

Supporting Information

for

**Evaluation of Nanoparticle Delivered Cisplatin in
Beagles[†]**

*Brittany Feldhaeusser^{b‡}, Simon R. Platt,^{b,e} Sean Marrache^{a‡}, Nagesh Kolishetti^{a,c}, Rakesh K. Pathak^a, David J. Montgomery^a, Lisa R. Reno^b, Elizabeth Howerth^{e,d}, and Shanta Dhar^{*a,e}*

^aNanoTherapeutics Research Laboratory, Department of Chemistry

^bDepartment of Small Animal Medicine and Surgery, College of Veterinary Medicine

^cPartiKula LLC, 7777 Davie Rd., Hollywood, FL 33024

^dDepartment of Pathology, College of Veterinary Medicine

^eRegenerative Bioscience Center

University of Georgia, Athens, GA, 30602

‡Equally contributed authors

**To whom correspondence should be addressed.*

E-mail: shanta@uga.edu

Materials and Methods. All chemicals were received and used without further purification unless otherwise noted. Cisplatin was purchased from Strem Chemicals, Inc. Dimethylaminopyridine (DMAP), K_2PtCl_4 , KCl, N-hydroxysuccinimide (NHS), triethylamine, 5-bromopentanoic acid, 6-bromohexanoic acid, sodium azide, N,N'-dicyclohexylcarbodiimide (DCC), hydrogen peroxide solution (30 wt.% in H_2O), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Dibenzocyclooctynes (DBCO)-amine was procured from Click Chemistry Tools Bioconjugate Technology Company. Carboxy terminated PLGA (dL/g, 0.15 to 0.25) was procured from Lactel and OH-PEG-OH of molecular weight 3350 was purchased from Sigma Aldrich. Triphenylphosphine (TPP), oligomycin, rotenone, antimycin-A, and trifluorocarbonyl cyanide phenylhydrazone (FCCP) were purchased from Sigma Aldrich. XF24-well cell culture microplates and Seahorse XF-24 cell Mito Stress Test Kit were purchased from Seahorse Bioscience.

Distilled water was purified by passage through a Millipore Milli-Q Biocel water purification system (18.2 $M\Omega$) containing a 0.22 μm filter. Cells were counted using a Countess® Automated cell counter procured from Invitrogen life technology. Gel permeation chromatographic (GPC) analyses were performed on a Shimadzu LC20-AD prominence liquid chromatographer equipped with a refractive index detector and Waters columns; molecular weights were calculated using a conventional calibration curve constructed from narrow polystyrene standards using tetrahydrofuran (THF) as an eluent at a temperature of 40 °C. Dynamic light scattering (DLS) and zeta potential measurements were carried out using a Malvern Zetasizer Nano ZS system. Inductively coupled plasma mass spectrometry (ICP-MS) studies were performed on a VG PlasmaQuad 3 ICP mass spectrometer. Plate reader analyses were performed on a Bio-Tek Synergy HT microplate reader. Bioenergetic assays were carried out using a Seahorse XF24

analyzer (Seahorse Biosciences, North Billerica, MA, USA). Clinical chemistry analyses were performed at UGA College of Veterinary Medicine using a Hitachi P-module biochemical analyzer. Hematological analyses were carried out on a Bayer-Advia 120 hematology analyzer.

Cell Culture and Handling. Canine J3TBG glioma and canine SDT3G glioblastoma cells were generously donated from the Dickinson Lab at UC Davis School of Veterinary Medicine. Cells were grown in complete media, consisting of Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% 200 mM L-glutamine. Cells were cultured in 75 cm² Falcon® flasks with vented caps and stored in a VWR® Symphony water-jacketed incubator at 37°C and 5% CO₂. When harvested, cells were washed and treated with phosphate buffered saline (PBS) and 0.05% trypsin, collected in 15 mL Corning® conical vials, and counted using a Countess® Automated cell counter from Invitrogen life technology. Cells were passaged every 3 to 4 days.

Synthesis of Platin-M, T-Platin-M-NP, NT-Platin-M-NPs. Construction of Platin-M, targeted polymer PLGA-*b*-PEG-TPP,¹ non-targeted polymer PLGA-*b*-PEG-OH, T-Platin-M-NPs, and NT-Platin-M-NPs with 30% Platin-M feed were carried out by following methods reported by us.²

MTT Cytotoxicity Assays. The cytotoxicity of cisplatin, Platin-M, NT-Platin-M-NPs, and T-Platin-M-NPs was tested in both J3TBG and SDT3G cell lines. Cells were washed, trypsinized, and plated at 3,000 cells/well in 96-well Corning® flat-bottomed tissue culture plates, then allowed to attach overnight at 37 °C, 5% CO₂. The media was changed and compounds were added at varied concentrations and incubated for either 24 or 72 h. The wells for 24 h studies had their media changed after 24 h and then were allowed to incubate for the remaining 48 h. After the incubation period, 20 μL (5 mg/mL) MTT was added to each well and incubated for 5 h to

allow cellular uptake. The media was removed and 100 μL DMSO per well was added to lyse cells and dissolve the formazan to elicit a color change. Absorbance levels were recorded after 5 min of gentle shaking on a Biotek Synergy HT plate reader at 550 nm with 800 nm background. Data was fitted to a sigmoidal curve and IC_{50} values reported at $\pm 99\%$ confidence interval using a three parameters logistics model in GraphPad Prism (San Diego, USA).

Mito-stress Test Analysis: To get the insight for mitochondrial profile upon treatment with cisplatin, Platin-M, NT-Platin-M-NP, and T-Platin-M-NP formulations, four parameters of mitochondrial functions, rate of basal respiration, ATP linked respiration, proton leak and spare respiratory capacity were investigated by using Seahorse XF-24 cell Mito Stress Test Kit. Prior to the assay, XF sensor cartridges were hydrated. To each well of an XF utility plate, 1.5 mL of Seahorse Bioscience XF calibrant (pH=7.4) was added and the XF sensor cartridges were placed on top of the utility plate, and kept at 37 °C incubator without CO_2 for a minimum of 12 h. SD3TG, and J3TBG cells were cultured in XF24-well cell culture microplates (Seahorse Bioscience) at a density of 40×10^3 cells/well in 200 μL growth medium and then incubated for 24 h at 37 °C in 5% CO_2 atmosphere. The cells were treated with Platin-M (1 μM), cisplatin (1 μM), T-Platin-M-NPs, NT-Platin-M-NPs (1 μM with respect to Platin-M) for 36 h at 37 °C in 5% CO_2 atmosphere. After 24 h, 150 μL of the culture medium was removed from each well and the cells were rinsed three times with 450 μL of XF DMEM assay media pre-warmed to 37 °C and finally 450 μL of XF DMEM assay media was added to each well and the plate was placed at 37 °C without CO_2 for 1 h prior to assay. Different parameters of respiration were calculated by subtracting the average respiration rates before and after the addition of the electron transport inhibitors oligomycin (1.0 μM), trifluorocarbonylcyanide phenylhydrazone or FCCP (1.0 μM), an ionophore that is a mobile ion carrier, and a mixture of antimycin-A (1.0 μM) which is a

complex III inhibitor and rotenone (1.0 μM), a mitochondrial inhibitor that prevents the transfer of electrons from the Fe-S center in Complex I to ubiquinone. The parameters calculated included: basal respiration (baseline respiration minus antimycin-A post injection respiration), ATP turnover (baseline respiration minus oligomycin post injection respiration), maximal respiratory capacity (FCCP stimulated respiration minus antimycin-A post injection respiration) and reserve respiratory capacity (FCCP stimulated respiration minus baseline respiration). Test articles on each well had three or four replicates.

In Vivo Safety Study and BioD. For initial bioD and safety studies, two female purpose bred beagles, aged 7-8 years old and weighing 8.9-13.3 kg, were used for the study in accordance with institutional approval. Prior to their use in the study, a physical and neurologic examination, complete blood count, serum chemistry panel, and CSF analysis were performed. Dogs were anesthetized with 6 mg/kg intravenous propofol in order to obtain CSF. On day 0, a single intravenous injection of T-Platin-M-NPs at 0.5 mg/kg with respect to Platin-M was diluted to 1 mL and given intravenously. Dogs were monitored for 6 h post-injection, recording heart rate, respiration rate, temperature, and blood pressure. On day 1, complete examinations were performed and blood and CSF were collected to repeat the clinical pathology. Daily physical and neurologic exams were performed until day 7, when the complete blood count, serum chemistry panel, and CSF analysis were repeated and additional plasma and CSF stored. Examinations were then performed every other day until day 14 when the aforementioned parameters were repeated again and the study was terminated by intravenous injection of euthanasia solution (1 mL/10 lbs). Necropsy was performed immediately. Spleen, kidneys, liver, heart, lungs, and brain were removed, cleaned of excess material and weighed. Samples for histopathology were fixed in 10% buffered formalin, routinely processed and embedded in paraffin, sectioned at 4 μm and

stained with hematoxylin (H) and eosin (E). Sections of liver and kidney were also stained with Perls iron stain. Representative samples weighing approximately 1 g were taken from each organ, dissolved in 70% nitric acid with gentle heating and shaking, and analyzed for Pt contents using ICP-MS to determine bioD of the drug. To evaluate the distribution of the drug in the plasma and CSF, the frozen plasma and CSF samples were thawed and submitted for ICP-MS for Pt contents.

Dose Dependent 14-Day Safety Study in Dogs. Safety studies were carried out in beagles (7-8 years old) at 2-2.2 mg/kg of T-Platin-M-NPs for 14 days following methods described above. Briefly, prior to dosing, a physical and neurologic examination, complete blood count, serum chemistry panel, and CSF analysis were performed after which a single injection of T-Platin-M-NPs at 2-2.2 mg/kg with respect to Platin-M was diluted to 20 mL and given intravenously over a period of 15 min. Post-injection monitoring of the animals for 14 days and examinations were performed in the same manner as mentioned above.

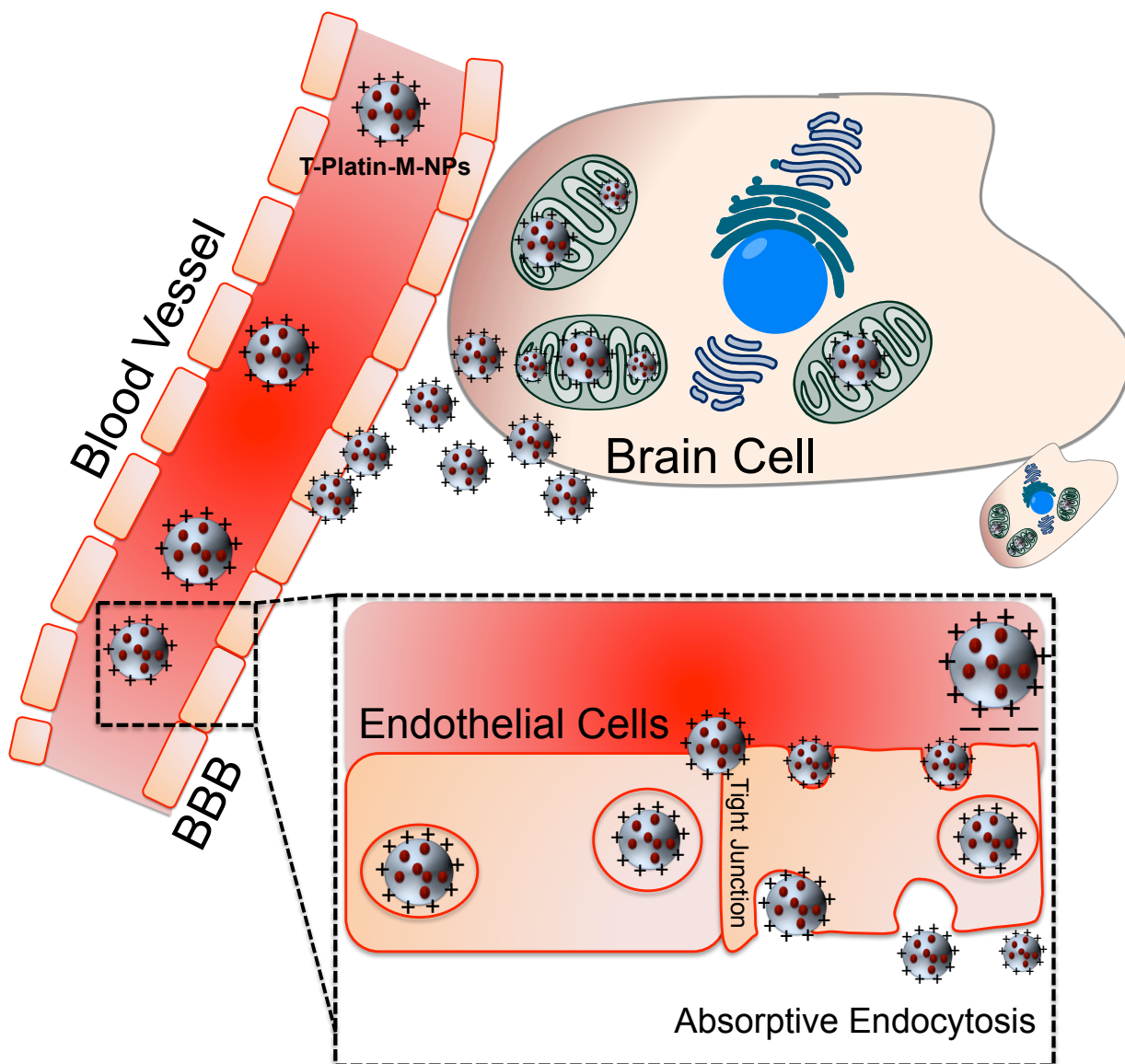


Figure S1. Proposed transport mechanisms of T-Platin-M-NPs through the BBB via absorptive interactions based on the surface charge, lipophilicity, and size of T-Platin-M-NPs, and subsequent association with mitochondria of brain cells.

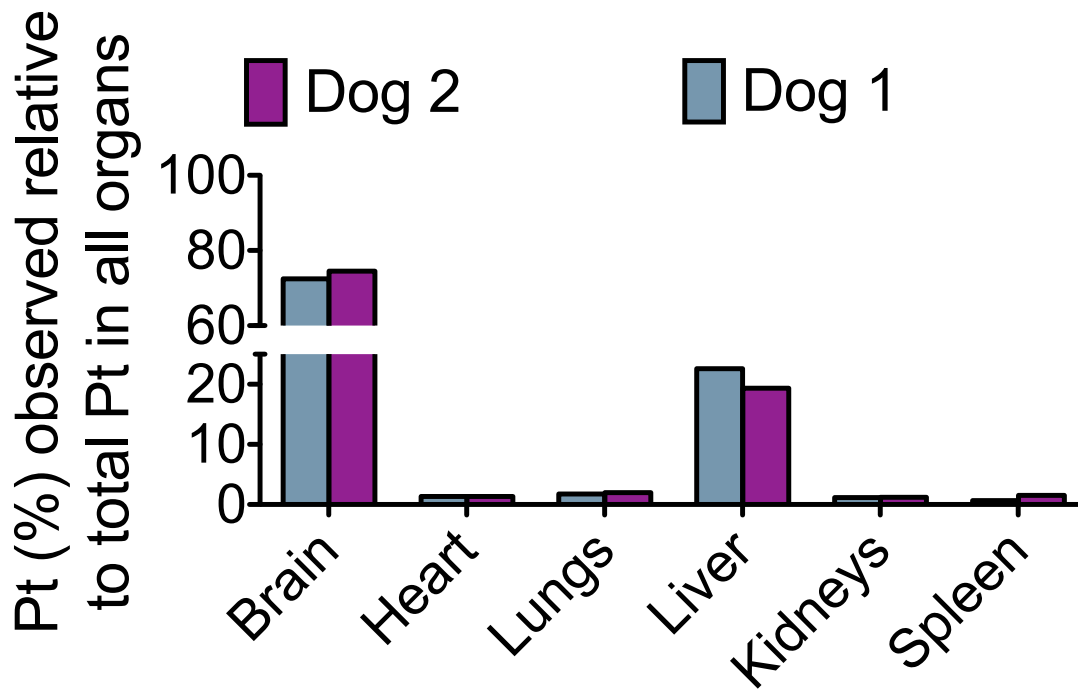


Figure S2. Platinum concentration in organs 14 days after single intravenous injection of T-Platin-M-NPs in two beagles from Day 14 post-injection.

References:

1. Marrache, S.; Dhar, S. Engineering of Blended Nanoparticle Platform for Delivery of Mitochondria-Acting Therapeutics. *Proc Natl Acad Sci USA* **2012**, *109*, 16288-16293.
2. Marrache, S.; Pathak, R. K.; Dhar, S. Detouring of Cisplatin to Access Mitochondrial Genome for Overcoming Resistance. *Proc Natl Acad Sci USA* **2014**, *111*, 10444-10449.