Regenerative Engineering and Translational Medicine

Altered Biodistribution and Tissue Retention of Nanoparticles Targeted with P-glycoprotein Substrates

--Manuscript Draft--

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Abstract:	Low molecular weight substrates of the efflux transporter, P-glycoprotein, alter the biodistribution and tissue retention of nanoparticles following intravenous administration. Of particular interest is the retention of the targeted nanoparticles in the brain. Drug delivery to the brain is hindered by the restricted transport of drugs through the blood-brain barrier (BBB). Drugs that passively diffuse across the BBB also have large volumes of distribution; therefore, alteration of their biodistribution to increase their concentration in the brain may help to enhance efficacy and reduce off-target side effects. In this work, targeted nanoparticles were used to explore a new approach to target drugs to the brain--the exploitation of the P-glycoprotein efflux pump. The retention of nanoparticles containing a strong P-glycoprotein substrate, rhodamine 6G, tethered to a PLA nanoparticle through a PEG spacer was greater than two-fold relative to untargeted nanoparticles and to nanoparticles tethered to a weaker P-glycoprotein substrate, rhodamine 123. In a P-glycoprotein knockout mouse model (mdr1a (-/-)), there were no significant differences in brain accumulation between rhodamine 6G targeted particles and controls, strongly supporting the role of P-glycoprotein. This proof of concept report shows the potential applicability of low molecular weight P-gp substrates to alter nanoparticle biodistribution.
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Response to Reviewers:	Reviewer #1: A majority of candidates developed for CNS diseases never make it to the clinic. The failure is primarily attributed to the drugs' inability to cross the blood-brain barrier (BBB) at levels sufficient for a therapeutic effect. The tight junctions that connect the endothelial cells and the involvement of specific transporters (such as the P-glycoprotein, P-gp) dynamically regulate the movement of molecules across the BBB into the CNS. The P-gp is is responsible for actively moving drugs out of the brain. Over the years, drug delivery scientists have actively worked on manipulating the P-gp to enhance drug-retention within the brain, although to limited success. Here, the

	authors propose a new strategy to improve the local retention of nanoparticles at the BBB, in contrast to crossing it. This could have significant implications in the clinic, especially in terms of achieving efficacy. The manuscript is well written and the experiments are well designed. Comments: 1)Include more details on the clinical/biological role of P-gp substrates in the introduction section. We agree that it is important for the reader to understand the role of P-gp. We have clarified the biological/clinical role of P-gp in the Introduction, as requested, by including the following, “The biological role of P-gp is to protect tissues from the accumulation of toxins. Clinically, P-gp can serve to prevent drug accumulation in a desired anatomical site, or as a mechanism of drug resistance.” 2)The TEM images aren't clear. Please include images with a better contrast. We have increased the contrast for the TEM images in Figure 2. 3)Please include details on long-term stability (at least 2-3 weeks) analysis of Rho6G-PEG-PLA NPs. While we have not conducted official pharmaceutical stability studies on the nanoparticles, in our hands the lyophilized particles were re-suspended easily many weeks after formulation. The following text has been added to the formulation section in the materials and methods, “Nanoparticles were re-suspended in this fashion without trouble for at least four weeks after fabrication.” 4)If possible, please include images of the whole mouse brain (wide field of view) to show NP accumulation at the site. Unfortunately, we do not have these images available. The point is a good one and is well-taken, but the expertise to capture these images no longer exists in our lab. 5)This is a proof-of-concept study and demonstrating therapeutic efficacy is beyond the scope of this manuscript. However, addressing key issues pertaining to CNS drug delivery is necessary to justify this approach scientifically and generate excitement. (a)P-gp expression has been shown to be expressed at higher levels in glioma compared to neurodegenerative disorders. This approach could enhance NP specificity to the BBB in general. Therefore, please include more details on how P-gp substrate mediated drug delivery could be of clinical value. The reviewer is correct that this approach will not be amenable to all diseases that manifest in the CNS. We have included the following paragraph in the discussion section to make this clearer to the reader, “The incidence of neurologically-based diseases is continually growing. According to the 2013 Center for Disease Control report, Mortality Multiple Cause Micro-data Files, Alzheimer’s disease and stroke are among the top 10 leading causes of death in the United States. The American Cancer Society reports brain cancer having only a 35% five-year survival rate. As of June 2009 only 8.2% of drugs developed for activity in the central nervous system have been approved for clinical use, making it the lowest percentage of all therapeutic classes. However, therapies for CNS diseases are among the top targets for drug development. The translation from drug discovery to development and clinical success is greatly dampened by delivery challenges to the CNS.” (b)However, reaching the BBB is only the first step in CNS delivery. P-gp is primarily expressed at the luminal surface of brain endothelial cells - implying its closer to the blood and away from the brain tissue. Tethering NPs to the P-gp necessarily don't imply that this will improve drug concentrations at the disease site. The drugs have to be released first and then traverse the BBB to reach the tissue. Since, there is no concrete evidence to justify this approach therapeutically, please list its possible limitations among others. We do realize and appreciate the limitations that are associated with this approach to

drug delivery to the brain. We also appreciate that the design we report is unlikely to be the most applicable delivery system. We used these architectures to demonstrate the potential opportunity available to the drug delivery community to use P-gp and its substrates as part of the targeted drug delivery arsenal. We included this text in the manuscript to focus the potential therapeutic opportunities for the field, “From a practical perspective, such a targeted delivery system could be used to deliver low molecular weight therapeutics designed to passively transport down a concentration gradient into the brain for treatment of CNS-based pathologies. Traditional low molecular weight therapeutics for the CNS are designed with octanol/water partition coefficients that permit sufficient solubility in both aqueous and non-aqueous solvents and can thereby passively diffuse across the BBB. One shortcoming of this small molecule design paradigm is that the compounds can passively diffuse across all cell membranes, leading to high volumes of distribution and adverse off-target effects. Biodistribution alteration of these compounds to increase their local concentration in the brain over time could serve to enhance the efficacy of some compounds, or perhaps rescue some therapeutics that have failed in the clinic due to poor side effect profiles.”

(c)In addition, please include details on the molecular basis of BBB and how transporters including P-gp could increase or decrease solute permeability. This could serve as a template for future engineering based upon P-gp substrate mediated drug delivery.
We agree that the effects of transporters including P-gp can influence solute permeability across the BBB. In addition to the paragraph in the Discussion about the work with transferrin and insulin transporters, and the work focusing on GPCR-targeting, we have included the following text to emphasize these points specific to P-gp, “Of course, this approach will not work for therapeutic compounds that are themselves P-gp substrates, making the judicious pairing of the disease and the desired therapeutic imperative for therapeutic success.”

(d)The authors highlight how sufficient levels of NPs are retained for 3h. However, the graphs also depict that nanoaprticles are eliminated from the BBB following 6h. This implies that therapeutic levels of drugs must be achieved within a time frame of 6h. Please address this as a limitation or explain briefly citing examples on how systems may be engineered to fine-tune drug release.
The reviewers are correct that the delivery system (targeted nanoparticles) may or may not be the best selection for the treatment of some CNS-based diseases. To this end, we have specifically stated in the manuscript that the purpose of the work was to demonstrate the potential opportunities for members of the drug delivery field to further explore and create new systems that build on these results to further the field.

Reviewer #2: Review Summary

Overall, this proof of concept research paper covers an important area of interest to the biomedical research community. In specific, the delivery of drug carrier devices across the blood brain barrier can be enhanced through their decoration with a P-glycoprotein substrate. The authors do a very good job of providing data that supports their targeted objective while also demonstrating the specificity of their approach. The paper is very well written with only minor errors and concerns detailed. The paper will be ready for publication after addressing these concerns and completing a final grammatical edit.

Comments

1)Page 4, Lay Summary - The word "target" or "targeted" is used a total of five times in this five sentence paragraph. Please choose some synonyms for some of these references to vary word choice.
Thank you. We have revised the Lay Summary accordingly, “The efficacy of medicines can be improved by diverting drugs to specific tissues. Finding new ways to target medicines to diseased tissue is an active area of research across disciplines. Drug-loaded nanoparticles, delivered to tissues of interest, are one way to accomplish this goal. The work reported in this manuscript explores the possibility of using small molecules to get nanoparticles to bind to a drug efflux pump, P-glycoprotein (P-gp) that is present in various tissues in the body. P-gp functions to remove drugs from tissues,

and it is usually considered a hindrance to drug targeting. The research in this paper shows that natural function of P-gp can be used favorable to retain nanoparticles in various tissues."

2)Page 10, Line 48 - Page 11, Line 16 - It is unclear to the reader why Europium needs to be added in addition to the P-glycoprotein target as an imaging agent since Rhodamine 6G is already fluorescent. This also brings up a large question regarding what non-fluorescent P-glycoprotein targets may be available to facilitate similar brain drug delivery without providing fluorescence which may be not preferred for some applications.
Thank you for requesting this clarification. We have modified the text in the Results "In vivo biodistribution" section to make the use of Europium more clear, "Time-resolved fluorescence of europium encapsulated within the nanoparticles was used to track their biodistribution following intravenous administration. Although the fluorescence of the rhodamine conjugates could be used to quantify nanoparticle concentration in various tissues, the tissues themselves autofluoresce at similar wavelengths and on the same time scale, leading to a high background signal. However, elements in the lanthanide III series, like europium, exhibit large Stokes shifts and long fluorescent decay lifetimes and are amenable to time resolved fluorescence wherein their fluorescent signal remains detectable even after tissue autofluorescence and rhodamine fluorescence are completed, resulting in a very low background signal and high signal to noise ratio."

3)It may not be readily apparent to the reader, especially if they are new to the drug delivery field, why the PEG shell is necessary for PLA particles as well as why a PLA-only particle with not PEG was not tested as a control. A sentence or two explaining the rationale behind this would be helpful.
We agree. The following text was added in the discussion section to make this clear, "The function of PEG in this study was three-fold, first to serve as a tether for the rhodamine P-gp substrates, second to prevent non-specific aggregation of the nanoparticles, and third to prevent non-specific sequestration of the nanoparticles by the liver and spleen."

4)Page 16, Lines 36 - 56 - The rationale for retention of nanoparticles at the blood brain barrier instead of transport across the membrane is helpful for the reader to know in the introduction as well as being reiterated here. With this in mind, additional content should be added to the introduction.
We agree and have included the following text in the Introduction, "The work presented herein establishes that P-gp substrates (i.e., small molecules that are actively transported by P-gp out of the CNS) can enhance the retention of nanoparticles in the brain. The premise is that a P-gp substrate tethered to the surface of a nanoparticle through a water soluble polymer linker binds to the P-gp expressed in the endothelium, leading to nanoparticle retention in the capillary lumen. For this work, the nanoparticles were not designed to be transported across the blood-brain barrier. Instead, they were designed to be captured by P-gp at the capillary endothelium and retained within the bloodstream. In this way, drugs could be release within the capillaries and enter the brain by passive diffusion down the concentration gradient."

5)Page 21, Figure 1 - The cartoon shows a singular Rhodamine 6G attached to the PEG-PLA particle surface. As there are expected to be many molecules, it would be more accurate to show a PEG corona with many substrate molecules.
The reviewer is correct that the particles are multivalent; however, we have drawn the figure both ways (single rhodamine and multiple rhodamines) and found the multiple rhodamine figure to be more confusing than clarifying. However, we have modified the legend of Figure 1 to account for the multivalent nature of the particle. The legend now reads, "Figure 1 The binding site for rhodamine on P-gp is on the inner intracellular leaflet of the transmembrane protein [37]. While counter intuitive (and energetically unfavorable) the tissue retention of the targeted nanoparticles suggests that the tethered substrate might transverse the cell membrane to bind to its receptor site. A possible mechanism, which requires additional investigation, is shown here where the PEG linker/P-gp substrate on a drug carrier (A) spans the cell membrane (B) allowing the P-gp substrate to bind to P-gp (C) and retain the drug carrier in the capillary. Please note that only one P-gp substrate is drawn on the nanoparticle to make the potential mechanism visually clear, whereas in practice there are multiple P-gp substrates in each nanoparticle."

	6)Page 28, Table 2 - While the increased accumulation at the brain is > 2x, the enhanced delivery to other tissues (i.e. ~ 4.5 x for liver and kidney ~ 3x for heart, and ~ 2.5x for lung) is greater. The concerns and/or opportunities with the build-up of nanoparticles in these other tissues should be more clearly discussed in the text. Also, opportunities to modify the targeting molecule to preferentially target brain P-glycoprotein should be mentioned. This is correct, and we have included the following text in the same section that speaks to the values in Table 2, “It is also important to acknowledge that the AUC of the targeted nanoparticles is also enhanced in other organs (i.e., liver, spleen, kidney, heart, lung) and that this accumulation should be taken into consideration when exploring this targeting approach with specific drugs and disease states.”

Altered Biodistribution and Tissue Retention of Nanoparticles Targeted with P-glycoprotein Substrates

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Abstract

Low molecular weight substrates of the efflux transporter, P-glycoprotein, alter the biodistribution and tissue retention of nanoparticles following intravenous administration. Of particular interest is the retention of the targeted nanoparticles in the brain. Drug delivery to the brain is hindered by the restricted transport of drugs through the blood-brain barrier (BBB). Drugs that passively diffuse across the BBB also have large volumes of distribution; therefore, alteration of their biodistribution to increase their concentration in the brain may help to enhance efficacy and reduce off-target side effects. In this work, targeted nanoparticles were used to explore a new approach to target drugs to the brain--the exploitation of the P-glycoprotein efflux pump. The retention of nanoparticles containing a strong P-glycoprotein substrate, rhodamine 6G, tethered to a PLA nanoparticle through a PEG spacer was greater than two-fold relative to untargeted nanoparticles and to nanoparticles tethered to a weaker P-glycoprotein substrate, rhodamine 123. In a P-glycoprotein knockout mouse model (mdr1a (-/-)), there were no significant differences in brain accumulation between rhodamine 6G targeted particles and controls, strongly supporting the role of P-glycoprotein. This proof of concept report shows the potential applicability of low molecular weight P-gp substrates to alter nanoparticle biodistribution.

Lay summary

The efficacy of medicines can be improved by diverting drugs to specific tissues. Finding new ways to target medicines to diseased tissue is an active area of research across disciplines. Drug-loaded nanoparticles, delivered to tissues of interest, are one way to accomplish this goal. The work reported in this manuscript explores the possibility of using small molecules to get nanoparticles to bind to a drug efflux pump, P-glycoprotein (P-gp) that is present in various tissues in the body. P-gp functions to *remove* drugs from tissues, and it is usually considered a hindrance to drug targeting. The research in this paper shows that natural function of P-gp can be used favorable to *retain* nanoparticles in various tissues.

Future work

The data reported in this manuscript serves to establish a proof-of-concept that low molecular weight P-gp substrates and be used to alter the biodistribution of nanoparticles. Future work includes: 1) understanding the targeting mechanism(s) that lead to these results; 2) identifying FDA-approved drugs that can target nanoparticles; and 3) evaluating how nanoparticle biodistribution is altered by using P-gp substrates with different binding constants.

Introduction

The alteration of nanoparticle biodistribution to enhance their tissue-specific retention is an active area of research. Diseases of the central nervous system (CNS) present consistent challenges to the field of drug development and delivery. Drug development for pathologies of the CNS has among the lowest clinical approval rates [1], partially stemming from challenges in the delivery of therapeutics across the blood-brain barrier (BBB). The BBB serves a natural protective function with tight endothelial junctions and active efflux mechanisms that limit transport of potential therapeutics into the brain, making drug delivery problematic [2].

To enhance the concentration of low molecular weight drugs in the brain, medicinal chemists alter the partition coefficient to maximize their passive diffusion through cell membranes, like the BBB. However, the drawback of this approach is that the drugs also diffuse promiscuously across all cell membranes, leading to large volumes of distribution, the need for high doses, and the induction of off-target side effects. A host of strategies to enhance drug delivery to the CNS have been proposed and include focused ultrasound [3], intranasal delivery [4], and receptor mediated (RM) targeting [5]. RM drug targeting has been explored as a method to enhance the concentration of small molecules, antibodies, peptides, proteins, and other therapeutics in the CNS with varying degrees of success and challenges [6,7]. For example, both transferrin [8-11]and insulin [12]have been investigated as CNS targeting agents owing to their ability to initiate receptor mediated endocytosis in the endothelium of the BBB; however, the systemic circulation of both endogenous transferrin and insulin compete with the targeted constructs for the target receptors [13]. Antibodies against CNS-specific targets, antibody-protein fusions and cell-penetrating peptides are other predominant approaches used to target drugs to

the brain. All these avenues for drug delivery to the brain have excellent potential, although some reports suggest off-target effects and transport challenges may limit their use [14,15]. Nanoscale systems for drug delivery have also been reported that function through their active transport across the BBB [16,17]. These examples depict the important advances made by leaders in the field, as well as the therapeutic promise of CNS-specific delivery. The literature also suggests that there is additional opportunity for new approaches to target drugs to the brain.

In this work, we explore the proof of concept for how low molecular weight substrates of P-glycoprotein (P-gp), a membrane-bound cellular efflux pump, can be used to alter the biodistribution of nanoparticles, with particular emphasis on nanoparticle retention in the capillaries of the brain. P-gp is a 170 kDa transmembrane protein that belongs to a large family of endogenous ATP-dependent transport systems that are constitutively expressed in tissues throughout the body as well as in multidrug resistant tumors [18]. The biological role of P-gp is to protect tissues from the accumulation of toxins. Clinically, P-gp can serve to prevent drug accumulation in a desired anatomical site, or as a mechanism of drug resistance. In the brain, P-gp is responsible for the active transport of a wide variety of substances from the CNS into the capillaries (i.e., out of the brain) and presents a significant challenge for the delivery of drugs developed to treat neurologically-derived diseases [18]. Antibodies against P-gp have been explored as a means to target drugs for cancer therapy [19] but CNS targeting using low molecular weight P-gp substrates is yet unexplored.

The work presented herein establishes that P-gp substrates (i.e., small molecules that are actively transported by P-gp out of the CNS) can enhance the retention of nanoparticles in the brain. The premise is that a P-gp substrate tethered to the surface of a nanoparticle through a water soluble polymer linker binds to the P-gp expressed in the endothelium, leading to

nanoparticle retention in the capillary lumen. For this work, the nanoparticles were not designed to be transported across the blood-brain barrier. Instead, they were designed to be captured by P-gp at the capillary endothelium and retained within the bloodstream. In this way, drugs could be release within the capillaries and enter the brain by passive diffusion down the concentration gradient.

Our findings show that when rhodamine 6G, a high affinity P-gp substrate, was tethered to the surface of nanoparticles formulated from polylactic acid-polyethylene glycol diblock copolymers, the nanoparticle accumulation in the brain (as measured by the area under the curve) was two-fold greater than untargeted nanoparticles and nanoparticles targeted with a lower affinity P-gp substrate (rhodamine 123). Also, accumulation of rhodamine 6G-tethered nanoparticles at 3 h in wild-type mice was two-fold greater relative to accumulation in P-gp knockout (i.e., mdr1a (-/-)) transgenic mice. These results establish the opportunity of using P-gp substrates to alter nanoparticle biodistribution and facilitate their retention in the brain, supporting their potential as a useful tool to deliver therapeutics to the CNS. With this initial report it is our intention to establish the potential opportunity of P-gp targeting low molecular weight substrates to the field, and we recognize that the specific nanoparticle construct reported herein is only a first example to be further improved upon by us and others in the field of drug delivery.

Materials and Methods

O-(2-aminoethyl) polyethylene glycol (HO-PEG-NH_2, M_n 5000), rhodamine 123 (Rho123), rhodamine 6G (Rho6G), triethylamine, dimethyl formaldehyde (DMF), monomethoxy polyethylene glycol (mPEG, M_n 5000), anhydrous toluene, stannous octoate,

europium chloride hexahydrate, 4,4,4-trifluoro-1-(2-naphthyl-1,3-butanedione) (NTA), acetone, dichloromethane (DCM), magnesium sulfate, poloxamer 188 solution and RIPA buffer were purchased from Sigma Aldrich (Saint Louis, MO). D,L-lactide was purchased from TCI America (Portland, OR). Phosphate buffered saline (PBS) was purchased from Corning (Corning, NY). Ammonium hydroxide was purchased from Alpha Aesar (Ward Hill, MA). Diethyl ether was purchased from Fisher Chemicals (Waltham, MA). Methanol was purchased from Macron Fine Chemicals (Avantor Performance Materials, Center Valley, PA).

PEG azeotropic distillation

Residual water was removed from mPEG by azeotropic distillation. mPEG (50 g, M_n 5000) was dissolved in 200 mL of anhydrous toluene with slight heating. Azeotropic distillation was conducted under reflux at 128 °C for 2 hours via a Dean Stark trap apparatus. Dry product was obtained from remaining toluene by rotoevaporation with mild warming followed by drying under high vacuum.

Synthesis of Rho6G-PEG and Rho123-PEG

Conjugates of PEG with Rho6G or Rho123 on one end and an alcohol group on the other were synthesized with minor variation of methods previously published by our group [20]. For the synthesis of Rho6G-PEG, HO-PEG-NH_2 (M_n 5000, 241.6 mg, $4.8x10^{-5}$ mol) was dissolved in anhydrous DMF (2 mL) with excess TEA at room temperature. Excess TEA was added to Rho6G powder (77.2 mg, $1.6x10^{-4}$ mol) and then dissolved in anhydrous DMF (12 mL) with slight heating. For the synthesis of Rho123-PEG, HO-PEG-NH_2 (M_n 5000, 438.3 mg, $8.7x10^{-5}$ mol) was dissolved in anhydrous DMF (2 mL) with excess TEA at room temperature. Excess

TEA was added to Rho123 powder (100 mg, $2.6x10^{-4}$ mol) which was then dissolved in anhydrous DMF (2 mL) at room temperature. For both Rho6G-PEG and Rho123-PEG production, the two DMF solutions were combined and stirred at 30 °C for 1 week while protected from light with an aluminum foil cover. The respective solutions were then diluted with Milli-Q water (38 mL) and dialyzed (MWCO 2000g/mol) in the dark against deionized water with 14 water exchanges over 1 week at room temperature. Following dialysis the solution was dried to a light pink powder by lyophilization in the dark for 3 days. Product yields for Rho6G-PEG and Rho123-PEG were 58% and 79.4%, respectively. ^{1}H NMR was conducted on an Inova 600 MHz spectrometer at 25 °C. For clarity and reproducibility of the conjugate synthesis and characterization, the ^{1}H NMR (to characterize conjugate chemical composition) as well as diffusion ordered NMR (to ensure conjugation of the rhodamine and PEG) spectra of each conjugate are provided in the supplemental information (Figure S3 and Figure S4). Rho6G-PEG conjugate ^{1}H NMR (600 MHz, DMSO-d_6) δ(ppm): 4.55 (t, 1H, **H**O PEG), 3.5 (s, 4H, C**H**$_2$C**H**$_2$O PEG), 6.06 (s, 2H, ArH Rho6G), 6.26 (s, 2H, ArH Rho6G), 6.97 (d, 1H, ArH Rho6G), 7.50 (m, 2H, Ar**H** Rho6G), 7.77 (d, 1H, Ar**H** Rho6G), 1.86 (s, 6H, ArC**H**$_3$), 1.21 (t, 6H,NCH$_2$C**H**$_3$). Rho123-PEG conjugate ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 4.55 (t, 1H, **H**O PEG), 3.44 (s, 4H, C**H**$_2$C**H**$_2$O PEG), 6.16 (s, 1H, Ar**H** Rho123), 6.17 (s, 1H, Ar**H** Rho123), 6.23 (d, 1H, Ar**H** Rho123), 6.32 (d, 1H, Ar**H** Rho123), 7.00 (d, 1H, Ar**H** Rho123), 7.50 (m, 4H, Ar**H** Rho123), 7.74 (d, 1H, Ar**H** Rho123)

mPEG-PLA, Rho6G-PEG-PLA, Rho123-PEG-PLA diblock copolymer synthesis and characterization

Lactide was recrystallized once from methanol and dried under high vacuum prior to reaction. Monomethoxy-PEG (mPEG, 400 mg, 0.08 mmol) and lactide (1843 mg, 12.8 mmol) were dissolved in anhydrous toluene (6 mL) under argon in a 25 mL Schlenk flask. Rho6G-PEG (54.3 mg, 0.01 mmol) and lactide (250.2 mg, 1.7 mmol) were dissolved in anhydrous toluene (1 mL) under argon in a 10 mL Schlenk flask. Rho123-PEG (100 mg, 0.02 mmol) and lactide (428.25 mg, 3.0 mmol) were dissolved in anhydrous toluene (1 mL) under argon in a 10 mL Schlenk flask. For all reactions the reaction vessels were placed in a 111 °C silicon oil bath with stirring. Upon the initiation of toluene reflux (111 °C), toluene (2 mL for mPEG-PLA, 1 mL for Rho6G-PEG-PLA and Rho123-PEG-PLA) containing stannous octoate (1.1 mol% lactide) was added all at once with stirring. The reaction was allowed to reflux with stirring over 24 hours, after which excess toluene was removed by rotoevaporation until an oily liquid remained and the product collected by precipitation into excess diethyl ether with stirring. The product was collected by filtration, dried overnight under high vacuum and stored at room temperature in a vacuum desiccator until characterization and nanoparticle formulation. The molecular weight of each diblock copolymer was determined by GPC (Waters, Milford, MA) using polystyrene standards with a THF mobile phase. The diblock copolymers were characterized by ^{1}H NMR. ^{1}H NMR for mPEG-PLA (600 MHz, DMSO-d_6) ppm: 5.20 (m, C**H** PLA), 3.51 (s,C**H**$_2$C**H**$_2$ PEG), 3.24 (s, C**H**$_3$ PEG), 1.45 (m, C**H**$_3$ PLA). ^{1}H NMR for Rho6G-PEG-PLA (600 MHz, DMSO-d_6) ppm: 5.20 (m, C**H** PLA), 3.24 (s, C**H**$_3$ PEG), 1.45 (m, C**H**$_3$ PLA), 1.83 (s, 6H, ArC**H**$_3$), 6.03 (s, 2H, ArH Rho6G), 6.22 (s, 2H, ArH Rho6G), 6.93 (d, 1H, ArH Rho6G), 7.46 (m, 2H, Ar**H** Rho6G), 7.74 (d, 1H, Ar**H** Rho6G). ^{1}H NMR for Rho123-PEG-PLA (600 MHz, DMSO-d_6) ppm: 5.20 (m, C**H** PLA), 3.24 (s, C**H**$_3$ PEG), 1.45 (m, C**H**$_3$ PLA), 6.60 (d, 1H, Ar**H** Rho123), 7.53 (d, 1H, Ar**H** Rho123), 7.74 (m, 4H, Ar**H** Rho123), 7.82 (d, 1H, Ar**H** Rho123)

Europium(III)tris(4,4,4-trifluoro-1-(2-naphthyl-1,3-butanedione)) chelate synthesis

The europium(III)tris(4,4,4-trifluoro-1-(2-naphthyl-1,3-butanedione)) ($Eu(NTA)_3$) chelate was made as previously reported [21]. In brief, NTA (800 mg) was dissolved in ethanol (75 mL) and ammonium hydroxide (20.4 mL of 28%) with stirring. On complete dissolution, a solution of europium chloride hexahydrate (366 mg) in DI water (10 mL) was added dropwise to the NTA solution. The chelate was allowed to form overnight by ethanol evaporation with stirring at room temperature. The resulting solution was then extracted once with an equal volume of DCM, the organic layer isolated and washed with DI water three times. The DCM solution was dried over magnesium sulfate, DCM removed by rotoevaporation, and the resulting solid dried under high vacuum. Time-resolved fluorescence of the europium chelate corresponded to previously reported wavelengths and intensities [22].

Nanoparticle fabrication and characterization

mPEG-PLA (100 mg) was dissolved in DCM (1 mL) then mixed with a DCM solution of $Eu(NTA)_3$ (1 mg/mL, 1 mL total). The solution was then diluted with acetone (8 mL), vortexed and added dropwise into milliQ water (10 mL) with stirring. The organic solvents were removed by rotoevaporation and the nanoparticles collected by ultracentrifugation using a Bruker LE-80 ultracentrifuge at 20,000 rpm for 30 minutes. The nanoparticles in the pellet were washed with milliQ water and ultracentrifuged again at 20,000 rpm for 30 minutes. This process was repeated once more and the final pellet was resuspended in a final volume of 1 mL of milliQ water. Poloxamer 188 (20 μL of 10% solution) was added to prevent aggregation prior to lyophilization overnight. For 10% Rho6G-PEG-PLA and 10% Rho123-PEG-PLA nanoparticles, the same

procedure was followed but with 90 mg of mPEG-PLA and 10 mg of Rho6G-PEG-PLA or Rho123-PEG-PLA, respectively.

Colloidal suspensions of the nanoparticles were made with milliQ water (1 mg/mL) and sonicated for 10 minutes. After sonication the suspensions were passed through a 0.45 μm filter and diluted 1:2 into a final suspension in HEPES (1 mM, pH 7.0) buffer. Size, polydispersity, and zeta potential were measured using a Malvern NanoZS (United Kingdom). The morphology of the particles was evaluated by TEM. The 1 mg/mL nanoparticle suspension was negatively stained with 2% uranyl acetate on titanium grids and imaged using a FEI T12 spirit TEM. To visualize the incorporation of $Eu(NTA)_3$ into the particles, solutions of unloaded and $Eu(NTA)_3$ loaded nanoparticles were imaged under natural and UV light. Nanoparticles were re-suspended in this fashion without trouble for at least four weeks after fabrication.

In vivo biodistribution

Seven week old ND4 Swiss Webster wild-type mice (WT mice) were purchased from Harlan Sprague Dawley. P-glycoprotein knockout mice (mdr1a (-/-)) were purchased from Charles River Laboratories. A suspension of nanoparticles (1 mg/mL) in sterile PBS was sonicated for 10 - 30 minutes until the solution was clear and then flowed through a 0.45 μm filter. A 200 μL aliquot of the nanoparticle suspension was injected intravenously through the tail vein. For WT mice, at 0.5, 3, 6, and 9 hours, five mice were euthanized by CO_2 inhalation for each formulation under an approved IACUC protocol (Cornell University, protocol number 2012-0034). For mdr1a (-/-) mice at 3 hours, five mice were also euthanized for each formulation. Blood was drawn via cardiac puncture and the liver, kidneys, heart, lungs, spleen and brain removed. Each organ was weighed, homogenized in 2 mL of RIPA buffer and the resulting mixtures were

centrifuged at 800 x g for 10 minutes. Time-resolved fluorescence was measured at an excitation and emission of 340 and 610 nm, respectively, for three separate samples of the homogenate supernatant for each of the five mice per organ. Percent injected dose was determined according to our previous studies that reported the utility of time-resolved fluorescence of europium to quantify nanoparticle content in tissues [22]. In brief, percent injected dose was calculated using Equation 1, where RD= raw data, SL= sensitivity limit, ID= injected dose, IF= interference factor, g tissue = gram of tissue and TF= time factor. The sensitivity limit is defined as average background + 3 standard deviations, the interference factor is the measure of signal attenuation caused by individual tissues, and time factor represents the signal decay kinetics at 37 °C for each time point. Area under the curve from 30 minutes to 9 hours was calculated by the trapezoid rule.

$$\left[\frac{(RD-SL)}{ID*IF*g\ tissue} * TF\right] * 100 = \frac{\%\ injected\ dose}{g\ tissue} \qquad \text{(Eq. 1)}$$

Fluorescent images of brain tissue slices were obtained for 10% Rho6G-PEG-PLA and mPEG-PLA nanoparticles under an approved IACUC protocol (Cornell University, protocol number 2009-0043). To obtain the fluorescent images, mice were anesthetized and perfused with formaldehyde 3 hours after nanoparticle injection. The brain was cryosectioned and imaged on a Zeiss Axio Examiner fluorescent microscope under DAPI and FITC filters. The FITC filter showed background tissue autofluorescence and the DAPI filter was used to visualize nanoparticles containing the europium chelate.

Results

Nanoparticle formulation

Copolymers of mPEG-PLA, Rho6G-PEG-PLA and Rho123-PEG-PLA were successfully synthesized with an overall M_n of 13,700, 12,400 and 14,400 respectively and PDI values of 1.6, 1.4 and 1.4, respectively. Particles were fabricated from mPEG-PLA, Rho6G-PEG-PLA and Rho123-PEG-PLA with and without entrapped $Eu(NTA)_3$. Particle characteristics are summarized in Table 1. All particles were ~100 nm in diameter and not statistically different from one another as determined by Student's t-test. Zeta potential values are also equivalent and approximately -27 mV. Targeted nanoparticles were formulated at a w:w ratio of 10:90 (Rho-PEG-PLA:mPEG-PLA) to ensure sufficient P-gp substrate content, but avoid nanoparticle aggregation from excessively hydrophobic surfaces. Fluorescence analysis of unloaded (no europium) nanoparticles (λ_{ex}=526 nm, λ_{em}=555 nm), confirmed rhodamine on both Rho6G and Rho123 nanoparticles, and its absence on mPEG nanoparticles. For qualitative reference, a 1mg/mL solution with 10% Rho6G-PEG or Rho123-PEG and 90% mPEG gave fluorescent readings of 306±45 and 23±4, respectively. TEM images (Figure 2a) showed all particle formulations to be spherical in shape. The incorporation of $Eu(NTA)_3$ into the nanoparticles was evident by the characteristic red fluorescence of the nanoparticles under UV light and its absence in unloaded nanoparticles (Figure 2b).

In vivo biodistribution

Time-resolved fluorescence of europium encapsulated within the nanoparticles was used to track their biodistribution following intravenous administration. Although the fluorescence of the rhodamine conjugates could be used to quantify nanoparticle concentration in various tissues,

the tissues themselves autofluoresce at similar wavelengths and on the same time scale, leading to a high background signal. However, elements in the lanthanide III series, like europium, exhibit large Stokes shifts and long fluorescent decay lifetimes and are amenable to time resolved fluorescence wherein their fluorescent signal remains detectable even after tissue autofluorescence and rhodamine fluorescence are completed, resulting in a very low background signal and high signal to noise ratio. The biodistribution of Rho6G-PEG-PLA, Rho123-PEG-PLA and mPEG-PLA nanoparticles was evaluated at 0.5, 3, 6 and 9 hours (Figure 3). Rho6G-terminated particles showed overall greater retention in liver, kidneys, heart, lungs and brain relative to Rho123 or methoxy-terminated particles. The 3 h time point is particularly interesting with nanoparticle retention in organs and blood throughout the body for all targeted and control formulations, with the exception of the brain where notably only the Rho6G-targeted nanoparticles were retained. Specifically, at 3 h, 0.16 ± 0.02 % ID/g were present in the brain whereas there was no accumulation for both methoxy or Rho123-targeted nanoparticles. These results suggest the particles are retained in the brain through the action of Rho6G. Representative brain tissue slices taken at the 3h time point (Figure 4) show nanoparticle accumulation in the brain with Rho6G-PEG-PLA nanoparticles (Figure 4a) relative to mPEG-PLA nanoparticle accumulation (Figure 4b). It is important to note that these images were obtained from perfused brains and are not merely nanoparticles passively retained in the lumen of the brain capillaries. To further support the retention of the nanoparticles in the brain, area under the curve (AUC) values were calculated (Table 2). AUC_{brain} for Rho6G-PEG-PLA exceeded both Rho123-PEG-PLA and mPEG-PLA nanoparticles by over 2-fold. It is also important to acknowledge that the AUC of the targeted nanoparticles is also enhanced in other organs (i.e., liver, spleen, kidney, heart, lung) and that this accumulation should be taken into

consideration when exploring this targeting approach with specific drugs and disease states. The relevance of this biodistribution pattern will depend on the specific pharmacologic characteristics of the intended drug cargo, but the retention in the brain remains significant given the absence of untargeted nanoparticle retention at the 3h time point and beyond. Whether the nanoparticles transverse the BBB is yet unknown and their tissue-specific localization in the brain is the focus of ongoing work.

To ascertain if the enhanced accumulation of Rho6G-targeted nanoparticles in the brain could be attributed to interactions with P-gp, brain accumulation was measured at 3h in a P-glycoprotein knockout mouse model, mdr1a (-/-), and directly compared to the WT mouse model. Brain accumulation of Rho6G, Rho123 and methoxy-terminated nanoparticles were compared in WT mice vs. mdr1a (-/-) at 3h (Figure 5). There is a significant decrease in the percent of injected dose/g tissue for Rho6G- terminated particles from 0.16 ± 0.02 % in WT mice to 0.08 ± 0.02% in mdr1a (-/-) mice (Figure 5c), whereas there are no significant differences in brain accumulation between WT and mdr1a (-/-) mice for either Rho123 (Figure 5b) or methoxy- (Figure 5a) terminated particles. Comparison of Rho6G-terminated nanoparticle accumulation in liver, kidneys, heart, lungs, spleen and blood between WT and mdr1a (-/-) mice (Figure 6) show that the only other significant difference, in the spleen, is an increase. This finding, that the Rho6G-terminated nanoparticles selectively accumulated in the brain of the mice that express P-gp relative to the mdr1a(-/-) knockout (and relative to the other insignificant changes with Rho123-terminated and untargeted nanoparticles, Figures S1 and S2, respectively) confirm the potential utility of P-gp as an untapped targeting opportunity using low molecular weight P-gp substrates.

Discussion

Targeted drug delivery systems are becoming increasingly useful as a means to alter the biodistribution or half-lives of therapeutics. In large part, the success of targeting to specific tissue types is dependent on the unique localization of the targeted receptor, as well as the substrate/receptor binding constant. Strategies through which to target drugs are varied [23] and range from antibodies [24] to aptamers [25,26] to peptides [27]. Nanoparticle targeting using small molecules is also reported. Examples include folic acid used to target folate receptor-positive cancers [28,29], galactose to target the asialoglycoprotein receptor of hepatocytes [30] and mannose to target the DC-SIGN lectin of dendritic cells [31]. Discovery of small molecules to target a variety of tissues was also reported using a high-throughput screening approach [32].

The incidence of neurologically-based diseases is continually growing. According to the 2013 Center for Disease Control report, Mortality Multiple Cause Micro-data Files, Alzheimer's disease and stroke are among the top 10 leading causes of death in the United States [33]. The American Cancer Society reports brain cancer having only a 35% five-year survival rate [34]. As of June 2009 only 8.2% of drugs developed for activity in the central nervous system have been approved for clinical use, making it the lowest percentage of all therapeutic classes [35]. However, therapies for CNS diseases are among the top targets for drug development [35]. The translation from drug discovery to development and clinical success is greatly dampened by delivery challenges to the CNS [36].

The targeted nanoparticles described herein are not designed to facilitate nanoparticle transport across the blood-brain barrier. Rather, they were expressly designed to ascertain whether low molecular weight substrates of P-gp had the potential to facilitate retention of a drug delivery system, nanoparticles in this particular case, within the brain. Nanoparticles targeted to

the brain capillary endothelium using the P-gp substrate, Rhodamine 6G, were retained longer (>3 hrs) and at a higher concentration (AUC greater than 2-fold) than non-targeted nanoparticles. Additionally, the design of the targeting mechanism to promote retention of nanoparticles within the brain, rather than to promote nanoparticle transport across the capillary endothelium that makes up the blood brain barrier, prevents nanoparticle scavenge by microglia.

From a practical perspective, such a targeted delivery system could be used to deliver low molecular weight therapeutics designed to passively transport down a concentration gradient into the brain for treatment of CNS-based pathologies. Traditional low molecular weight therapeutics for the CNS are designed with octanol/water partition coefficients that permit sufficient solubility in both aqueous and non-aqueous solvents and can thereby passively diffuse across the BBB. One shortcoming of this small molecule design paradigm is that the compounds can passively diffuse across all cell membranes, leading to high volumes of distribution and adverse off-target effects. Biodistribution alteration of these compounds to increase their local concentration in the brain over time could serve to enhance the efficacy of some compounds, or perhaps rescue some therapeutics that have failed in the clinic due to poor side effect profiles. Of course, this approach will not work for therapeutic compounds that are themselves P-gp substrates, making the judicious pairing of the disease and the desired therapeutic imperative for therapeutic success.

A historically effective way to target molecules to the CNS is to exploit the endogenous receptor-mediated transport mechanisms to facilitate passage across the BBB. Among the many receptor-ligand pairs, transferrin continues to be the most widely studied. The transferrin receptor was one of the first receptors used to shuttle therapeutic agents into the CNS and significant improvements in CNS targeting have been demonstrated *in vivo* [37]. Recent studies

report ~2 fold increase in drug concentration within rodent brain 2 to 4 h post administration with a transferrin conjugated nanoparticle [38,39]. Although transferrin-targeted systems are promising, overall efficacy is challenged by competitive binding with endogenous transferrin [9]. Recent reports also suggest that in biologically relevant media, protein adsorption can form a corona around the transferrin and inhibit its receptor binding constant [10]. Further development, codified by the term "Trojan Horse", included antibody-protein fusions and peptidomimetic monoclonal antibodies directed toward transferrin and insulin receptors for the delivery of proteins and nucleic acids to the CNS [15,40]. While antibody approaches are valuable, this study recognized the need for new approaches to target drugs to the CNS.

The study reported herein specifically queried whether the drug efflux transporter, P-gp, which is highly expressed in the endothelial cells that comprise the BBB, could be exploited to enhance the accumulation of nanoparticles in the brain. The efflux mechanism of P-gp is an under-explored drug delivery target that is endogenous to the BBB endothelia, and actively transports small molecules from the CNS into the blood. The conceptual design of the targeting strategy is shown in Figure 1, wherein a P-gp substrate is tethered to a polylactic acid nanoparticle through a polyethylene glycol spacer. As a first approach, Rho6G and Rho123 were used to establish a proof of concept for the use of P-gp substrates as targeting agent to the CNS. The specific mechanism(s) though which the tethered rhodamine interacts with P-gp is unknown and warrants further investigation. It is a thought-provoking fundamental question because the binding site locale for free rhodamines in on the inner leaflet [41], which suggests that the tethered substrate could transverse the cell membrane to bind to its receptor site as depicted in Figure 1; however, we currently do not have data to support this supposition and pose the

question to the field as a straw-man suggestion to encourage further inquiry by other investigators.

Nanoparticles were formulated from diblock copolymers of PEG and PLA with the PEG chain terminated with either Rho6G or Rho 123. The function of PEG in this study was three-fold, first to serve as a tether for the rhodamine P-gp substrates, second to prevent non-specific aggregation of the nanoparticles, and third to prevent non-specific sequestration of the nanoparticles by the liver and spleen. Previous reports from our group established that rhodamine-PEG conjugates retained their affinity to P-gp and remained substrates, which prompted their use for the present investigation [20]. The presence of rhodamine on the nanoparticle surface was verified by fluorescence. The quantum efficiency of Rho6G exceeds Rho123 leading to the fluorescence difference reported in Table 2 [42], whereas the fluorescence of both rhodamine-containing nanoparticles exceeded methoxy-terminated PEG nanoparticles. While not directly comparable as the nanoparticles are in suspension and have rhodamine molecules within close proximity to one another, the fluorescence of a 10% solution of Rho123-PEG is also significantly lower than a 10% solution of Rho6G-PEG, due to the lower quantum yield of Rho123 [43] and the decrease in quantum yield for both Rho123 and Rho6G upon conjugation [44]. To begin to understand the influence of P-gp binding affinity on nanoparticle targeting and accumulation, both Rho6G and Rho123 were investigated as targeting moieties, as they possess different affinities for P-gp with reported K_m values of approximately 2 μM and 40 μM respectively [45]. Rho6G is the stronger P-gp substrate [45], which supports the greater accumulation of Rho6G-PEG-PLA nanoparticles in the brain relative to the Rho123-terminated nanoparticles.. Accumulation of Rho6G-terminated nanoparticles remained high in the brain at 3 h relative to controls; a phenomenon that was not seen in the P-gp deficient knockout mice.

These findings suggest that the Rho6G-terminated particles are able to effectively target P-gp, leading to an enhanced accumulation in the brain. Fluorescent images of perfused brain cryosections qualitatively confirm the enhanced accumulation of the Rho6G-PEG-PLA nanoparticles in the brain relative to untargeted control nanoparticles as measured by the time-resolved fluorescence of europium. Specific brain compartments were not considered in this study; however, the favored brain compartments for P-gp targeted nanoparticles to accumulate is of particular interest and is currently under investigation.

There are potential opportunities to modulate the nanoparticle locale at the cellular level as well. In the recent literature, the binding and internalization of nanoparticles containing agonists or antagonists of G protein coupled receptors was explored. The results of this innovative study found that agonists bind and trigger cellular internalization, where antagonists bind and remain externally located at the cell surface [46]. These authors introduced the idea of a multi-ligand particle, which could also be an attractive approach for using cross-ligand targeting to P-gp and other receptors in the CNS. Another forward-thinking concept is that because P-gp has multiple binding pockets, only one of which involves rhodamine binding [47], if a delivery system were created that contained both agonist and an antagonist toward opposing binding pockets, perhaps an increase in particle retention and an extension of therapeutic exposure time could be achieved.

Our findings define a unique approach toward enhancing the local concentration of drug-loaded nanoparticles in the capillaries of the brain. We explored a new way to target drug delivery vehicles to the brain through the application of low molecular weight substrates that are actively transported out of the brain. Specifically, PEG-PLA nanoparticles containing Rho6G on the surface effectively targeted P-gp in the brain capillaries and enhanced retention greater than

two-fold over untargeted controls. There are a number of P-gp substrates that are approved for administration into humans, and these first results set the groundwork for a potentially new way to target drugs to the brain through exploitation of the function of P-gp.

Figure 1

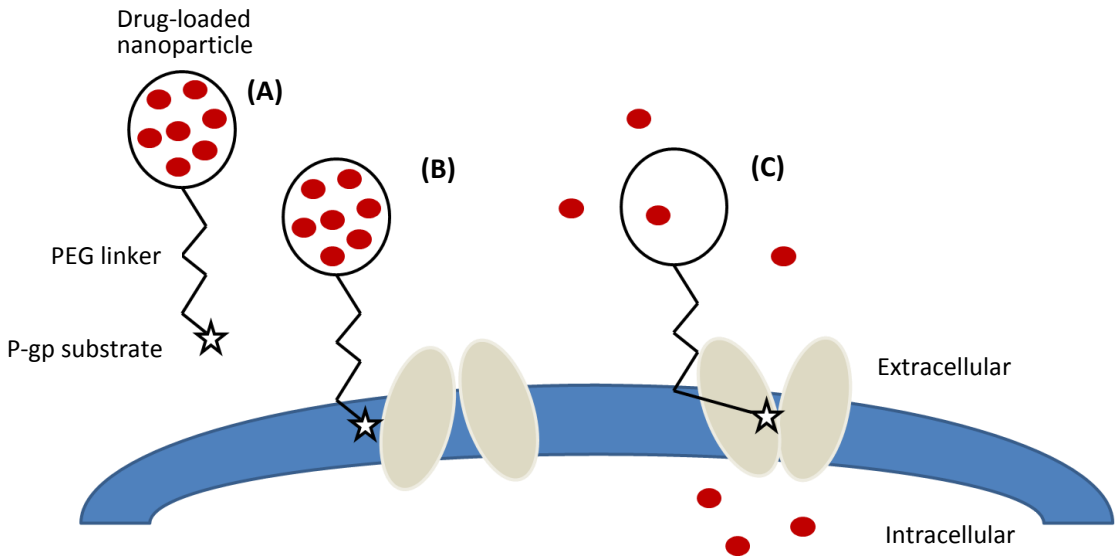

Figure 1 The binding site for rhodamine on P-gp is on the inner intracellular leaflet of the transmembrane protein [37]. While counter intuitive (and energetically unfavorable) the tissue retention of the targeted nanoparticles suggests that the tethered substrate might transverse the cell membrane to bind to its receptor site. A possible mechanism, which requires additional investigation, is shown here where the PEG linker/P-gp substrate on a drug carrier (A) spans the cell membrane (B) allowing the P-gp substrate to bind to P-gp (C) and retain the drug carrier in the capillary. Please note that only one P-gp substrate is drawn on the nanoparticle to make the potential mechanism visually clear, whereas in practice there are multiple P-gp substrates in each nanoparticle.

Figure 2

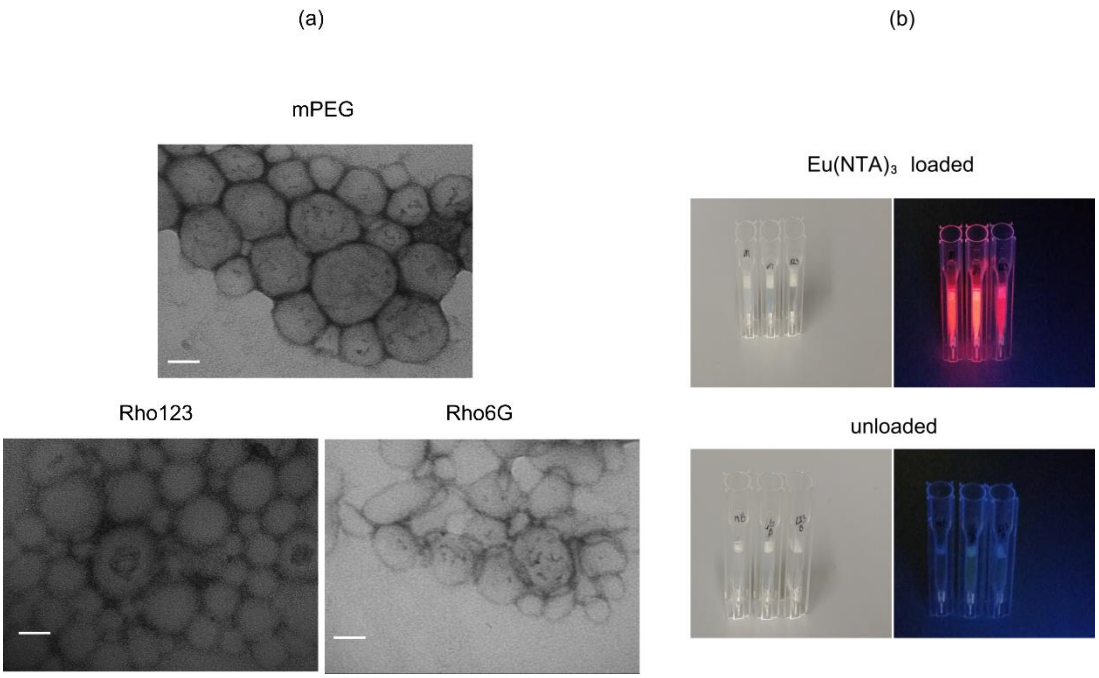

Figure 2 (a) TEM images of mPEG-PLA, 10% Rho123-PEG-PLA and 10% Rho6G-PEG-PLA nanoparticles. Scale bar represents 50 nm. All particle types show polydispersity between 50-100 nm in diameter. (b) Fluorescent glow after exposure to UV light of $Eu(NTA)_3$ loaded nanoparticles compared to unloaded particles. Cuvettes from left to right in (b): mPEG-PLA, 10% Rho6G-PEG-PLA, 10% Rho123-PEG-PLA

Figure 3

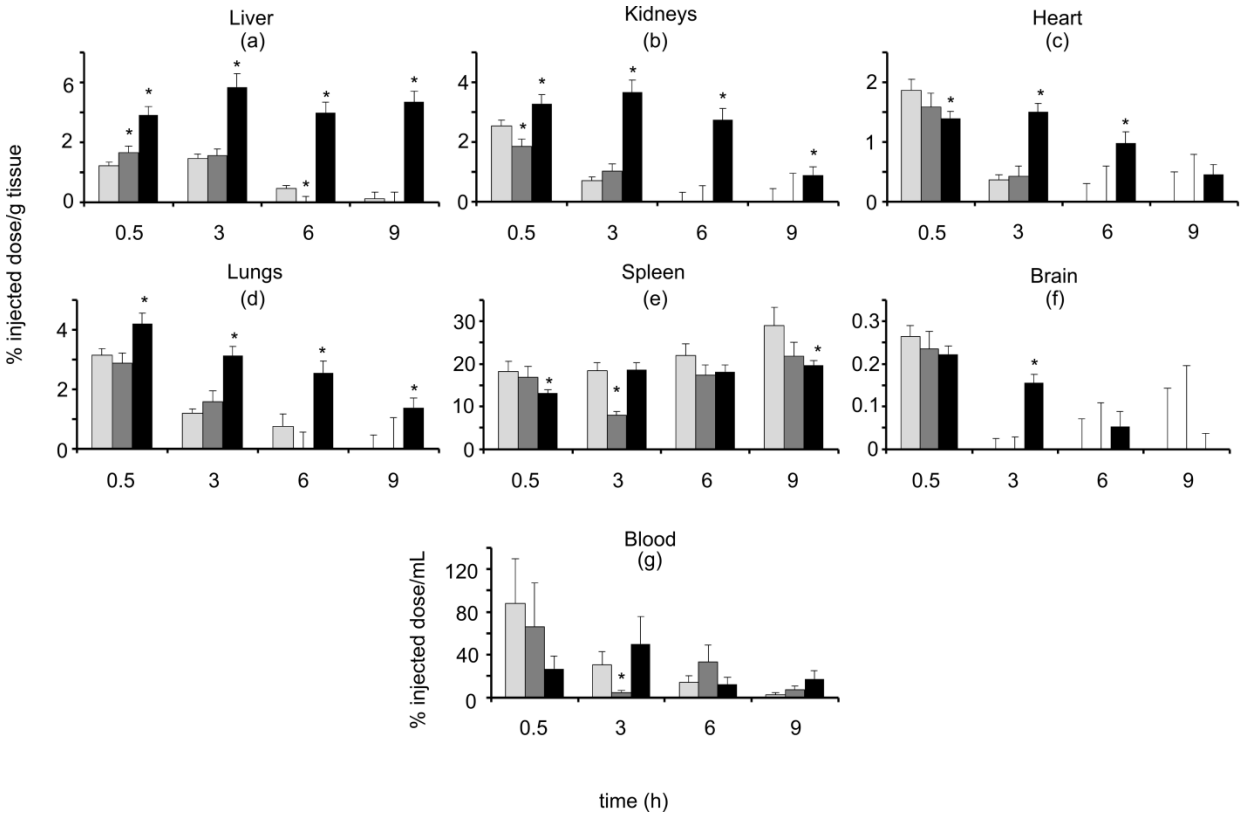

Figure 3 Biodistribution of mPEG-PLA (light gray), 10% Rho123-PEG-PLA(dark gray), and 10% Rho6G-PEG-PLA (black) particles in (a) liver, (b) kidneys, (c) heart, (d) lungs, (e) spleen, (f) brain and (g) blood. Values are % injected dose (%ID) per gram of tissue or mL. Rho6G-terminated particles remain in the brain at 3h compared to mPEG and Rho123 terminated particles. * statistical significance compared to mPEG-PLA nanoparticles at $p < 0.01$. Error bars depict +/- one standard deviation

Figure 4

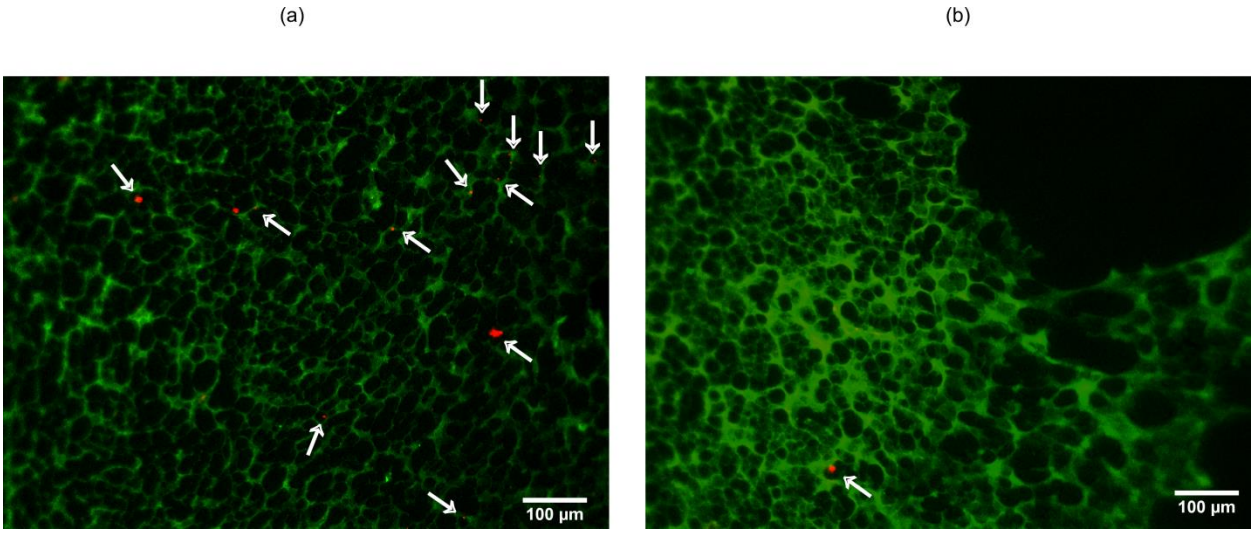

Figure 4 Representative brain tissue sections from mice injected with (a) Rho6G-PEG-PLA nanoparticles and (b) mPEG-PLA nanoparticles. Scale bars are 100 µm. Tissue autofluorescence is shown in green and the nanoparticles are in red. There are visibly particles in the brain after 3h in the Rho6G-PEG-PLA sample but only one aggregate visible in the mPEG-PLA sample

Figure 5

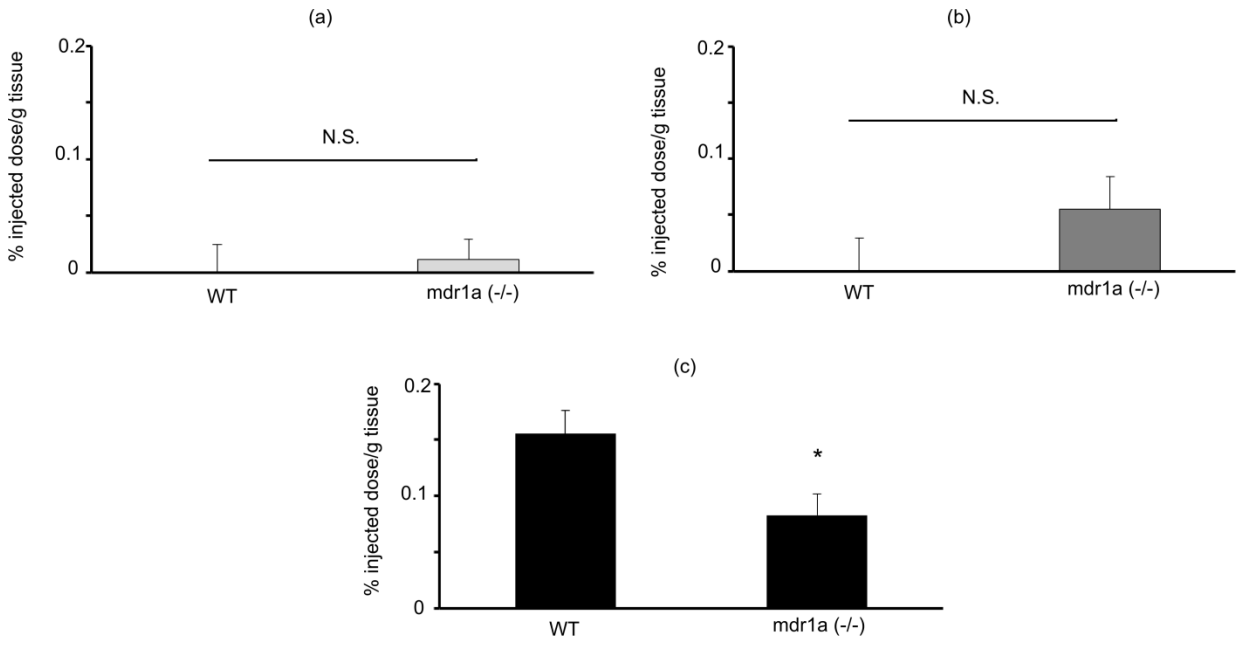

Figure 5 Brain accumulation of (a) mPEG-PLA nanoparticles, (b) Rho123-PEG-PLA nanoparticles and (c) Rho6G-PEG-PLA nanoparticles in WT mice compared to a P-gp knockout mouse, mdr1a (-/-), at 3h. Values are % injected dose (%ID) per gram of tissue. There is a significant decrease seen in with the Rho6G-PEG-PLA particle accumulation in the P-gp knockout model. * represents statistical significance from the WT model at $p < 0.001$. Error bars depict +/- one standard deviation

Figure 6

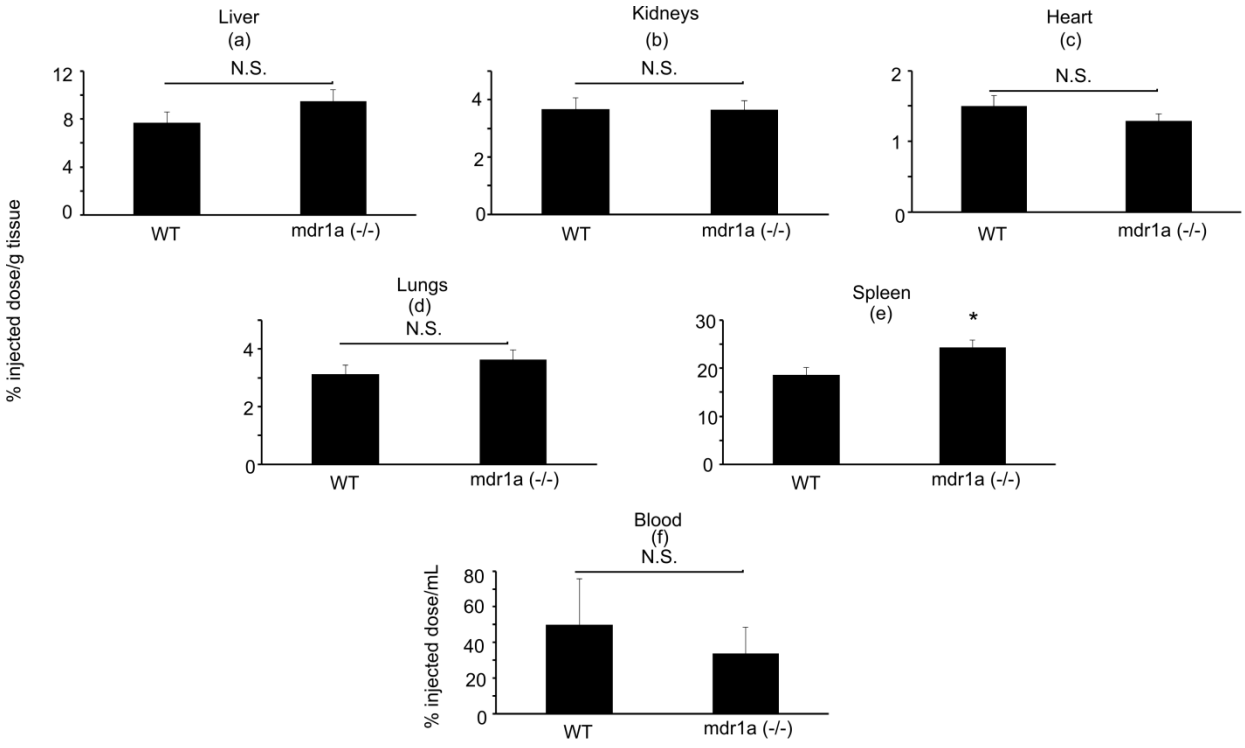

Figure 6 Comparison between WT mice to P-gp knockout mice of 6G-PEG-PLA accumulation at 3h in (a) liver, (b) kidneys, (c) heart, (d) lungs, (e) spleen and (f) blood. Values are % injected dose (%ID) per gram of tissue or mL. Only the spleen shows a significant difference between the two mouse models. * statistical significance from WT model at $p < 0.01$. Error bars depict +/- one standard deviation

Table 1 Size, PDI and zeta potential of nanoparticle formulations in HEPES (1 mM, pH 7.0) buffer. All sizes are ~100 nm in diameter (not statistically different from each other) with equivalent zeta potentials. Greater fluorescence at excitation/emission of 526/555 nm from rhodamine particle formulations show rhodamine attachment to particle.

Formulation	Loading	size (nm)	PDI	Zeta potential (mV)	Fluorescence (526/555 nm)
mPEG	---	121.5 ± 9.0	0.109 ± 0.004	-28.4 ± 0.29	13.2 ± 0.85
10% 6G	---	106.3 ± 5.0	0.111 ± 0.01	-26.9 ± 0.36	794 ± 50
10% 123	---	93.9 ± 4.3	0.121 ± 0.02	-26.7 ± 0.47	38.7 ± 2.0
mPEG	$Eu(NTA)_3$	120.3 ± 9.6	0.069 ± 0.03	-27.5 ± 0.50	---
10% 6G	$Eu(NTA)_3$	99.6 ± 1.1	0.103 ± 0.01	-26.3 ± 0.40	---
10% 123	$Eu(NTA)_3$	108.2 ± 4.0	0.073 ± 0.02	-26.8 ± 0.72	---

Table 2 Area under the curve from 30 minutes to 9 hours. *blood values represented as % ID/mL.

AUC (%ID-h/g)			
Organ	**mPEG**	**Rho123**	**Rho6G**
Liver	14.1 ± 1.7	12.7 ± 2.8	56.2 ± 4.6
Kidney	5.11 ± 1.6	5.14 ± 2.9	23.7 ± 2.1
Heart	3.33 ± 1.6	3.15 ± 2.8	9.46 ± 1.0
Lung	9.43 ± 1.9	7.94 ± 3.4	23.5 ± 2.2
Spleen	183 ± 16	128 ± 13	151 ± 8.6
Brain	0.33 ± 0.4	0.29 ± 0.6	0.87 ± 0.2
Blood*	240 ± 85	204 ± 100	236 ± 90

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Compliance with Ethical Standards

The authors declare no real or apparent conflicts of interest. All research involving animals was conducted under IACUC approval at Cornell University. Specific approved protocol numbers are provided in the text of the manuscript in the relevant sections.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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Supporting Information

Title: Exploitation of P-glycoprotein Agonists to Target Nanoparticles to the Brain

Short title: P-gp Agonist Targeting to the Brain

Authors: Lindsey A. Crawford[1], Hannah Watkins[2], Elizabeth Wayne[2], and David Putnam[1,2*]

SI Figure 1

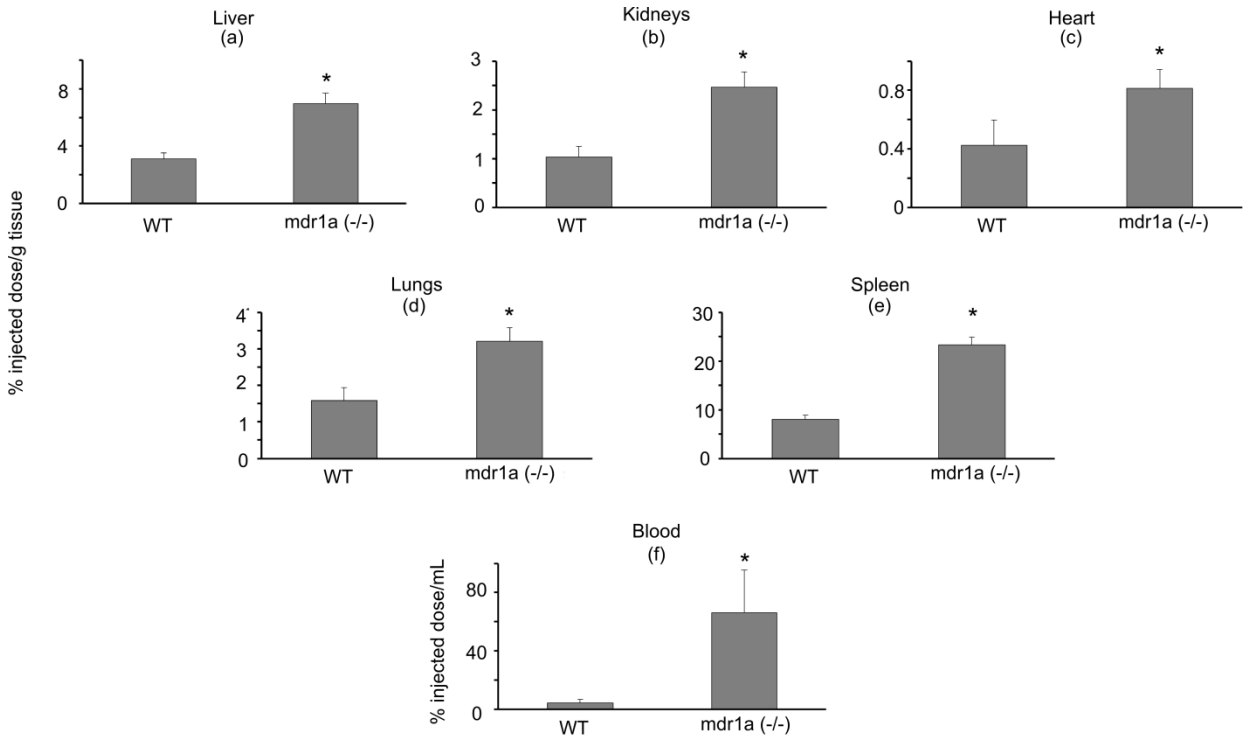

Figure S1. Comparison between WT mice to P-gp knockout mice of 123-PEG-PLA accumulation at 3h in (a) liver, (b) kidneys, (c) heart, (d) lungs, (e) spleen and (f) blood. All organs show a significant difference between the two mouse models. * statistical significance from WT model at $p < 0.01$. Error bars depict +/- one standard deviation.

SI Figure 2

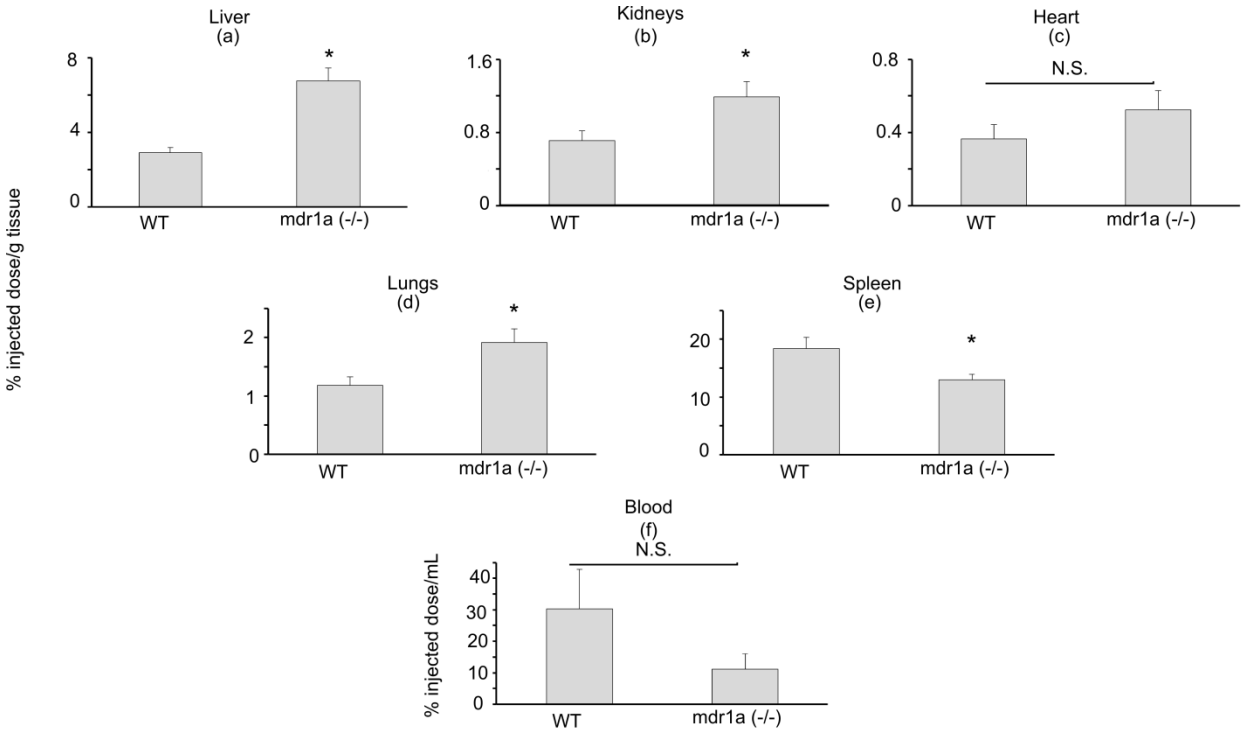

Figure S2. Comparison between WT mice to P-gp knockout mice of mPEG-PLA accumulation at 3h in (a) liver, (b) kidneys, (c) heart, (d) lungs, (e) spleen and (f) blood. Liver, kidneys, lungs and spleen show a significant difference between the two mouse models. * statistical significance from WT model at $p < 0.01$. Error bars depict +/- one standard deviation.

SI Figure 3

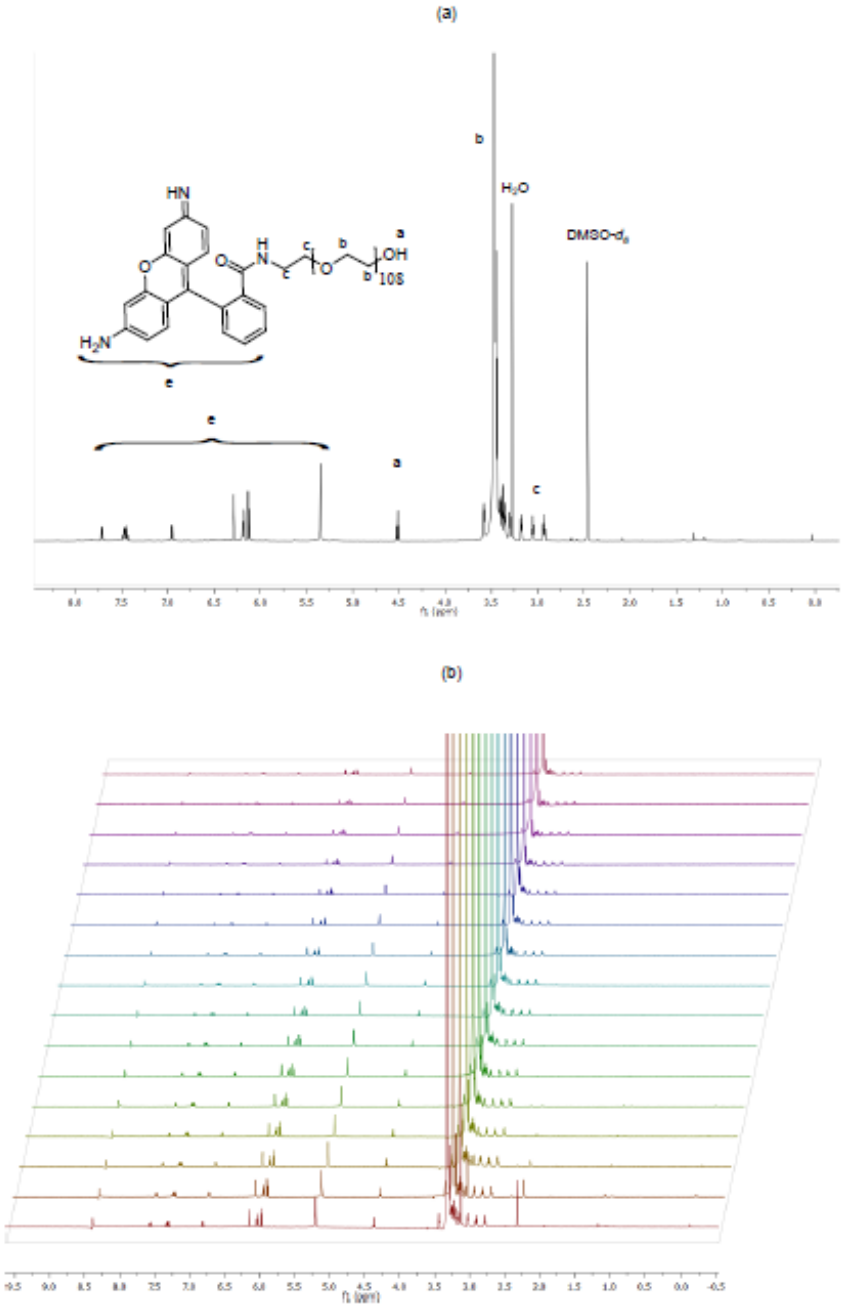

Figure S3. (a) ^{1}H NMR of the PEG-Rho123 conjugate in DMSO-d_6 at 600 MHz. Peaks in the group e represent Rho123 while peaks at a and b are the peaks from PEG. (b) Diffusion ordered NMR of the PEG-Rho123 conjugate in DMSO-d_6 at 600 MHz. Rho123 associated peaks decay at the same rate as PEG peaks assuring conjugation.

SI Figure 4

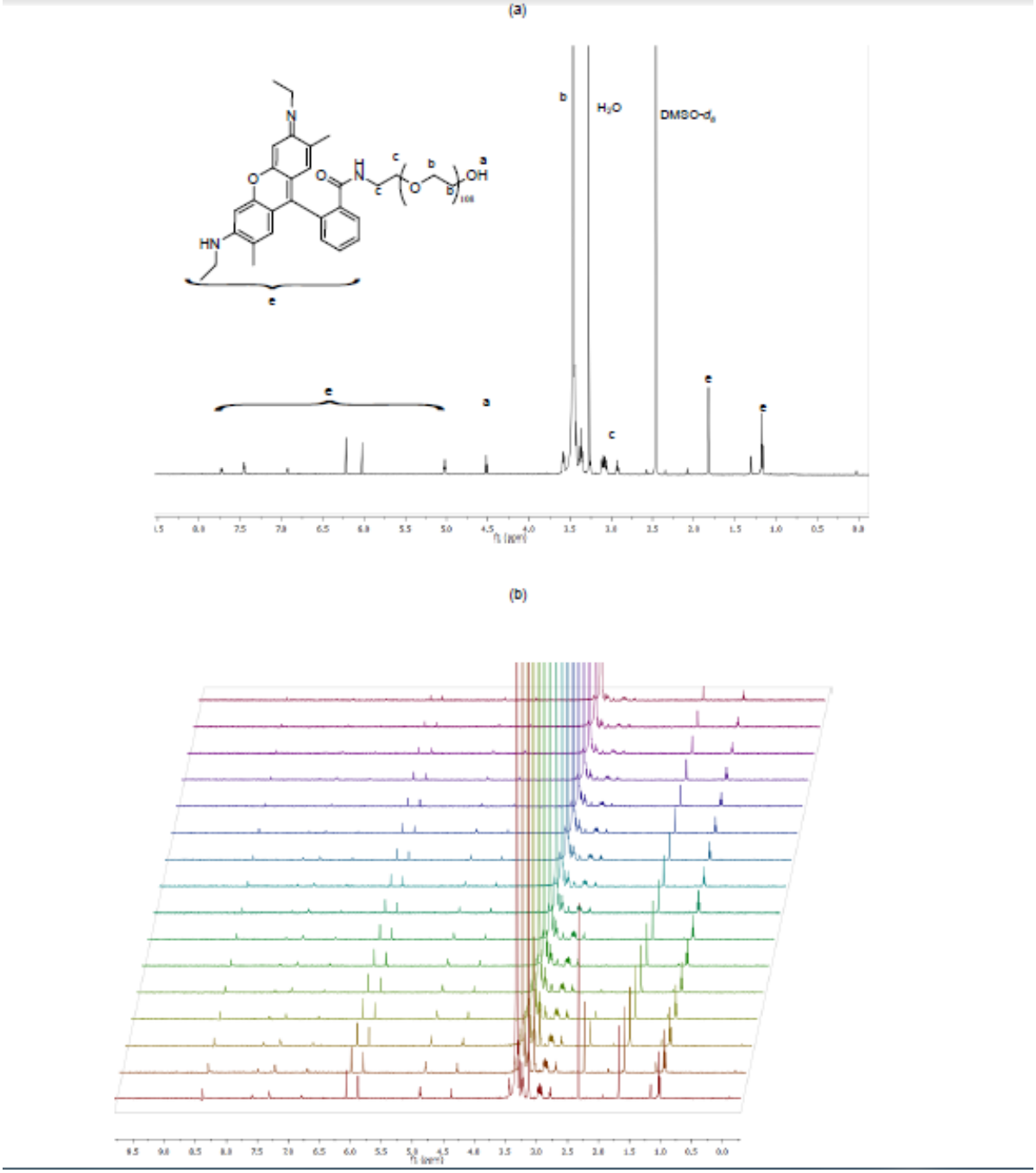

Figure S4. (a) ^{1}H NMR of the PEG-Rho6G conjugate in DMSO-d_6 at 600 MHz. Peaks in group e represent Rho6G while peaks at a and b are the peaks from PEG. (b) Diffusion ordered NMR of the PEG-Rho6G conjugate in DMSO-d_6 at 600 MHz. Rho6G associated peaks decay at the same rate as PEG peaks assuring conjugation.

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May 14, 2019

Dear Professor Laurencin,

Thank you for the opportunity to revise and resubmit our manuscript, "Altered Biodistribution and Tissue Retention of Nanoparticles Targeted with P-glycoprotein Substrates" to *Regenerative Engineering and Translational Medicine*. We have addressed each reviewer's comments/concerns and detailed the manuscript changes below. We hope that the paper is now suitable for publication in your journal

Sincerely,

David Putnam

COMMENTS TO THE AUTHOR:

Reviewer #1: A majority of candidates developed for CNS diseases never make it to the clinic. The failure is primarily attributed to the drugs' inability to cross the blood-brain barrier (BBB) at levels sufficient for a therapeutic effect. The tight junctions that connect the endothelial cells and the involvement of specific transporters (such as the P-glycoprotein, P-gp) dynamically regulate the movement of molecules across the BBB into the CNS. The P-gp is is responsible for actively moving drugs out of the brain. Over the years, drug delivery scientists have actively worked on manipulating the P-gp to enhance drug-retention within the brain, although to limited success. Here, the authors propose a new strategy to improve the local retention of nanoparticles at the BBB, in contrast to crossing it. This could have significant implications in the clinic, especially in terms of achieving efficacy. The manuscript is well written and the experiments are well designed.

Comments:

1) Include more details on the clinical/biological role of P-gp substrates in the introduction section.
We agree that it is important for the reader to understand the role of P-gp. We have clarified the biological/clinical role of P-gp in the Introduction, as requested, by including the following, "The biological role of P-gp is to protect tissues from the accumulation of toxins. Clinically, P-gp can serve to prevent drug accumulation in a desired anatomical site, or as a mechanism of drug resistance."

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2) The TEM images aren't clear. Please include images with a better contrast.
We have increased the contrast for the TEM images in Figure 2.

3) Please include details on long-term stability (at least 2-3 weeks) analysis of Rho6G-PEG-PLA NPs.
While we have not conducted official pharmaceutical stability studies on the nanoparticles, in our hands the lyophilized particles were re-suspended easily many weeks after formulation. The following text has been added to the formulation section in the materials and methods, "Nanoparticles were re-suspended in this fashion without trouble for at least four weeks after fabrication."

4) If possible, please include images of the whole mouse brain (wide field of view) to show NP accumulation at the site.
Unfortunately, we do not have these images available. The point is a good one and is well-taken, but the expertise to capture these images no longer exists in our lab.

5) This is a proof-of-concept study and demonstrating therapeutic efficacy is beyond the scope of this manuscript. However, addressing key issues pertaining to CNS drug delivery is necessary to justify this approach scientifically and generate excitement.

(a) P-gp expression has been shown to be expressed at higher levels in glioma compared to neurodegenerative disorders. This approach could enhance NP specificity to the BBB in general. Therefore, please include more details on how P-gp substrate mediated drug delivery could be of clinical value.
The reviewer is correct that this approach will not be amenable to all diseases that manifest in the CNS. We have included the following paragraph in the discussion section to make this clearer to the reader, "The incidence of neurologically-based diseases is continually growing. According to the 2013 Center for Disease Control report, Mortality Multiple Cause Micro-data Files, Alzheimer's disease and stroke are among the top 10 leading causes of death in the United States. The American Cancer Society reports brain cancer having only a 35% five-year survival rate. As of June 2009 only 8.2% of drugs developed for activity in the central nervous system have been approved for clinical use, making it the lowest percentage of all therapeutic classes. However, therapies for CNS diseases are among the top targets for drug development. The translation from drug discovery to development and clinical success is greatly dampened by delivery challenges to the CNS."

(b) However, reaching the BBB is only the first step in CNS delivery. P-gp is primarily expressed at the luminal surface of brain endothelial cells - implying its closer to the blood

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and away from the brain tissue. Tethering NPs to the P-gp necessarily don't imply that this will improve drug concentrations at the disease site. The drugs have to be released first and then traverse the BBB to reach the tissue. Since, there is no concrete evidence to justify this approach therapeutically, please list its possible limitations among others.

We do realize and appreciate the limitations that are associated with this approach to drug delivery to the brain. We also appreciate that the design we report is unlikely to be the most applicable delivery system. We used these architectures to demonstrate the potential opportunity available to the drug delivery community to use P-gp and its substrates as part of the targeted drug delivery arsenal. We included this text in the manuscript to focus the potential therapeutic opportunities for the field, "From a practical perspective, such a targeted delivery system could be used to deliver low molecular weight therapeutics designed to passively transport down a concentration gradient into the brain for treatment of CNS-based pathologies. Traditional low molecular weight therapeutics for the CNS are designed with octanol/water partition coefficients that permit sufficient solubility in both aqueous and non-aqueous solvents and can thereby passively diffuse across the BBB. One shortcoming of this small molecule design paradigm is that the compounds can passively diffuse across all cell membranes, leading to high volumes of distribution and adverse off-target effects. Biodistribution alteration of these compounds to increase their local concentration in the brain over time could serve to enhance the efficacy of some compounds, or perhaps rescue some therapeutics that have failed in the clinic due to poor side effect profiles."

(c) In addition, please include details on the molecular basis of BBB and how transporters including P-gp could increase or decrease solute permeability. This could serve as a template for future engineering based upon P-gp substrate mediated drug delivery.

We agree that the effects of transporters including P-gp can influence solute permeability across the BBB. In addition to the paragraph in the Discussion about the work with transferrin and insulin transporters, and the work focusing on GPCR-targeting, we have included the following text to emphasize these points specific to P-gp, "Of course, this approach will not work for therapeutic compounds that are themselves P-gp substrates, making the judicious pairing of the disease and the desired therapeutic imperative for therapeutic success."

(d) The authors highlight how sufficient levels of NPs are retained for 3h. However, the graphs also depict that nanoaprticles are eliminated from the BBB following 6h. This implies that therapeutic levels of drugs must be achieved within a time frame of 6h. Please address this as a limitation or explain briefly citing examples on how systems may be engineered to fine-tune drug release.

The reviewers are correct that the delivery system (targeted nanoparticles) may or may not be the best selection for the treatment of some CNS-based diseases. To this end, we have

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specifically stated in the manuscript that the purpose of the work was to demonstrate the potential opportunities for members of the drug delivery field to further explore and create new systems that build on these results to further the field.

Reviewer #2: Review Summary

Overall, this proof of concept research paper covers an important area of interest to the biomedical research community. In specific, the delivery of drug carrier devices across the blood brain barrier can be enhanced through their decoration with a P-glycoprotein substrate. The authors do a very good job of providing data that supports their targeted objective while also demonstrating the specificity of their approach. The paper is very well written with only minor errors and concerns detailed. The paper will be ready for publication after addressing these concerns and completing a final grammatical edit.

Comments

1) Page 4, Lay Summary - The word "target" or "targeted" is used a total of five times in this five sentence paragraph. Please choose some synonyms for some of these references to vary word choice.
Thank you. We have revised the Lay Summary accordingly, "The efficacy of medicines can be improved by diverting drugs to specific tissues. Finding new ways to target medicines to diseased tissue is an active area of research across disciplines. Drug-loaded nanoparticles, delivered to tissues of interest, are one way to accomplish this goal. The work reported in this manuscript explores the possibility of using small molecules to get nanoparticles to bind to a drug efflux pump, P-glycoprotein (P-gp) that is present in various tissues in the body. P-gp functions to remove drugs from tissues, and it is usually considered a hindrance to drug targeting. The research in this paper shows that natural function of P-gp can be used favorable to retain nanoparticles in various tissues."

2) Page 10, Line 48 - Page 11, Line 16 - It is unclear to the reader why Europium needs to be added in addition to the P-glycoprotein target as an imaging agent since Rhodamine 6G is already fluorescent. This also brings up a large question regarding what non-fluorescent P-glycoprotein targets may be available to facilitate similar brain drug delivery without providing fluorescence which may be not preferred for some applications.
Thank you for requesting this clarification. We have modified the text in the Results "In vivo biodistribution" section to make the use of Europium more clear, "Time-resolved fluorescence of europium encapsulated within the nanoparticles was used to track their biodistribution following intravenous administration. Although the fluorescence of the rhodamine conjugates could be used to quantify nanoparticle concentration in various tissues, the tissues themselves autofluoresce at similar wavelengths and on the same time

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scale, leading to a high background signal. However, elements in the lanthanide III series, like europium, exhibit large Stokes shifts and long fluorescent decay lifetimes and are amenable to time resolved fluorescence wherein their fluorescent signal remains detectable even after tissue autofluorescence and rhodamine fluorescence are completed, resulting in a very low background signal and high signal to noise ratio."

3) It may not be readily apparent to the reader, especially if they are new to the drug delivery field, why the PEG shell is necessary for PLA particles as well as why a PLA-only particle with not PEG was not tested as a control. A sentence or two explaining the rationale behind this would be helpful.
We agree. The following text was added in the discussion section to make this clear, "The function of PEG in this study was three-fold, first to serve as a tether for the rhodamine P-gp substrates, second to prevent non-specific aggregation of the nanoparticles, and third to prevent non-specific sequestration of the nanoparticles by the liver and spleen."

4) Page 16, Lines 36 - 56 - The rationale for retention of nanoparticles at the blood brain barrier instead of transport across the membrane is helpful for the reader to know in the introduction as well as being reiterated here. With this in mind, additional content should be added to the introduction.
We agree and have included the following text in the Introduction, "The work presented herein establishes that P-gp substrates (i.e., small molecules that are actively transported by P-gp out of the CNS) can enhance the retention of nanoparticles in the brain. The premise is that a P-gp substrate tethered to the surface of a nanoparticle through a water soluble polymer linker binds to the P-gp expressed in the endothelium, leading to nanoparticle retention in the capillary lumen. For this work, the nanoparticles were not designed to be transported across the blood-brain barrier. Instead, they were designed to be captured by P-gp at the capillary endothelium and retained within the bloodstream. In this way, drugs could be release within the capillaries and enter the brain by passive diffusion down the concentration gradient."

5) Page 21, Figure 1 - The cartoon shows a singular Rhodamine 6G attached to the PEG-PLA particle surface. As there are expected to be many molecules, it would be more accurate to show a PEG corona with many substrate molecules.
The reviewer is correct that the particles are multivalent; however, we have drawn the figure both ways (single rhodamine and multiple rhodamines) and found the multiple rhodamine figure to be more confusing than clarifying. However, we have modified the legend of Figure 1 to account for the multivalent nature of the particle. The legend now reads, "Figure 1 The binding site for rhodamine on P-gp is on the inner intracellular leaflet of the transmembrane protein [37]. While counter intuitive (and energetically unfavorable) the tissue retention of the targeted nanoparticles suggests that the tethered substrate might

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transverse the cell membrane to bind to its receptor site. A possible mechanism, which requires additional investigation, is shown here where the PEG linker/P-gp substrate on a drug carrier (A) spans the cell membrane (B) allowing the P-gp substrate to bind to P-gp (C) and retain the drug carrier in the capillary. Please note that only one P-gp substrate is drawn on the nanoparticle to make the potential mechanism visually clear, whereas in practice there are multiple P-gp substrates in each nanoparticle."

6) Page 28, Table 2 - While the increased accumulation at the brain is > 2x, the enhanced delivery to other tissues (i.e. ~ 4.5 x for liver and kidney ~ 3x for heart, and ~ 2.5x for lung) is greater. The concerns and/or opportunities with the build-up of nanoparticles in these other tissues should be more clearly discussed in the text. Also, opportunities to modify the targeting molecule to preferentially target brain P-glycoprotein should be mentioned.
This is correct, and we have included the following text in the same section that speaks to the values in Table 2, "It is also important to acknowledge that the AUC of the targeted nanoparticles is also enhanced in other organs (i.e., liver, spleen, kidney, heart, lung) and that this accumulation should be taken into consideration when exploring this targeting approach with specific drugs and disease states."