**Ratio of fluorescence in tumor/non-tumor brain tissue (T/NB)**
P/LLL(40%)	0.25X	10.0±0.301	1.0±0.059	9.7
	1X	42.7±1.230	5.1±0.165	8.3
P/LLL/AP2	0.25X	13.5±0.481	2.1±0.068	6.5
	1X	148.2±2.722	9.4 ±0.433	15.7
P/LLL/B6	0.25X	3.0±0.099	1.2±0.082	2.5
	1X	69.2±2.050	6.8±0.197	10.2
P/LLL/D1	1X	86.6±1.883	5.3±0.176	16.4
P/LLL/ACI89	1X	37.1±0.714	4.3±0.108	8.6
P/LLL/D3	1X	92.6±1.803	7.8±0.266	11.9
P/LLL/M4	1X	86.2±1.665	3.8±0.132	22.8

Table S4: Summary of hierarchy in the cortex of normal brain (BL/6 and Balb/c mice, Balb/C were measured previously),[1] glioblastoma-inoculated BL/6 mouse and AD-model 5XFAD mouse. The experimental protocol for fluorescence microscopic assay was identical for all systems.

Mouse model - dose	**Hierarchy of BBB crossing**
Normal - Balb/c - 1X [11]	AP2>B6>M4
Normal - BL/6 - 1X	D1>D3≈ACI89>AP2
Normal - BL/6 - 2X	AP2>D3>B6
Tumor - BL/6-tumor – 1X	AP2>D3>D1≈M4>B6>P/LLL(40%)>ACI89
AD* - ADtg 5XFAD - 2X	D3≈D1≈AP2>P/LLL(40%)≈B6≈M4

Table S5: Fluorescence intensities in the contralateral hemisphere of tumor-bearing BL/6-mice compared to the intensities in the cortex of BL/6 mice **in fixed brain sections, after subtraction of PBS background fluorescence**. * AD: D3 and D1 nanoconjugates were also measured in 2XTg mouse model.

		Fluorescence intensity	
Conjugate	**Dose**	**Tumor mice Contralateral brain tissue ±SEM**	**BL/6 Normal mice brain ±SEM**

P/LLL/AP2	1X	7.78±0.35	9.23±0.19
P/LLL/D3	1X	6.96±0.22	13.29±0.61
P/LLL/M4	1X	5.73±0.13	8.6±0.23
P/LLL/D1	1X	6.03±0.17	14.5±0.66
P/LLL/B6	1X	5.01±0.16	9.65±0.62

Methods:

Characterization of nanoconjugates [1]:

SEC-HPLC analysis: The analysis was performed using a Hitachi L-2130 pump with a Hitachi L-2455 detector with EZChrome Software. The SEC-HPLC column was Polysep 4000, at 1ml/min flow rate, PBS 1X (pH 7.4).

ζ Potential. ζ potential of nanoconjugates was measured using a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK). Ten µL aliquots of nanoconjugate samples were diluted in 0.99 mL PBS, and the voltage applied was 150 mV. Data represent the mean of three measurements.

Rhodamine (rh) quantification of the final nanoconjugates: *Prior* to lyophilization, 10 µL sample of rhodamine labeled nanoconjugate was diluted with 990 µL PBS pH 7.4. Absorbance was scanned at wavelength 570 nm (Flexstation, Molecular Devices, Sunnyvale, CA, USA). The dye concentration was calculated from A570 measurements using the molar absorbance coefficient 119000 $M^{-1}cm^{-1}$. In addition, fluorescence scans

(excitation 570 nm / emission 600 nm, cutoff 590 nm) confirmed the presence of rhodamine in the samples.

Transwell-based *in Vitro* model of the BBB

Immortalized human cerebral microvascular endothelial cells (hCMEC/D3) were used in Transwell plates to establish an *in Vitro* model of the BBB, as previously described [2]. Cells were seeded between passages 25 and 35 on the apical side of 12-well polystyrene Transwell plates (0.4 µm sized pores, 1.12 cm^2 inserts) and grown in differentiation medium (Endothelial cell growth basal medium (EBM-2) supplemented with 5% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1.4 µM hydrocortisone, 5 µg/ml ascorbic acid, 1% lipid concentrate, 10 mM HEPES buffer and 1 ng/mL basic fibroblast growth factor (bFGF)) for 3 days (500 µL in apical compartment, 1 mL in basolateral compartment) at 37 ^oC with saturated humidity and 5% CO_2. After 3 days, medium was replaced with growth medium (EBM-2 supplemented with 5% FBS, 1% penicillin/streptomycin, 1.4 µM hydrocortisone, 5 µg/mL ascorbic acid, 1% lipid concentrate, 10 mM HEPES buffer and 10 mM LiCl) for 3-4 days, and medium was changed every 2-3 days. Trans endothelial electrical resistance (TEER) was measured every day to monitor tight junction formation using an epithelial volt ohm meter (EVOM2) (World Precision Instruments, FL, USA).

Cell viability assay (MTS): [3]

Cell proliferation assay kit (Abcam) was used to measure cell viability following incubation with each compound. hCMEC/D3 cells were seeded on clear 96-well plates (10,000 cells/well) and grown for 2-3 days until they reached confluence. Cells were incubated with compounds at concentrations equivalent to 0.25X, 0.5X or 1X (26.6, 53.2 and 106.4

μg/mL rhodamine dye) suspended in 100 μL differentiation media per well for 3 h at 37 ^{o}C. Compounds were then removed, and cells were washed twice with 50 μL phosphate buffered saline (PBS). Fresh differentiation medium (100 μL) was added to each well and supplemented with 10 μL (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) solution. Cells were incubated for 2 h at 37 ^{o}C. Plate was briefly shaken, and absorbance of each sample was read using a plate reader (specified above for Rh quantification) at 490 nm wavelength. Cell viability was calculated as a percentage of the viability of cells incubated in differentiation medium alone.

In Vitro BBB-*permeability of nanoconjugates.* Following 6-7 days of cell growth on Transwell inserts, cell medium was removed from each well. Growth medium without FBS was added to the basolateral compartment (1 mL) to replicate the protein-deficient cerebrospinal fluid (CSF). Rhodamine-labeled compounds (P/LLL(40%), P/LLL/D3, P/LLL/AP2, P/LLL/M4, P/LLL/D1) were added to the apical compartment at a concentration equivalent to 0.25X (26.6 μg/mL dye quantified on nanoconjugate using UV) in growth medium (500 μL). TEER was measured. Cells were incubated with compounds for 3 h at 37^{o}C and 20 μL samples were removed from the basolateral compartment every 30 min and replaced with 20 μL differentiation media without FBS. After 3 h, TEER was measured again, all media was removed, and cells were washed with warm DPBS with $CaCl_2$ and $MgCl_2$. Fluorescence was measured in white, flat bottomed 96-well plates (Costar Location *etc*) using a SpectraMax M2 fluorescence plate reader (Molecular Devices, CA, USA), with ex570/em600. Endothelial permeability was calculated as previously described [4].

Animal procedures

In Vivo *protocols using normal (healthy) mice*

Eight to 14-week-old C57BL/6J (BL/6) mice were obtained from Charles River Laboratories (Wilmington, MA, USA) and used for permeation experiments representing the healthy brain. Mouse maintenance and experimental procedures followed the guidelines established by the Cedars Sinai Institutional Animal Care and Use Committee (IACUC Protocol #7416). Three to four mice of each sex were used for each experiment. Nanoconjugates were dissolved in sterile PBS (pH 7.4) prior to each experiment and injected intravenously (IV) into the lateral tail vein. Mice were anesthetized using isoflurane procedure, and their tails were briefly warmed in water at 40-42^{o}C to dilate veins and facilitate injections. All conjugates were administered as a single dose, at final concentrations ranging from 0.274 (4X) to 0.548 (8X) μmol of total nanoconjugate per kg body weight, or as indicated for each experiment. The drug injection volume was kept constant at 150 μL. For normal and tumor bearing mice experiments, 15 minutes before euthanasia, mice were injected with 100 μL tomato lectin (DyLight 488 Lycopersicon Esculentum (Tomato) Lectin, catalog # DL-1174 Vector laboratories, 1mg/ml) to stain blood vessels [1].

After euthanasia, the brains were harvested and frozen in optimal cutting temperature compound (OCT). Brain sections (14 μm) were fixed with 1% paraformaldehyde for 5min and then rinsed with PBS. Nuclei were stained with DAPI for the optical analysis. Optical imaging and data analysis are described below [1].

AD-Mouse Model

Three mouse models of AD were used in this study. Firstly, for imaging studies, the double transgenic (2XTg-AD) mice [B6.Cg-Tg (APP_{SWE}/$PS1_{\Delta E9}$)85Dbo/Mmjax]; MMRRC stock# 34832-JAX|APP/PS1) were obtained from Koronyo-Hamaoui laboratory (Department of Neurosurgery, Cedars-Sinai Medical Center, Los Angeles, USA). The 5XFAD transgenic mice [B6.Cg-Tg(APPSwFlLon,PSEN1*M146L*L286V)6799Vas /Mmjax]; MMRRC stock# 34848-JAX|5xFAD) and the model used for RNA-*Seq* analysis, 3XTg, [B6;129-Tg(APPSwe,tauP301L)1Lfa Psen1tm1Mpm/Mmjax] were obtained from Jackson Laboratories. All strains are on a C57BL/6J congenic background. Three to four mice (mixed gender, 6-8 month) were used for each imaging experiment. IV nanoconjugate and tomato lectin administration is as described above for normal BL/6 mice. Mouse maintenance and experimental procedures followed the guidelines established by the Cedars Sinai Institutional Animal Care and Use Committee (IACUC Protocols #7416 and #8475).

P/LLL/D3/rh nanoconjugate was injected to 2XTg-ADmice at the doses of 1X (0.274 µmol/Kg), 1.5X (0.411 µmol/Kg) and 2X (0.55 µmol/Kg), as well as P/LLL/D1 at a dose of 2X (0.55 µmol/Kg).

P/LLL/D1, P/LLL/D3, P/LLL/B6, P/LLL/ACI89, P/LLL/M4 and P/LLL/AP2 were also injected at a dose of 2X (0.55 µmol/Kg), to 5XFAD mice.

The brains were harvested and a 14 µm thick slices which were fixed and stained with DAPI to envision the nuclei. Optical imaging and data analysis were done by the same procedure described below.

Brain tumor mouse models of intracranial glioblastoma [5]

Mouse glioblastoma cell line GL261 was a gift from Dr. Badies lab (UC San Diego, San Diego, CA) and was cultured in Dulbecco's modified Eagle medium (DMEM) (ATCC, Manassas, VA) containing 10% FBS with 1% penicillin (100 u/mL), streptomycin (100 µg/mL), and amphotericin B (0.25 µg/mL) at 37°C with 5% CO_2. All animal experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Cedars-Sinai Medical Center (Los Angeles, CA, USA).

Twenty thousand GL261 cells in a volume of 2 µL, were implanted intracranially into the right basal ganglia in 45 females 8-week-old C57BL/6J mice (from Jackson Laboratory, Sacramento, CA). 3 mice per nanoconjugate treatment group were randomized into 15 groups and were injected once intravenously in 21 days after cell implantation, with either PBS, PMLA/LLL/Rh (1%), PMLA/LLL/Rh (1%), PMLA/AP2 (2%)/ LLL/Rh(1%), PMLA/ LLL/AP2 (2%)/Rh (1%), PMLA/ AP2 (1%)/Rh (1%), PMLA/ LLL/AP2 (1%)/B6 (1%)Rh (1%), PMLA/ /B6 (1%)/Rh (1%), PMLA/LLL /B6 (2%)/Rh (1%), PMLA/LLL /B6 (2%)/Rh (1%), PMLA/LLL/D1(2%)/Rh (1%), PMLA/LLL/ACI89(2%)/Rh (1%), PMLA/LLL/D3 (2%)/Rh (1%) or PMLA/LLL/Miniap4 (2%)/Rh (1%). The nanoconjugates were injected at a dose of 0.067 µmol/Kg-0.274 µmol/Kg.

Animal drug injections – tumor mouse model

Nanoconjugates were IV tail-injected into 11-week-old C57BL/6J (BL/6)-mice with intracranial glioblastoma, cell line GL261, as described above in normal mice and ADtg-mice. After injection, mice were promptly returned to their cages. At times 15 min before euthanasia, mice were injected with a mix of 75 µL Tomato Lectin (DyLight 488 Lycopersicon Esculentum (Tomato) Lectin, catalog # DL-1174 Vector laboratories, 1mg/ml) and 50µl Ricin Lectin (Fluorescein Ricinus Communis Agglutinin I (RCA 120),

catalog # FL-1081 Vector laboratories, 5mg/mL) to label tumor and brain vessels. Mice were anesthetized and euthanized by cervical dislocation followed by decapitation at 2 h after the injection of nanoconjugates. Brains were collected, saved in OCT and used for optical analysis.

Image acquisition and optical analysis – glioblastoma mouse model

To identify drug distribution in the vessels of tumor tissue and in extravascular tumor vessels compared to normal brain, the extent of penetration of nanoconjugates through BBB was measured under condition of frozen tissue (no fixation) in 14 μm thick sections which were prepared with a Leica CM3050 S cryostat (Leica Biosystems, Inc., Buffalo Grove, IL, USA). The prepared frozen sections were air-dried at room temperature, then mounted with ProLongGold Antifade (Thermo Fisher Scientific) mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) to counterstain cell nuclei. Images were captured using a Leica DM6000 B microscope (Leica Microsystems, Inc., Buffalo Grove, IL, USA). For both tumor and normal brain in the non-tumor hemisphere, 5 images were quantified per mouse using 20 ROI each 10 x 10 μm^2. Since tumors were inoculated in the cortex only, the contra-lateral hemisphere measurement was taken only from the cortex of that hemisphere. Tumors and anatomy could be identified without difficulties under the microscope DAPI channel. Rhodamine-labeled nanoconjugates were visualized with a 534-558 nm wavelength excitation and 560-640 nm wavelength emission filter set, viewed with a 20X Leica HC Plan Apo 0.70 N.A. and a 40X Leica HCX Plan Apo 0.85 N.A. lens, and recorded with a Leica DFC 360 FX camera. The camera was controlled with Leica LAS X software. Images were acquired with 2.0 sec + 1.0 gain

exposures with the 40X lens. These parameters were held constant to enable image-to-image comparisons across specimens. To compare between normal and ADtg mice an additional set of sections were prepared after tissue fixation and imaging in the same way described above for ADtg and normal mice.

Factorial Analysis [6]

The Factorial Analysis (DoE study) is a statistically designed set of experiments which allows a better understanding of the studied system as well as optimization of that system using a minimal number of *in Vivo* experiments. For software design and analysis see Minitab 17 (Minitab, LLC, State College, Pennsylvania, USA). In the present investigation, input "DoE -factors" were **factor A**, the percent loading of vectors (shuttle peptides) attached to P/LLL(40%), and **Factor B** indicates the doses of the injected nanoconjugate. **Combination AB** was defined as the interaction of the two factors when co-ligated on the delivery platform as described in Table 2 (main text). For both factors, minimum and maximum values were set. For vector loading (factor A), the minimum value was 0% and the maximum value 2% (% refers to the fraction of the total malic acid content). The maximum value of 2% was chosen after a crowding effect in normal mice was observed when AP2 loading was increased from 2% to 4% [7]. For the dose (factor B) the lowest value was set 0.25X while the highest was set 1X. "Center points" nanoconjugates had a loading of 1% and the injected dose 0.625X. The factors A and B can be coupled by the co-ligation AB with the delivery platform. The extent of this interaction can be measured in the DoE-study in terms of an **"**interaction plot", which graphically displays the coupling [12]. In addition to the definition of the input factors, the output of the study is the

“response**”** which needs to be defined. We defined two main responses, the tumor uptake of the nanoconjugates in relative fluorescence units corrected by the fluorescence intensity of the PBS control, and the specificity of the nanoconjugate uptake by the tumor (“tumor or T”) in terms of the ratio of fluorescence intensity uptake in the tumor divided by the fluorescence intensity uptake in the contralateral hemisphere (“normal brain or NB”, ratio: “T/NB**”**). The “brain” fluorescence was measured in the contralateral hemisphere of the tumor-bearing mice but in the same location in the cortex.

Pareto charts (**Fig. S6B**) present the calculated statistical significance of the chosen response using bar graphs. The calculation includes both factors tested, and the interaction (or coupling) between the two. The red line in the graph, indicates the calculated standardized effect needed for a factor to reach statistical significance for a given output or response. The standardized effects are t-statistics that test the null hypothesis that the effect is 0. An example for the selectivity (T/NB) is shown in **Fig. S6B** for both combined AP2/B6 and AP2 only matrixes. A factor is significant if the bar crosses the red line. For the AP2-matrix, both percent of AP2 peptide loading on the polymer and the injected dose are not significant factors (**Fig. S6B**). When B6-peptide is added to the system (**combined-matrix, P/LLL/AP2/B6**), the vector peptide loading percentage is still not a significant factor, but the dose for this nanoconjugate is significant (crossing the red line). These observations bear on the reliability of observations made earlier on the basis of dose variations in the contour plots. When a B6 matrix was tested (P/LLL/B6, data not shown), similarly to the AP2 matrix, the injected dose was not significant for the T/B response. Overall, it means that when a single peptide is attached to the nanoconjugate (either AP2 to target LRP1 (or LAT1, and B6 to target TfR) the dose is not a significant

factor for tumor selectivity. However, when the system is combined, and the nanoconjugate can target both LRP1 (or LAT1) and TfR, then the dose becomes significant for the achievement of higher selectivity.

Supporting evidence for coupled transcytosis of the AP2- and B6- vectors when loaded together is obtained by the impact of different doses 0.25X and 1X in plot **Fig. S6C** for achieving selectivity towards the **tumor (T/NB**). The computed slopes of the lines in both graphs according to DoE software have opposite signs which indicates coupling between the “percent of AP2 peptide loading” and the “dose” in generating the T/NB response. There would be no coupling if the lines run parallel or overlapping. A coupling is supported by the allosteric interactions between vectors and P/LLL(40%) binding to proposed different subsites at the two-site receptor models gating access to the transcytosis pathways. And the data here confirm a coupling between the LRP1- and TfR-transcytosis pathways via the co-ligation of AP2 and B6 on the same platform molecule. DoE-analysis was chosen as the method to investigate tumor selectivity as a function of the nanoconjugate ligand-loading and the injected dose.

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