Permeability of the Blood-Brain Barrier to a Rhenacarborane

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Received October 1, 2008; accepted January 28, 2009

ABSTRACT

The treatment of brain malignancies with boron neutron capture therapy depends on their ability to cross the blood-brain barrier (BBB). An especially promising class of boron-containing compounds is the rhenacarboranes that, if able to cross the BBB, could act as delivery vehicles as well as a source of boron. Here, we examined the ability of the 3-NO-3,3- κ^2 -(2,2'-N₂C₁₀H₆(Me){[(CH₂)₇¹³¹I]-4,4'}-closo-3,1,2-ReC₂B₉H₁₁ (rhenacarborane) labeled with iodine-131 to be taken up into the bloodstream after subcutaneous administration and to cross the BBB. The ¹³¹I-rhenacarborane was quickly absorbed from the injection site and reached a steady state in arterial serum of 2.59%/ml of the administered dose. Between 73 and 95% of the radioactivity in serum 6 h after administration represented intact ¹³¹I-rhenacarborane. Its octanol/buffer partition coefficient was 1.74, showing it to be lipophilic. Tissue/serum ratios

for brain, lung, and liver showed classic patterns for a lipid-soluble substance with high levels immediately achieved and rapid redistribution. For brain, a steady state of approximately 0.107% of the administered dose/gram-brain was rapidly reached, and 71% of the radioactivity in brain 6 h after subcutaneous administration represented intact ¹³¹I-rhenacarborane. Steady-state values were 1.53 and 0.89% of the injected dose per gram for lung and liver, respectively. ¹³¹I-Rhenacarborane was quickly effluxed from brain by a nonsaturable system after its injection into the lateral ventricle of the brain. In conclusion, these results show that a rhenacarborane was enzymatically resistant and able to cross the BBB by transmembrane diffusion and accumulate in brain in substantial amounts. This supports their use as therapeutic agents for targeting the central nervous system.

Boron neutron capture therapy (BNCT) holds promise as a treatment for various malignancies of the central nervous system (CNS). For example, the method is currently used as an adjunct to surgery in the treatment of glioblastoma multiforme (Yamamoto et al., 2008). The effectiveness of BNCT relies on its ability to eradicate micrometastases in the brain that are otherwise undetectable by surgical methods (Pisarev et al., 2007). BNCT uses an isotope of boron to deliver targeted bursts of radiation to malignant cells. Upon uptake into the cells, boron-10 is externally irradiated with neutrons and becomes unstable, decaying to lithium and releasing a

high-energy α particle. A further advantage is that boron can form polyhedral clusters that allow for the delivery of multiple boron-10 atoms in a single molecule. The potency of treatment as well as the selectivity of neutron captures by the boron atom makes this option an encouraging alternative to other more toxic therapies.

An especially promising class of boron compounds are the closo-3,1,2-ReC₂B₉ rhenacarboranes, which consist of nido-icosahedral carborane cages η^5 -bonded to the *d*-block transition element rhenium (Hawthorne, 1968; Blandford et al., 1998; Fischer et al., 2005; Sogbein et al., 2005; Armstrong and Valliant, 2007). Besides containing multiple boron vertices in a three-dimensional superaromatic structure, these compounds have the ability to bind additional ligands at the metal center, which can in turn be designed to tether pharmacologically active molecules. Thus, rhenacarboranes could potentially act as delivery vehicles for a wide range of diag-

This work was supported in part by the Veterans Affairs Merit Review; the National Institutes of Health [Grants R01-NS051334, R01-NS050547, R01-AG029839]; and Beaumont Research Grant.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.108.146878.

ABBREVIATIONS: BNCT, boron neutron capture therapy; CNS, central nervous system; BBB, blood-brain barrier; THF, tetrahydrofuran; PPh₄Br, tetraphenylphosphonium bromide; IR, infrared; TLC, thin layer chromatography; DMSO, dimethyl sulfoxide; %Inj/g, percentage of an intravenous dose taken up per gram of brain; %Inj/brain, residual in brain expressed as a percentage of the dose injected into the lateral ventricle of the brain; vis., visible; PC, partition coefficient; complex **1**, 3,3-(CO)₂-3-NO-closo-3,1,2-ReC₂B₉H₁₁; complex **2**, PPh₄[3-Br-3-CO-3-NO-closo-3,1,2-ReC₂B₉H₁₁]; complex **3**, 3-NO-3,3- κ^2 -(2,2'-N₂C₁₀H₆(Me){[(CH₂)₇Br]-4,4'}-closo-3,1,2-ReC₂B₉H₁₁]; complex **4**, 3-NO-3,3- κ^2 -(2,2'-N₂C₁₀H₆(Me){[(CH₂)₇¹³¹I]-4,4'}-closo-3,1,2-ReC₂B₉H₁₁].

nostic or therapeutic agents. Indeed, Valliant and colleagues have investigated the chemistry of rhenacarborane species as a means to develop convenient synthetic pathways to imaging reagents that incorporate the rhenium congener technetium, the most commonly used imaging radioisotope (Sogbein et al., 2004; Green et al., 2006; Causey et al., 2008).

Optimal utility of rhenacarboranes would require them to cross the blood-brain barrier (BBB). The BBB represents several monolayer barriers that regulate the entry of substances from the blood into the CNS (Neuwelt et al., 2008). The vascular BBB, formed of brain endothelial cells, is generally the most important of these for delivery of drugs to deep brain tissues and represents a significant obstacle for penetration by chemotherapeutics into the CNS. Hydrophobic, low-molecular-weight compounds can diffuse through these membranes with great success (Oldendorf, 1974). Boron-based molecules such as the rhenacarboranes are probably very lipid-soluble because of their low ionic charges. However, many lipid-soluble drug candidates are unable to accumulate in the CNS because they are substrates for brain-to-blood transport systems such as *p*-glycoprotein, or they are concentrated and excreted by liver (Begley, 2004). The capacious metallacarborane cage structure in combination with the presence of the heavy *d*-block rhenium raises the possibility that the volumetric size, molecular weight, or both of the rhenacarboranes might exclude them from crossing the BBB. Here, we characterize the ability of a rhenacarborane to be taken up by brain in comparison with other tissues.

Materials and Methods

Syntheses and Radioactive Labeling

General Considerations. Solvents (CH_2Cl_2 , hexanes, Et_2O , and THF) were distilled over appropriate drying agents under argon before use. Acetone (ASC reagent grade) was purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. All reactions were carried out under a dry, oxygen-free argon atmosphere using Schlenk line and glove-box techniques. Complex **1** and ligand 2,2'- $\text{N}_2\text{C}_{10}\text{H}_6(\text{Me})[(\text{CH}_2)_7\text{Br}]-4,4'$ (4-methyl-4'-bromoheptyl-2,2'-bipyridyl) were prepared using literature methods (Ellison and Iwamoto, 1983; Ellis et al., 1999). The reagent tetraphenylphosphonium bromide (PPh_4Br) was purchased from Aldrich Chemical Co. and used as received.

Instrumentation. All solution measurements were made at 298 K. IR measurements were made using solution cells in an RX-I FTIR spectrometer (PerkinElmer Life and Analytical Sciences, Boston, MA). NMR measurements were recorded using a 300-MHz spectrometer (Varian, Inc., Palo Alto, CA): ^1H (300.0 MHz), ^{13}C (75.4 MHz), and ^{11}B (96.3 MHz). NMR chemical shifts, δ , are given in ppm and referenced versus SiMe_4 (^{11}B versus external $\text{BF}_3\cdot\text{OEt}_2$). Coupling constants, J , are given in Hertz. The bipyridyl ring numbering scheme is given in Scheme 1.

Synthesis of Precursor Complex 2. Complex **1** (0.27 g; 0.67 mmol) was combined with PPh_4Br (0.37 g; 0.88 mmol), and the reactants were dissolved in THF (50 ml) in a two-necked Schlenk flask-fitted with a condenser and attached to an Ar/Schlenk line. The

reaction mixture was refluxed under Ar for 48 h, after which time solvent was removed in vacuo. The residue was recrystallized from $\text{CH}_2\text{Cl}_2/\text{Et}_2\text{O}$ (20 ml; 1:1) affording red microcrystals of complex **2** (0.40 g; 74%). Data for **2** (298 K): IR (CH_2Cl_2) (cm^{-1}) $\nu_{\text{max}}(\text{BH})$ 2550m, $\nu_{\text{max}}(\text{CO})$ 1974s, $\nu_{\text{max}}(\text{NO})$ 1715s; ^1H NMR (CD_2Cl_2) δ 7.93, 7.76, 7.63 (m $\times$ 3, 20 H, Ph), 3.62, 2.59 (s br $\times$ 2, 2 H, cage CH); $^{13}\text{C}\{^1\text{H}\}$ NMR (CD_2Cl_2) δ 206.3 (s, CO), 136.1 (d, *p*-C, Ph, $J_{\text{PC}} = 3$), 134.8 (d, *o*-C, Ph, $J_{\text{PC}} = 10$), 130.9 (d, *m*-C, Ph, $J_{\text{PC}} = 12$), 117.9 (d, *i*-C, Ph, $J_{\text{PC}} = 90$), 50.6, 46.5 (s br $\times$ 2, cage CH); $^{11}\text{B}\{^1\text{H}\}$ NMR (CD_2Cl_2) δ -3.7, -5.5, -6.9, -8.2, -9.5, -11.9, -16.6, -19.3, -20.6 (s br $\times$ 9, 9 B); UV-visible (CH_2Cl_2) λ_{max} (nm) ($\epsilon \times 10^{-3} (\text{M}^{-1} \text{cm}^{-1})$) 338 (1.5), 413 (0.32), 519 (0.41). Analysis Calculated for $\text{C}_{27}\text{H}_{31}\text{B}_9\text{BrNO}_2\text{PRe}$: C, 22.5; H, 5.3; N, 4.8; Br 13.5%. Found: C, 22.8; H, 5.4; N, 4.7; Br 13.3%.

Synthesis of Complex 3. Complex **2** (0.25 g; 0.29 mmol) was combined with the ligand 2,2'- $\text{N}_2\text{C}_{10}\text{H}_6(\text{Me})[(\text{CH}_2)_7\text{Br}]-4,4'$ (0.11 g; 0.31 mmol) in a two-necked Schlenk flask. The reagents were dissolved in THF (30 ml), and the solution was refluxed for 24 h, during which time the color changed from the deep red of the starting compound **2** to dark green. Solvent was removed in vacuo, and the compound was then chromatographed on silica gel. Elution with CH_2Cl_2 /hexanes (1:1) afforded a deep green fraction. Solvent was removed in vacuo and the residue crystallized from CH_2Cl_2 /hexanes (10 ml; 1:2) to afford dark green microcrystals of complex **3** (0.12 g, 54%). Data for **3** (298 K): IR (CH_2Cl_2) (cm^{-1}) $\nu_{\text{max}}(\text{BH})$ 2560m, $\nu_{\text{max}}(\text{NO})$ 1664s; ^1H NMR (CD_2Cl_2) δ 9.30, 9.28 (d $\times$ 2, 2 H, $\text{H}^{6,6'}$, $J_{\text{HH}} = 6$, 6), 8.17, 8.14 (s $\times$ 2, 2H, $\text{H}^{3,3'}$), 7.42 (d, 2 H, $\text{H}^{5,5'}$, $J_{\text{HH}} = 6$), 3.43 (t, 2 H, CH_2Br , $J_{\text{HH}} = 7$), 2.90 (t, 2 H, 4- CH_2 , $J_{\text{HH}} = 8$), 2.67 (s, 3 H, 4'-Me), 1.86, 1.80 (m $\times$ 2, 4 H, $\text{bipyCH}_2\text{CH}_2$ and $\text{CH}_2\text{CH}_2\text{Br}$), 1.76 (s br, 2 H, cage CH), 1.43 [m, 6 H, $\text{bipyCH}_2\text{CH}_2(\text{CH}_2)_3$]; $^{13}\text{C}\{^1\text{H}\}$ NMR (CD_2Cl_2) δ 160.8, 160.7 (s $\times$ 2, $\text{C}^{2,2'}$), 159.7, 159.2 (s $\times$ 2, $\text{C}^{6,6'}$), 157.9, 153.5 (s $\times$ 2, $\text{C}^{4,4'}$), 128.7, 128.0, 123.9, 123.2 (s $\times$ 4, $\text{C}^{3,3'}$ and $\text{C}^{5,5'}$), 40.8 (s br, cage C), 38.2 (s, 4- CH_2), 34.6 (s, CH_2Br), 33.3, 31.1, 30.0, 29.0, 28.5 [s $\times$ 5, $\text{bipyCH}_2(\text{CH}_2)_3$], 22.1 (s, 4'-Me); $^{11}\text{B}\{^1\text{H}\}$ NMR (CD_2Cl_2) δ -2.8 (s br, 2B), -4.1 (s br, 1B), -6.1 (s br $\times$ 2, 3B), -16.1 (s br $\times$ 2, 3B); UV-visible (CH_2Cl_2) λ_{max} (nm) ($\epsilon \times 10^{-3} (\text{M}^{-1} \text{cm}^{-1})$) 250 (19.5), 305 (20.1), 383 (6.4), 663 (0.70); Analysis Calculated for $\text{C}_{20}\text{H}_{34}\text{B}_9\text{BrN}_3\text{ORe}$ (695.9 Da): C, 34.4; H, 4.9; N, 6.0; Br 11.5%. Found: C, 34.0; H, 4.7; N, 6.0; Br 11.3%.

Radioactive Labeling. Complex **3** (1–2 mg) was radioactively labeled by treatment of an acetone solution (10 ml) with 2 mCi of Na^{131}I in a test tube. After being stirred overnight, the solution was spotted on a TLC plate and eluted in a chamber containing CH_2Cl_2 /hexanes (1:1) as solvent. The iodo-substituted product (complex **4**) (742.9 Da) was readily identified as a dark green fraction and was consequently scraped off into a beaker. The fraction was then dissolved in CH_2Cl_2 and purified using a syringe filter to remove the silica. The excess solvent was evaporated with air, and the remaining product was reconstituted in DMSO. Unlabeled complex **4** could be produced in the same manner using NaI.

Subcutaneous Injection Studies

For this and all other animal studies, 2-month-old CD-1 male mice from our in-house colony (VA Medical Center, St. Louis, MO) were used. All animal studies were approved by the local animal use committee. For this analysis, tissue/serum ratios for time points ranging from 2 min to 6 h after injection were plotted against time. A 50- μl sample of complex **4** dissolved in DMSO was injected under the skin. At each time point (*t*), arterial blood was collected from the carotid artery; the mouse was decapitated; and the brain, liver, injection site, kidney, retroperitoneal fat, and lung were harvested. A 50- μl sample of arterial serum was collected from the blood after centrifugation at 5000 rpm for 10 min at 4°C. All tissue and serum samples were then placed in a gamma counter to detect the level of radioactivity in each. Tissue/serum ratios were plotted against time to determine the rate of uptake of complex **4** into various tissues. The tissue/serum ratio in units of microliters per gram was calculated as follows:

Scheme 1. Numbering scheme for bipyridyl ligands.

$$\frac{\text{tissue}}{\text{serum}} = \frac{C_t \times 50 \mu\text{l}}{C_s \times (\text{tissue wt. (g)})} \quad (1)$$

where C_t represents the level of radioactivity in the respective tissue, and C_s represents the level of radioactivity in the 50- μl serum sample. The percentage of the injected dose of complex **4** that was present in a milliliter of serum (%Inj/ml) was calculated from the following equation:

$$\% \text{Inj/ml} = 100(20 \times C_s)/(C_t) \quad (2)$$

where C_i represent the cpm injected subcutaneously. The percentage of the injected dose that was present in a gram of tissue (%Inj/g) was calculated from the following equation:

$$\% \text{Inj/g} = 100 \times C_t/(\text{tissue wt.} \times C_i) \quad (3)$$

Determination of the Octanol/Buffer Partition Coefficient

Lipid solubility of the boron compound was measured by determination of an octanol/buffer partition coefficient. This protocol involved adding 5 μl of complex **4** (73,625 cpm) to 500 μl of 0.25 M chloride-free sodium phosphate buffer, pH 7.5, and then vigorously mixing with 500 μl of octanol for 1 min. The two phases were separated by centrifugation at 5000 rpm for 10 min. Then, 100- μl aliquots were taken from each phase and counted in a gamma counter. This procedure was done in triplicate, and a mean partition coefficient (PC) obtained, was expressed as follows:

$$\frac{\text{octanol}}{\text{buffer}} \text{ PC} = \frac{(\text{octanol phase cpm})}{(\text{chloride} - \text{free sodium phosphate buffer phase cpm})} \quad (4)$$

The log value for this coefficient can be either positive or negative. A positive log value indicates a lipophilic compound, whereas a negative value represents a lipophobic (hydrophilic) compound.

Effect of DMSO on BBB Integrity

Previous work has found that DMSO may (Broadwell et al., 1982) or may not (Neuwelt et al., 1983) disrupt the BBB. We determined whether our subcutaneous injection of DMSO altered the BBB to albumin, the substance traditionally used to measure BBB disruption that has a diameter similar to that of complex **4**. For this study, 50 μl of either saline or DMSO was injected subcutaneously followed by a 200- μl (500,000-cpm) intravenous injection into the jugular vein of ^{125}I -labeled albumin. Albumin was labeled by the chloramine-T method and purified on a Sephadex G-10 column as described previously (Banks et al., 1991). Between 10 min to 2 h after the intravenous injection of ^{125}I -albumin, brains and samples of carotid artery blood were collected as described above. Radioactivity in brain and serum samples was detected for ^{125}I using a gamma counter. Brain/serum ratios were determined and plotted against exposure time (Blasberg et al., 1983; Patlak et al., 1983). Exposure time (Expt) was calculated as follows:

$$\text{Expt} = \frac{\int_0^t C_p(t) dt}{C_p t} \quad (5)$$

where C_p represents the level of radioactivity in the serum and $C_p t$ is radioactivity in the serum at time t . Exposure time corrects for clearance of ^{125}I -albumin from blood over time; not correcting for clearance would overestimate the rate of entry into brain. The slope of the linear portion of the relation between brain/serum ratios and exposure times measures the blood-to-brain unidirectional influx rate (K_i) of ^{125}I -albumin. The intercept of the linearity measures the initial volume of distribution (V_i) in brain. Disruption or increased

leakage of the BBB can be reflected in either an increase in the K_i or V_i for albumin.

Identification of Radioactivity in Serum and Brain

Whole brain collected 6 h after the subcutaneous injection of complex **4** was homogenized in 1 ml of distilled water and then centrifuged at 4500g for 15 min. The resulting supernatant was lyophilized. Arterial serum and urine were lyophilized. The lyophilized materials were reconstituted in 1 ml of acetone and spotted on a TLC plate as described above. The percentage of radioactivity eluting in the same band as complex **4** was calculated and reported as representing intact complex **4**.

Brain-to-Blood Efflux

The intracerebroventricular injection method was used as described previously (Banks et al., 1997). Two-month-old male ICR mice were anesthetized on day of study with 0.15 ml of 40% urethane. The scalp was removed, and a hole was made into the lateral ventricle, 1.0 mm lateral and 0.2 mm posterior to the bregma, with a 26-gauge needle with a tubing guard that kept the depth of the holes constant (2.0–2.5 mm). Mice received 1.0 μl of intracerebroventricular injections containing 5×10^3 cpm of complex **4**. Mice were decapitated at 2, 5, 10, and 20 min after injection. The whole brain was removed, the pituitary and pineal glands were discarded, and the level of residual radioactivity in the whole brain was determined from the counts after 3 min in a gamma counter. The level of radioactivity in whole brain at $t = 0$ was determined in mice overdosed with anesthetic as described previously (Banks et al., 1997). Mice were done in sets of three and the mean value of the cpm remaining in the brain was divided by the cpm injected and multiplied by 100 to yield the percentage of the injected dose remaining in brain (%Inj/brain). The log of %Inj/brain was regressed against time. A significant correlation between log(%Inj/brain) and time indicates a measurable efflux from brain to blood. The half-time clearance was calculated by multiplying the inverse slope of the slope by 0.301. Other mice were studied 10 min after an intracerebroventricular injection of complex **4** with or without 50 μg of unlabeled complex **4** included in the injection.

Statistics

Means are reported with their S.E.s. Linear and nonlinear regression lines were computed and compared statistically with the Prism 5.0 software package (GraphPad Software Inc., San Diego, CA). Linear regression lines are reported with their correlation coefficients and n values and, when significant, with their slopes and intercepts.

Results

Synthesis and Characterization of Rhenacarborane Complexes. The rhenacarborane complex (complex **1**) is a previously reported, highly lipophilic d -block metallacarborane complex (Ellis et al., 1999). With a view to exploiting its hydrophobicity, we have considered complex **1** as a valuable synthon for rhenacarborane-based drug delivery vehicles. Despite the general propensity for many metal-carbonyl complexes to labilize and substitute carbonyl ligands, complex **1** demonstrated some considerable resistance to binding other ligands either thermally or chemically. The current objective was nevertheless to introduce a replacement ligand architecture that would incorporate a remote functionality as a means to tether pharmacophores and/or radioactive labels. Our initial choice was to use the chelating ability of 2,2'-bipyridyl, which also bears a pendant alkyl bromide group at the 4-position of one of the pyridyl rings. We therefore synthesized the ligand 4-methyl-4'-bromoheptyl-2,2'-bipyridyl according to the literature method (Ellison et al., 1983). At-

tempts to bind this ligand via direct substitution of both carbonyl ligands in complex **1** were unsuccessful, however. To circumvent this apparent roadblock, we thus treated complex **1** with a source of bromide ions, PPh_4Br , in refluxing THF. This afforded the deep red compound (complex **2**) in reasonable yield as a consequence of monocarbonyl substitution (Scheme 2). Spectroscopic analysis revealed the structural identity of the complex anion, which was further confirmed by an X-ray structure determination. A more detailed description of the physicochemical properties of compound **2** will be deferred until a full report on this complex and its derivatives has been prepared (P. A. Jelliss, J. H. Orlando, N. P. Rath, and X. Shi, manuscript in preparation). In the interim, a brief discussion of the most pertinent spectroscopic data will suffice. Thus, the complex anion displayed absorptions in its CH_2Cl_2 solution IR spectrum at $\nu_{\text{max}}(\text{CO}) = 1974 \text{ cm}^{-1}$ and $\nu_{\text{max}}(\text{NO}) = 1715 \text{ cm}^{-1}$, characteristic of simple terminal ligation to a Re^{I} metal center for the carbonyl and nitrosyl ligands, respectively. The complex is produced as unresolved enantiomers, courtesy of the metal stereocenter. Thus, the absence of any rotation-reflection symmetry element is readily observed in the $^{11}\text{B}\{^1\text{H}\}$ NMR spectrum, which displays nine distinct resonances between $\delta -3.7$ and -20.6 , each integrating to one boron. This contrasts with similar complexes possessing pseudo mirror symmetry, which would produce a maximum of six observable signals (ideally in a ratio of 1:1:1:2:2:2). The presence of this chirality also produces two distinct resonances for the adjacent cage CH groups at $\delta 2.59$, 3.62 and at $\delta 46.5$, 50.6 in the ^1H and $^{13}\text{C}\{^1\text{H}\}$ NMR spectra, respectively.

Although not immediately apparent, compound **2** served as a much improved synthon for our target species. Thus, simple refluxing with the same 4-methyl-4'-bromoheptyl-2,2'-bipyridyl ligand in THF afforded the neutral complex (complex **3**) in respectable yield (Scheme 2). The observation that reaction of the ligand proceeds more easily with anionic complex **2** than it does with neutral complex **1**, even without the benefit of a halide-abstracting agent, is somewhat surprising and worthy of future discussion in a chemistry forum. Complex **3** was also comprehensively spectroscopically probed, with the IR spectrum of a CH_2Cl_2 solution confirming loss of the CO ligand by virtue of the disappearance of the characteristic $\nu_{\text{max}}(\text{CO})$ resonance, leaving only the resonance for the terminal nitrosyl ligand at $\nu_{\text{max}}(\text{NO}) = 1664 \text{ cm}^{-1}$. Al-

though the 4-methyl-4'-bromoheptyl-2,2'-bipyridyl moiety is an asymmetric ligand, the impact on the metal center stereochemistry is minimal as is evidenced by the $^{11}\text{B}\{^1\text{H}\}$ NMR spectral observation of just four resonances (integrating 2:1:3:3) between $\delta -2.8$ and -16.1 . This also accounts for single resonances for the cage CH groups in the ^1H ($\delta 1.76$) and $^{13}\text{C}\{^1\text{H}\}$ ($\delta 40.8$) NMR spectra. The asymmetry is evident, however, in the resonances for the 2,2'-bipyridyl ligand itself with discrete signals recorded for the $\text{H}^{3,3'}$ and $\text{H}^{6,6'}$ aromatic protons in the ^1H NMR spectrum as well as 10 distinct resonances for the aromatic carbons in the $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum between $\delta 160.8$ and 123.9 . All IR and NMR data for complex **3** are in accordance with the basic three-legged piano stool structure depicted in Scheme 2. The dark green color of the complex may be attributed to the moderately weak ($\epsilon = 700 \text{ M}^{-1} \text{ cm}^{-1}$), yet broad (peak width at half-height, $\Delta\lambda_{1/2} = 109 \text{ nm}$ for a 0.5 mM solution) absorption observed in the UV-visible spectrum in CH_2Cl_2 at $\lambda_{\text{max}} = 663 \text{ nm}$.

The conversion of complex **3** to radiolabeled **4** was achieved using routine organic synthetic methodology to substitute the alkyl bromide in acetone using Na^{131}I . The small quantities of complex **4** required for subsequent studies were isolated using thin layer chromatography and filtration, the complex being readily identified by its characteristic dark green color.

Subcutaneous Injection Studies. Figure 1 shows the loss over time from the site of injection and the entry into the blood. Loss from the site of injection was determined by excising the area of injection and was modeled on a one phase exponential decay (Fig. 1, top) and had a rate constant of 0.0252 , yielding a half-life of 27.5 min and a plateau of 28.6% injected dose ($r = 0.950$; $n = 5$). Uptake into blood was modeled as a one-site binding hyperbola (Fig. 1, bottom), which reached a maximum of 2.59% injection/ml with half of

Scheme 2. Synthetic pathway for rhenacarborane complex **4**.

Fig. 1. Absorption of complex **4** from site of injection into the blood. Top, absorption of complex **4** from the site of injection. Bottom, uptake of complex **4** into the blood.

this value achieved 35.8 min after injection ($r = 0.843$; $n = 15$).

Figure 2 (top) shows the uptake by brain expressed as brain/serum ratios. The highest value was found at the earliest time point (2 min after injection), and modeling with a one-phase exponential decay model predicted a maximal brain/serum ratio of $295 \mu\text{L/g}$ ($r = 0.986$; $n = 15$). The curve approached a steady state (plateau) of $41.8 \mu\text{L/g}$. Figure 2 (bottom) shows the results expressed as the %Inj/g and modeled to a one-site binding hyperbola ($r = 0.770$; $n = 15$). The percentage of the injected dose taken up by brain approached a calculated maximal value of 0.107% Inj/g and did so rapidly, reaching half of this value at 6.03 min.

For comparison, tissue/serum ratios (Fig. 3, top) and %Inj/g (Fig. 3, bottom) were calculated for liver and lung. As expected for a small, lipid-soluble molecule, maximum tissue/serum ratios were high and occurred early for liver ($26,432 \mu\text{L/g}$; $r = 0.9998$; $n = 6$) and lung ($16,984 \mu\text{L/g}$; $n = 15$). The %Inj/g did not change over time for either liver or lung and averaged $1.53 \pm 0.19\%$ Inj/g for lung and $0.89 \pm 0.13\%$ Inj/g for liver.

Octanol/buffer Partition Coefficient. The mean octanol/buffer partition coefficient for complex 4 was 1.74 ± 0.13 ($n = 3$ determinations). The log value for this number is 0.24, indicating that the compound is lipophilic.

Effect of DMSO on BBB Integrity. Figure 4 shows the brain/serum ratios for albumin pretreated with DMSO versus saline solution. The slopes for each line do not deviate significantly from zero, indicating that albumin was not being taken up by in a time-dependent manner by brain. The slopes also do not deviate significantly from each other indicating that the lines can be considered equal to each other. Together, these analyses demonstrate that DMSO did not

Fig. 2. Transport of complex 4 from blood to brain. Top, results plotted as brain/serum ratios versus time. A high initial uptake with rapid decline to a steady-state level is consistent with a lipid-soluble compound. Bottom, results plotted as percentage of the subcutaneously injected dose taken up by brain versus time. A steady-state level of 0.107% Inj/g was quickly reached and sustained for the study.

Fig. 3. Uptake of complex 4 by lung and liver. Top, results expressed as tissue/serum ratios versus time. Bottom, results expressed as %Inj/g.

Fig. 4. Effect of subcutaneous injection of DMSO on BBB integrity. Injection of DMSO did not disrupt the BBB to radioactively labeled albumin.

disrupt the BBB and the brain/serum ratios can be taken to represent the vascular space of the brain, which averaged $14.3 \pm 0.8 \mu\text{L/g}$ ($n = 8$) for the saline group and $13.3 \pm 0.6 \mu\text{L/g}$ ($n = 7$) for the DMSO group.

Identification of Radioactivity in Serum and Brain. Two separate samples of radioactivity recovered from serum 6 h after the subcutaneous injection of complex 4 showed that 73.2 and 94.9% of the radioactivity eluted in the position of complex 4. Single TLC plates for brain and urine showed 71.2 and 51.1%, respectively, of radioactivity eluted as complex 4.

Brain-to-Blood Efflux. Figure 5 shows clearance of complex 4 from brain after its intracerebroventricular injection ($r = 0.690$; $n = 13$; $p < 0.0001$; $n = 10$). The half-time clearance was calculated to be 18.4 min. The inset shows that addition of $50 \mu\text{g}/\text{mouse}$ unlabeled rhenacarborane did not affect the retention of complex 4 by brain, demonstrating that clearance was not likely by a saturable system.

Discussion

The rhenacarboranes, a class of boron-rich metal complexes, show promise for the treatment of CNS diseases both as direct agents and as delivery systems. However, the abil-

Fig. 5. Brain-to-blood transport of complex **4**. Results were expressed as the log of the percentage of residual counts injected into brain versus time. Complex **4** was effluxed from brain with a half-life of 18.4 min. Inset shows that unlabeled rhenacarborane (50 μ g/mouse) did not affect the clearance of complex **4** from brain consistent with a nonsaturable efflux mechanism.

ity of these compounds to be taken up by the brain has not been assessed. Here, we examined the ability of a parent complex **4** (Scheme 2) to be taken up by the brain. Because of the hydrophobicity of the compound, we administered it by subcutaneous injection in mice. We found that it was quickly taken up from the site of injection, with 50% of it absorbed 27.5 min after injection and approximately 72% of it taken up during the 6 h of the study (Fig. 1, top). Levels achieved in blood approached 2.59% of the injected dose per milliliter of arterial serum, reaching half of that level 35.8 min after injection (Fig. 1, bottom). TLC showed that 73 to 95% of the radioactivity in arterial serum 6 h after subcutaneous injection represented intact complex **4**. Overall, these results show that complex **4** is readily taken up into blood and is enzymatically stable. These characteristics of complex **4** allowed us to assess its ability to cross the BBB.

Figure 2 (top) shows a very high, early uptake by brain with a brain/serum ratio of almost 300 μ g/g, after which the brain/serum ratios rapidly declined to a plateau of approximately 42 μ g/g. This pattern is typical of substances that rapidly cross the BBB or that have significant efflux systems and represents rapid entry followed by equilibrium between brain and blood (Segal et al., 1990). The plateau level of 42 μ g/g is in excess of the vascular space of the brain (measured in Fig. 4 to be approximately 14 μ g/g in these animals) and so demonstrates prolonged, significant uptake by the brain. When the results were expressed as the percentage of the subcutaneously injected dose that had entered brain (Fig. 2, bottom), it was found that a steady state of approximately 0.107% Inj/g brain was approached and that half of this level was achieved by approximately 6 min after injection. This compares with levels of approximately 0.07% Inj/g for interleukin-1 α (Banks et al., 1991), 0.17% Inj/g for leptin (Banks et al., 1996), and less than 0.02% Inj/g for morphine (Advokat and Gulati, 1991; Banks and Kastin, 1994), all of which cross the BBB to exert effects within the CNS (Friedman and Halaas, 1998; Banks et al., 2001). TLC showed that 71.2% of the radioactivity obtained from brain 6 h after subcutaneous administration represented intact complex **4**.

The octanol/buffer partition coefficient of 1.74 confirms our hypothesis that boron compounds would be lipophilic and supports the idea that they are crossing the BBB by the mechanism of transmembrane diffusion. A partition coefficient

greater than unity demonstrates that complex **4** is more soluble in octanol than in aqueous media. A partition coefficient greater than 1 and less than 10 is usually taken to be ideal for BBB penetration for small lipid-soluble substances. Substances with an extremely high-lipid solubility, that are sticky, or that are highly charged can be trapped by brain endothelial cell membranes and so compromise their passage across the BBB (Martel et al., 1996, 1997).

The brain uptake of complex **4** does not seem to have been inhibited by its large molecular volume or mass. Although the molecular mass of complex **4** is 742.91 Da, it is often stated that compounds of greater than 400 Da cannot cross the BBB by transmembrane diffusion (Levin, 1980). However, this is based on work with compounds that cannot accumulate in brain because they are *p*-glycoprotein substrates (Begley, 2004). Substances as large as CINC1, which has a molecular mass of 7.8 kDa, can cross the BBB to some degree by transmembrane diffusion (Pan and Kastin, 2001). The ability of complex **4** to readily cross the BBB supports the idea that there is no absolute molecular mass cut-off for crossing the BBB.

The patterns of uptake for lung and liver (Fig. 3) are consistent with the distribution of a lipid-soluble substance. Steady-state uptake values were approximately 15 times higher for lung and approximately 9 times higher for liver. The finding that 51.1% of the radioactivity in urine represented intact complex **4** suggests that this compound is largely excreted intact from the body.

Because we injected complex **4** carborane in DMSO, we determined whether the BBB remained intact during the study. DMSO has been reported to disrupt the BBB after intraperitoneal injection (Broadwell et al., 1982) but not after intravenous and intracarotid injection (Neuwelt et al., 1983). We used radioactively labeled albumin, a substance typically used for such assessments, to determine whether there was any BBB disruption. We found no difference in the brain/serum ratios for albumin in DMSO versus saline-injected mice (Fig. 4). This shows that the ratios represented vascular space and provides no evidence for BBB disruption.

The rapid uptake by brain and stability in blood suggests that this and other carboranes might be useful in the delivery to brain of substances that enter the CNS poorly. The synthetic methodology used will require us to modify the terminal halo group of complexes **3** or **4** so as to produce a terminal primary amino or hydroxyl group. These groups can be used to bind with the carboxyl terminus of peptides in particular. Efficacy of the pharmacophore may or may not depend on its cleavage from the carborane. Additional linkages, such as an ester linkage, could also be added between the pharmacophore and the carborane that are either vulnerable to cleavage within the CNS or preserve pharmacophore activity (Genka et al., 1993).

Finally, we examined the ability of complex **4** to cross the BBB in the brain-to-blood direction. Consistent with its rapid decrease in brain/serum ratios seen in the top panel of Fig. 1, we found that it had a significant efflux rate from brain of approximately 18.4 min (Fig. 5). This is more rapid than can be attributed to the reabsorption of cerebrospinal fluid and is consistent with a saturable efflux transporter or with a lipid-soluble substance distributing out of brain (Taylor, 2002). The inset of Fig. 5 shows that a saturable component to efflux could not be demonstrated by including 50 μ g/mouse of un-

labeled compound in the intracerebroventricular injection. Thus, it is likely that the rapid brain-to-blood efflux is based on its ability to diffuse across the membranes comprising the BBB because of its lipid solubility. Partitioning between aqueous and lipid domains in the brain-to-blood direction involves similar considerations as those discussed above for the blood-to-brain direction with more lipid-soluble compounds moving across the BBB more rapidly than less lipid-soluble compounds (McQuay et al., 1989; de Lange et al., 1993).

In conclusion, we examined the ability of complex **4**, a ^{131}I -labeled rhenacarborane, to cross the BBB. We found rapid uptake and equilibrium phases with a steady state of approximately 0.1% of the injected dose being taken up per gram of brain. The compound was stable in brain and blood. These characteristics suggest that rhenacarboranes could be useful as therapeutic compounds for CNS diseases.

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