Evaluation of the Blood Brain Barrier Transport, Population Pharmacokinetics and Brain Distribution of Benztropine Analogs and Cocaine Using In Vitro and In Vivo Techniques

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BBB Transport and Distribution of Benztropine Analogs and Cocaine

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Abstract

Purpose. The N-substituted 3α[bis (4'-fluorophenyl) methoxy]tropanes (AHN 2-003, AHN 1-055, AHN 2-005, JHW 007) bind with high affinity to the dopamine transporter, and inhibit dopamine uptake more potently than cocaine, but demonstrate behavioral profiles in animal models of psychostimulant abuse that are unlike that of cocaine. The objective of this study was to characterize the in vitro permeability, brain distribution and pharmacokinetics of the benztropine (BZT) analogs. Methods. Transport studies of cocaine and the BZT analogs (10^{-4} M) were conducted across BBMECs. Male Sprague Dawley rats (~300g) were administered BZT analogs (10mg/kg) or cocaine (5 mg/kg) via the tail vein. Blood and brain samples were collected over 36 hours and assayed using UV-HPLC. Results. Transport of both AHN 1-055 (2.15 x 10^{-4} cm/sec) and JHW 007 (2.83 x10^{-4} cm/sec) was higher (p <0.05) than cocaine (1.63 x 10^{-4} cm/sec). The volume of distribution (12.3 – 30.5 L/kg) of the analogs was significantly higher than cocaine (0.9 l/kg). The BZT analogs displayed a > 8-fold higher elimination half-life (4.12 – 16.49 hrs) as compared to cocaine (0.49 hr). The brain-to-plasma partition coefficients were at least two-fold higher for the BZTs vs cocaine, except for AHN 2-003. Conclusions: The BZT analogs are highly permeable across the blood-brain barrier, and possess a pharmacokinetic profile different from that of cocaine. These characteristics, in addition to their distinctive behavioral profiles, suggest that the BZT analogs may be promising candidates for the treatment of cocaine abuse.
Cocaine exerts its psychomotor stimulant and reinforcing effects primarily through the blockade of the dopamine transporter (DAT) (Ritz et al 1987, Carroll et al 1992, Wise, 1996; Self and Nestler, 1995). The blockade of the DAT by cocaine yields synaptic accumulation of dopamine due to a reduction in the re-uptake of dopamine. Significant synthetic efforts for the development of effective therapeutic agents to treat cocaine abuse have focused on chemical entities that also interact with the dopamine transporter (Carroll, 1992, Carroll 1999, Newman, 1998, 2000). As such, these agents are sometimes referred to as “substitute” medications. It has been suggested that the ideal properties of a substitute therapeutic agent include high binding affinity to the DAT, slow onset of action, long duration of action and behavioral effects that may be distinctive from those of cocaine (Rothman and Glowa, 1995, Newman and Kulkarni, 2002). A slow onset of effect, characterized by a gradual increase in dopamine synaptic levels may not produce the profound psychomotor stimulant and mood enhancing effects associated with cocaine. Further, a long duration of action characterized by sustained and elevated dopamine levels would thus reduce the repeated administrations associated with cocaine abuse. Numerous therapeutic agents have been developed based on the substitute therapy strategy including, GBR 12909 and RTI 112, dopamine uptake inhibitors currently in clinical trials (Sogaard et al 1990, Rothman and Glowa, 1995, Carroll, 1999, Newman, 2000). However, these agents demonstrate cocaine-like actions in animal models and thus may have abuse liability themselves or lack effect in treating cocaine abuse or maintenance of abstinence (Tutton and Crayton 1993).

A series of the N-substituted 3α[bis (4’fluorophenyl) methoxy] tropanes, or benztropine (BZT) analogs, have been developed as potential substitute therapeutic agents for cocaine-abuse (Newman et al., 1994, 1995, Agoston et al., 1997, Robarge et al., 2000, Newman et al., 2001). The BZT analogs bind with higher affinity to the DAT (Ki_{BZT} = 11-30 nM vs Ki_{cocaine} = 187 nM)
and inhibit dopamine uptake with a higher potency, in comparison to cocaine (Table 1). (Agoston et al., 1997, Katz et al., 2001). These analogs are not efficacious locomotor stimulants in mice and also, do not fully substitute for cocaine in drug discrimination studies (Katz et al., 1999). Hence these analogs may have potential as substitute therapeutic agents for the treatment of cocaine abuse. As such, it is necessary for them to reach the brain in sufficient concentrations to produce this desired effect in vivo.

The BZT analogs are highly lipophilic (cLogP<sub>BZT</sub> = 3.1 - 5.5) as compared to cocaine (cLogP<sub>cocaine</sub> = 2.72), a physiochemical property known to promote blood-brain barrier (BBB) transport. However, the BBB transport characteristics of the BZT analogs have not been characterized. Further, it should be noted that the presence of efflux proteins such as P-glycoprotein may limit entry of these agents into the brain. (Ambudkar et al., 1999). As stated, another important characteristic of a potential “substitute therapeutic agent” is a slow onset and long duration of action (Gorelick 1998). As such, the ideal pharmacokinetic properties of a substitute agent would be slow input especially into the brain as well as a slow rate of elimination out of the body. This pharmacokinetic profile would most likely allow for a gradual increase in brain dopamine levels resulting in slow inhibition of dopamine re-uptake along with a sustained elevation of dopamine. Cocaine is rapidly absorbed and metabolized after administration (Wilkinson et al., 1990, Mets et al., 1999). Thus, a pharmacokinetic profile different from cocaine might also be a determining factor in predicting the therapeutic efficacy of a potential substitute agent.

Based on the above observations, investigations of the BBB permeability, brain distribution and pharmacokinetics of the BZT analogs are warranted to determine if they possess the requisite dispositional characteristics identified as ideal for substitute therapeutic agents. To investigate
this, the following two objectives were pursued: (1) determine the \textit{in vitro} permeability of BZT analogs across bovine brain microvessel endothelial cell (BBMEC) monolayer and (2) determine the brain distribution and pharmacokinetics of BZT analogs after intravenous administration to male Sprague Dawley rats using destructive sampling techniques. The physiochemical and pharmacologic properties of the BZT analogs evaluated in this study are presented in Table 1.
METHODS

Materials

Cell Culture medium components: Dulbecco’s modified Eagle medium (DMEM/F-12), phosphate buffered saline (PBS), gentamycin (100x), horse serum (from platelet-poor plasma), polymyxin B, amphotericin B, and L-Glutamine (100x) were obtained from Gibco (Grand Island, NY). [14C]Sucrose (516 mCi/mmol), [3H]Propranolol (27 mCi/mmol), Rhodamine 123, Heparin (sodium salt), oxprenolol, sodium phosphate dibasic, cocaine, triethylamine (TEA), and sodium fluoride were purchased from Sigma Chemical Co. (St. Louis, MO). Isolation and plating materials were supplied from the following sources; collagenase/dispsase, protease, percoll, fibronectin (bovine plasma), and dextran from Sigma Chemical Co and MEM (lx), MEM (10x), dimethyl sulfoxide (DMSO) and Hepes (IM) from Gibco. Rat tail collagen (type 1) was obtained from Fischer Scientific (Newark, DE). The BZT analogs were synthesized as previously described (Newman et al., 1994, 1995, Agoston et al., 1997). All chemicals and solvents were ACS analytical grade or HPLC grade.

Bovine Brain Microvessel Endothelial Cells Isolation and Seeding

Bovine brain microvessel endothelial cells (BBMEC) were isolated manually from the gray matter of cerebral cortices as previously described (Audus and Borchardt 1986, Cox et al., 2001). Primary bovine microvessel fragments were seeded onto 12 well Costar inserts (Transwell) 1cm² in diameter consisting of a 0.4 μm-pore polycarbonate filter. Filter membranes (apical surface) of the inserts were treated with 0.5 ml of rat tail collagen type 1, excess was aspirated off and the filters were allowed to dry. Fibronectin (0.5 ml) was then added to each insert, removed shortly thereafter (40 min), and allowed to dry. BBMECs were then seeded onto the collagen/fibronectin coated membranes at a density of approximately 70,000-80,000
cells/cm² after suspending the cells in plating medium consisting of DMEM F12 (1x) and gentamycin (50 µg/ml), supplemented with horse serum, polymyxin B and amphotericin B (50 µg/ml). Upper (apical, A) compartment received 0.5 mL of plating medium, and lower (basolateral, B) received 1.5 mL for the first 3 days of growth. On the third day after plating, the changing medium which consisted of DMEM F12 (1x), gentamycin (50 µg/ml), horse serum, heparin and amphotericin (50 µg/ml), was added to the cells. The changing medium was added every other day. Cells were placed in an incubator maintained at 37°C, with 5% CO2 and 95% humidity until confluence was reached within 10-14 days. Integrity of the BBMEC monolayer was assessed by determination of flux of radiolabeled markers, ¹⁴C-sucrose (paracellular) and ³⁵H-propranolol (transcellular) as well as transepithelial electrical resistance (TEER) values (100-150 ohm.cm²) for each 12 well Costar™ plate before the transport study.

**Benztropine Analogs and Cocaine Transport Studies**

Transport experiments were performed in triplicate in both the apical-to-basolateral (A→B) as well as basolateral-to-apical (B→A) directions. BZT analogs and cocaine solutions were prepared in phosphate-buffer saline (PBS) at a concentration of 1 x 10⁻⁴ M. On confluency of BBMECs, complete culture medium was removed from both the apical (A) and basolateral (B) sides and both sides were washed twice with sterile PBS. The inserts were then placed into tissue culture plates (Costar™) containing 1.5 mL of prewarmed PBS. All the transport studies were performed in triplicate and BBMECs were continuously agitated during the experiments on a shaker plate (100 rpm) at 37°C. For the A→B transport study, 0.4 mL of BZT analogs/cocaine/radiolabeled marker was added to the apical side at time t =0. After 5 minutes, the insert was removed and transferred to a new transwell containing fresh PBS. This procedure was repeated up to 120 minutes, with samples being collected from the basolateral compartment.
at 5, 10, 15, 20, 30, 45, 60, 75, 90 and 120 min. For the B→A transport study, 1.5 mL of the test agent was placed on the basolateral side at time t=0. The apical side contained 0.4 mL of fresh PBS. After 5 minutes, the PBS sample from the apical side was removed and replaced with an equal volume of fresh PBS. Samples were collected at similar time points and the samples were stored at –70°C until HPLC analysis.

**Benztropine Analogs and Cocaine Inhibition Studies**

To evaluate the possible interaction of BZT analogs with the efflux protein, Pgp, inhibition studies with a known Pgp inhibitor, PSC833, were carried out. PSC833, a nonimmunosuppressive analog of cyclosporin A, has been shown to be a potent and specific inhibitor of Pgp (Boesch et al 1991). Rhodamine 123, a known Pgp substrate was used to evaluate the functional activity of the efflux protein in BBMECs (Fontaine et al 1996). Bidirectional transport of rhodamine 123 (3.2 µM) was determined across the BBMEC monolayer and the efflux ratio was determined as a measure of Pgp activity. Rhodamine levels in the transport study samples were determined by HPLC. For inhibition studies with BZT analogs, medium was removed and inserts were washed with PBS. Cells were then pre-incubated with 0.5 µM PSC833 on the apical side for 30 minutes (Smith et al 1998), after which PSC833 was removed, cells were washed and PSC833 was added again along with the BZT analog/cocaine solution (1 x 10⁻⁴ M) at t =0. Apical-to-basolateral transport studies were carried out in the manner stated above and samples were collected at various time points up to 120 minutes. Samples were stored at –70°C until HPLC analysis.

**Animal Pharmacokinetic Studies**

Adult male Sprague Dawley rats (250-275 g) were used in this study and were purchased from Hilltop Animal Laboratories (Scottsdale, PA). The protocol was approved by the
Institutional Animal Care and Use Committee of the School of Pharmacy, University of Maryland. All the animals were housed in the animal facility at a room temperature of 72 ± 2°F and their care followed the “Guide for the care and use of laboratory animals” according to NIH specifications. Animals were allowed food (Purina 5001 rodent chow) and water ad libitum and were observed closely with respect to appearance, appetite, and waste elimination to ensure that they were healthy.

**Benztropine Analogs and Cocaine Dosing and Sampling**

BZT analogs (hydrochloride or oxalate salts) were dissolved in sterile water for injection with the aid of DMSO (9%) and heat and a dose of 10 mg/kg free base was administered iv (via the tail vein). The animals were fasted approximately 10 hours prior to dosing when all food was removed. Animals were not restrained during any phase of the study except during dosing, when the animals were placed in rodent restraining chambers. The total volume administered did not exceed 10% of the total blood volume. A destructive sampling study design was followed where cohorts of 3 animals were sacrificed by CO2 asphyxiation at pre-dose and post-dose at 5, 15, 30, 60, 120, 240, 360, 600, 720, 1080, 1440, and 2100 minutes. Blood was collected by heart puncture using heparinized syringes, centrifuged for 10 minutes at 3000 rpm, and plasma was stored at −70°C until HPLC analysis. Brain tissue was immediately excised, weighed, placed on ice, snap frozen in liquid nitrogen and stored at −70°C until analysis.

For comparison, brain distribution and pharmacokinetics of cocaine were also determined. Cocaine HCl was dissolved in sterile water for injection and a dose of 5 mg/kg free base was administered intravenously (via tail vein). Cohorts of 3 animals were sacrificed by CO2 asphyxiation predose and postdose at 2, 5, 10, 15, 30, 60, 120, and 240, minutes. Blood was collected by heart puncture using preheparinized syringes and 200 µL of saturated sodium
fluoride solution (stock = 50 mg/mL in water) was added to prevent the hydrolysis of cocaine by plasma cholinesterases. This was followed by centrifugation for 10 minutes at 3000 rpm, and plasma was stored at –70°C until HPLC analysis. Brain tissue was immediately excised and stored at –70°C until analysis.

**Benztropine Analog HPLC Analysis**

A valid specific high-performance liquid chromatography (HPLC) method to was used to quantify the BZT analogs in cell culture media, plasma and brain samples (Raje et al. 2001). Cell culture and plasma standards were analyzed using liquid-liquid extraction with hexane, followed by evaporation and reconstitution. Brain samples were homogenized with phosphate-buffered saline before extraction. The chromatographic conditions consisted of a Supelcosil LC-ABZ Plus column (250 x 4.6 mm, 5 µm), UV-detector (λ=220 nm), gradient mobile phases: (A) methanol:0.05M Na₂HPO₄ (pH = 3.0), 40:60 (v/v) and (B) methanol:0.05M Na₂HPO₄ (pH = 3.0), 80:20 (v/v) and a flow rate of 1 ml/min pumped over a 15 min gradient profile. The internal standard was oxprenolol. without any interference from endogenous peaks. The calibration curves were linear in the range of 100-50000 ng/ml, 25-10,000 ng/ml and 50-10,000 ng/ml (r² ≥ 0.999) for cell culture, plasma and brain, respectively.

**Cocaine HPLC Analysis**

A slight modification of a previously described HPLC method was used to quantitate cocaine in plasma and brain tissue samples (Pan et al., 1997). Reverse phase chromatography with ultraviolet detection (λ = 235 nm) was utilized to quantitate the eluate. The mobile phase consisted of 0.05M monobasic ammonium phosphate, methanol and acetonitrile (76:12:12 v/v) pumped at a flow rate of 1.0 mL/min. Oxprenolol served as the internal standard. Brain tissue was homogenized, diluted with an equal volume of phosphate buffered saline. To the brain
homogenate mixture, 100 µL of TEA was added after which the samples were vortexed briefly for 20-30 seconds. For plasma samples, six mLs of hexane were added to 0.6 mL of plasma, samples were vortexed (30-45 secs) and centrifuged at 3000 rpm for 5 min. The supernatant was evaporated to dryness at 40°C under a gentle stream of nitrogen. The resulting residue was reconstituted with 210 µL of mobile phase (0.05M monobasic ammonium phosphate: methanol: acetonitrile: 76:12:12 v/v), transferred to a micro-vial (300 µL capacity) and 200 µL were injected onto the HPLC system. The calibration curves were found to be linear in the range of 10-5000 ng/ml for plasma and 25 – 1000 ng/g for brain (r² ≥ 0.999). The intra- and inter-day variability and error were ≤ 10%.

**Rhodamine 123 HPLC Analysis**

Rhodamine transport study samples were analyzed by an HPLC system consisting of a Waters 2690 liquid chromatograph supplied with a LC 20 fluorescence detector (Perkin Elmer, PA) set at an excitation and emission wavelength of 498 and 525 nm, respectively. The chromatographic data was recorded using a model 3390A Hewlett Packard integrator (Rockville, MD). Separation was achieved at ambient temperature using a reverse phase C8 column (150 × 4.6 mm, 5 µm; Phenomenex, IL) fitted with a 5 µm LC-8, 2 cm guard column (Phenomenex, IL). The mobile phase consisted of a mixture of 0.025 mM Na₂HPO₄ and methanol (50/50 v/v) pumped at a flow rate of 0.5 mL/min, after filtration and degassing. The chromatographic run time was 15 min and 150 µL of the sample was injected onto the system. The calibration curve was found to be linear in the range of 62.5 – 5000 ng/ml (r² ≥ 0.999).

**Permeability Data Analysis**
Apparent permeability coefficients ($P_{app}$) alone or in the presence of inhibitor were calculated for each compound following triplicate experiments ($n = 3$). Permeability coefficients were determined at sink conditions from the following equation:

$$P_{app} = \frac{(dC / dt) \cdot V_r}{SA \cdot C_0}$$  \hspace{1cm} (1)

where $P_{app}$ is the apparent permeability coefficient in cm/sec, $dC/dt$ is the flux of drug across the monolayer, calculated as the linearly regressed slope through linear data, $V_r$ is the volume in the receiver compartment, $SA$ is the diffusion area (1 cm$^2$) and $C_0$ is the initial BZT analog or cocaine concentration in the donor chamber at $t = 0$. All values are represented as mean and standard deviation of the triplicate experiments performed under identical conditions and from the same preparation of cells.

Efflux ratios (Re) were calculated according to the following equation:

$$Re = \frac{P_{app} (B \rightarrow A)}{P_{app} (A \rightarrow B)}$$  \hspace{1cm} (2)

**Pharmacokinetic Analysis of Destructive Sampling Data**

Data obtained after the administration of the BZT analogs and cocaine to rats were initially analyzed by the Naïve Pooled Data Method (NPM). Plasma concentration vs time data from a given compound were pooled and analyzed according to nonlinear least squares. Compartment modeling was used to estimate various pharmacokinetic parameters ($V_d$, $kel$, $AUC_{inf}$, $t_{1/2}$, and $CL$) using WinNonlin$^\text{TM}$ (version 3.1, Pharsight Corp., Cary, NC). Both one and two compartment analysis was evaluated to determine the best model fit. Various
weighting schemes evaluated included a weight of 1, 1/y (where y is the drug concentration), 1/y², 1/Predicted concentration (iterative re-weighting) and 1/Predicted concentration². Goodness of fit was based on visual inspection, final residual sum of squares, weighted residual sum of squares, random distribution of residuals, Akaike’s Information Criteria and Swartz Criteria.

In order to evaluate inter-animal variability and to statistically compare the pharmacokinetic parameters of the BZT analogs and cocaine, population pharmacokinetic analysis was performed with WinNonmix™ Version 2.01 (Pharsight Corp., Cary, NC). (Cox et al., 2002). A two-stage analysis (naïve pooled data method) with the individual pharmacokinetic data was performed initially and it was determined that a two compartment model best described the disposition of both the BZT analogs and cocaine after a single iv bolus dose. Each structural parameter for the two-compartment model i.e. V₁, K₁₀, K₁₂ and K₂₁ was expressed as a function of the fixed effects as well as random effects i.e. inter-animal variability η using additive, proportional, and exponential relationships. The effect of the weight of each animal as the covariate was also assessed. The residual error (ε) was assumed to be identically and independently distributed with a mean of 0 and a variance of σ². The residual error was modeled using additive, proportional or exponential models, “Goodness of fit” was based on WSSR, % CVs for parameters, Akaike’s information criteria (AIC), Schwarz criteria (SC), and plots of observed and model-predicted concentration vs. time, time vs. residual concentration and observed vs. model-predicted concentrations. Local minima in the sum of squares surface were avoided by changing the values of the initial estimates and repeating the iterative process to achieve convergence.

**Brain Distribution Analysis**
Total brain concentrations of BZT analogs were determined and partition coefficients ($R_i$) were calculated based on the ratio of drug in brain to drug in plasma. The $AUC_{\text{inf}}$ (area under the curve) from time 0 to infinity were used to calculate $R_i$ according to the following formula:

$$R_i = \frac{AUC_{\text{inf(brain)}}}{AUC_{\text{inf(plasma)}}}$$  \hspace{1cm} (3)

The $AUC_{\text{inf}}$ for both plasma and brain tissue were determined by noncompartmental methods (WinNonlin™) utilizing the linear trapezoidal rule.

**Statistical Analysis**

Permeability coefficients obtained from the transport studies and the pharmacokinetic parameters were compared by one-way ANOVA followed by Tukey’s post hoc analysis. Statistical significance was set at $p < 0.05$. 
RESULTS

Benztropine Analogs and Cocaine Transport Across BBMECs

Permeability studies were conducted to evaluate the bi-directional transport of BZT analogs across the BBMEC monolayer. The flux of the BZT analogs as well as cocaine was linear as a function of time. Apparent permeability coefficients (P_app) of BZT analogs ranged from 1.54 to 2.83 x 10^{-4} cm/sec in comparison to a P_app value of 1.63 x 10^{-4} cm/sec for cocaine (Table 2). The P_app values for the BZT analogs ranked in the following order: AHN 2-003 < AHN 2-005 < Cocaine < AHN 1-055 < JHW 007. The BZT analogs showed very high permeability in the apical-to-basolateral (A→B) direction. The A→B transport of AHN 1-055 and JHW 007 was significantly higher than that of cocaine (p < 0.05). There was no significant difference between the A→B P_app for AHN 2-003 and AHN 2-005 vs. cocaine.

The BZT analogs as well as cocaine were significantly effluxed across BBMEC monolayers with P_app values in the basolateral-to-apical (B→A) direction being significantly higher than P_app values in the A→B direction (p < 0.05) and B→A/A→B ratios > 4. The B→A P_app values ranged from 8.51 to 22.8 x 10^{-4} cm/sec in comparison to a P_app value of 6.88 x 10^{-4} cm/sec for cocaine with the rank order: cocaine < AHN 2-003 < AHN 2-005 < AHN 1-055 < JHW 007. Except for JHW 007, there was no significant difference between the B→A P_app for cocaine and the other BZT analogs. The B→A/A→B ratio was smallest for cocaine (4.2) and highest for JHW 007 (8.0).

Transport of rhodamine 123 was evaluated to confirm the functional activity of Pgp in the cultured BBMEC monolayers. The B→A/A→B ratio for rhodamine 123 was ~ 4, a value which is in accordance with literature (Lee et al., 1994). To evaluate involvement of Pgp, cells were pre-incubated with PSC833 (0.5 µM). As seen in Table 2, A→B transport of all compounds,
including cocaine, increased in the presence of PSC-833 (p < 0.05). The A→B Papp values in the presence of PSC833 ranged from $1.84 \times 10^{-4}$ cm/sec in comparison to a Papp value of $2.12 \times 10^{-4}$ cm/sec for cocaine (Table 2).

**Benztropine Analogs and Cocaine Pharmacokinetics**

Figure 2A presents the mean plasma concentration vs time profile for the BZT analogs and cocaine after single iv bolus dosing in Sprague Dawley Rats. Pharmacokinetics of the BZT analogs appeared to follow a bi-exponential decay, with a 2-compartmental model providing the best fit. Figure 2B shows the Winnonlin™-predicted and observed concentration vs. time fits for the BZT analogs (best fits shown). The parameter estimates obtained from Winnonlin™ along with one concentration data point from each animal were used to obtain individual concentration vs. time profiles using the mixed effects population modeling program Winnonmix™. A two-compartment model provided the best fit for each animal. The inter-animal variability was best described by an exponential model and the residual error was described by an additive model.

**Benztropine Analogs and Cocaine Comparative Pharmacokinetics**

The primary goal in utilizing population analysis was to statistically compare the pharmacokinetic parameters obtained after the administration of the BZT analogs and cocaine given the destructive sampling study design. Table 3 summarizes the population pharmacokinetic parameters for the BZT analogs and cocaine after single dose iv administration. Dose-normalized AUC_{inf} for cocaine (5,932 mg/L·hr) was not significantly different from the AUC_{inf} for AHN 2-005 (7,296 mg/L·hr ) and JHW 007 (5,566 mg/L·hr). However, the AUC_{inf} was significantly higher (p <0.05) for AHN 2-003 (15,152 mg/L·hr ) and AHN 1-055 (10,699 mg/L·hr) as compared to cocaine. Cocaine is known to be rapidly metabolized by plasma esterases and has a significantly shorter t1/2 as discussed below (Wilkinson et al 1980, Mets et al
The low AUC_{inf} for cocaine in comparison to AHN 2-003 and AHN 1-055 is most likely due to this extensive metabolism.

The steady-state volume of distribution (V_{ss}) of the BZTs was ≥ 12-fold higher compared to that of cocaine (p < 0.05), with values ranging from 12.3 to 30.5 L/kg, in comparison to a value of 0.9 L/kg for cocaine. AHN 2-003 had the highest V_{ss}, which is about 30 times higher than that of cocaine. In general, the BZTs are more lipid soluble (cLogP 3.10 – 5.53) as compared to cocaine (ClogP 2.72) (Table 1). The significantly lower Vdss of cocaine vs the BZT analogs may be explained by the differences in lipophilicities of the compounds.

The BZT analogs displayed a ≥ 8 fold longer elimination half-life (t_{1/2}\beta) with the half-life of cocaine being significantly shorter than the BZT analogs (p < 0.05). The elimination half-lives ranged from 4.12 to 16.49 hrs for the BZT analogs in comparison to a t_{1/2}\beta of 0.49 hours for cocaine and ranked in the following order: AHN 2-005 < JHW 007 < AHN 1-055 < AHN 2-003. There was no significant difference between the half-lives of AHN 2-005 (4.12 hrs) and JHW 007 (5.35 hrs). Clearance for all the compounds in this series was significantly slower than that of cocaine (p < 0.05), except for JHW 007 which was found to have a clearance value of 3.29 L/hr/kg, in comparison with 3.05 L/hr/kg for cocaine.

**Brain Distribution of Benztropine Analogs and Cocaine**

Figure 3 illustrates the brain concentration vs. time profiles for the BZT analogs after naïve pooled averaging of the data whereas, the R_i values and brain t_{1/2}\beta values are specified in Table 3. In general, higher concentrations of BZTs as well as cocaine were seen in the brain tissue in comparison to plasma. Peak brain levels for all compounds were seen at the first sampling time after iv administration, indicating that there is no lag time for the entry of these compounds to their effect site. Except for AHN 2-003, the BZT analogs showed a ≥ 2-fold higher brain-to-
plasma ratio as compared to cocaine. Maximum brain-to-plasma ratios were seen after 1 hour for JHW 007 and AHN 2-005, 2 hours for AHN 1-055 and 4 hours for AHN 2-003, in comparison to 15 minutes for cocaine. The ratio for AHN 2-003, the least lipophilic of the series, was similar to that for cocaine ($R_i = 2.1$ for AHN 2-003 vs 2.0 for cocaine). AHN 2-005 was found to have the highest brain uptake ($R_i = 6.6$), followed by JHW 007 ($R_i = 5.6$), and AHN 1-055 ($R_i = 4.8$). Brain half-lives for all the compounds were comparable to plasma half-lives (Table 3).
DISCUSSION

The BZT analogs may be considered as potential substitute therapeutic agents and display significantly higher binding affinity to the DAT as compared to cocaine (Agoston et al., 1997). Despite potent inhibition of dopamine uptake, \textit{in vitro}, these agents have not been found to display significant cocaine-like behavioral effects in animal models of cocaine abuse (Agoston et al., 1997, Katz et al., 1999). It should be noted that the \textit{in vitro} binding affinities of these compounds may not necessarily reflect their abilities to inhibit dopamine uptake, \textit{in vivo}, as the BBB penetrability of these compounds, after dosing is of critical importance. As such, studies were performed to evaluate the BBB permeability, brain distribution and pharmacokinetics of the BZT analogs and cocaine.

The low molecular weight (365 – 422 Da) and the high lipophilicity (ClogP > 3.0) of the BZT analogs would suggest passive permeability across the BBB. As expected, the transport was very high for the analogs corresponding to permeability of other CNS agents which display good uptake into the brain (\(P_{\text{app}} \approx 0.5 \times 10^{-4} - 3 \times 10^{-4} \text{ cm/sec}\)) (Pagliara et al., 1999). Both AHN 1-055 and JHW 007 had significantly higher \(A \rightarrow B\) transport in comparison to cocaine. This may be due to the fact that both AHN 1-055 and JHW 007 are much more lipophilic than cocaine. Although more lipophilic than AHN 1-055, there was no significant difference between permeability of AHN 2-005 and cocaine. However, differences in the N-substituents (R group, Table 1, Figure 1) may alter their permeability across the BBB. Nonetheless, the high permeability observed for the BZT analogs indicates that they should effectively bind to DAT and inhibit dopamine uptake since the BBB does not minimize their transport.
Our *in vitro* studies suggest that the transport of the BZT analogs and cocaine may involve a Multi-Drug Resistance (MDR) protein system that efﬂuxes the agents out of the BBB (Ambudkar et al., 1999). The transport of BZT analogs and cocaine increased in the presence of the Pgp inhibitor, PSC833, indicating the possibility that these compounds may be substrates of an efﬂux membrane transporter. Nonetheless, their high passive permeability, and high brain-to-plasma ratios (2.1- 6.6) would suggest that the efﬂux system does not effectively minimize brain uptake. Furthermore, it should be noted that the BZT binding affinities (Table 1) are at least 14-fold higher than cocaine. Although the efﬂux of BZT analogs and cocaine appears to be similar, they should more effectively block dopamine uptake based on their higher binding affinity.

According to the pharmacokinetic or rate theory, it would be of therapeutic importance for an ideal “substitute therapeutic” to possess a pharmacokinetic profile different from cocaine (Gorelick 1998). It is clear that the high abuse potential of cocaine is in part due to its rapid onset and short duration of action which reﬂects its pharmacokinetic (PK) and pharmacodynamic (PD) properties (Quinn et al., 1997). Positron Emission Tomography (PET) studies have shown that the rate at which cocaine enters the brain and blocks the DAT is the variable associated with the “high” rather that the presence of the drug in the brain (Volkow et al., 1997, 1999). Thus, a substitute therapeutic agent should provide a slower onset of action to decrease the rapid mood-enhancing effects associated with cocaine. Another critical property required for a substitute therapeutic agent is a longer residence time and slower elimination to minimize repeated cocaine administration. Taken together the potential substitute therapeutic should possess a slow input into the CNS as well as a slow clearance in comparison to cocaine.

The BZT analogs entered the brain rapidly with peak levels occurring at the first sampling time point. Maximum brain levels were observed within two minutes for cocaine. Maximu
brain-to-plasma ratios were observed after one hour for JHW 007 and AHN 2-005, two hours for AHN1-055, and four hours for AHN 2-003, in comparison to 15 minutes for cocaine. Interestingly, in drug discrimination studies wherein rats are trained to discriminate 10 mg/kg of cocaine from saline, cocaine dose-dependently and fully substitutes for the cocaine cue, with a pretreatment time of 5 min (Katz et al., 1999). Conversely, at a pretreatment time of 5 min, AHN1-055 does not produce cocaine-like discriminative stimulus effects, even at the highest doses tested. However, at a pretreatment time of 90 min., AHN 1-055 fully generalizes to the cocaine discriminative stimulus (Katz et al., 1999). Thus, although, AHN 1-055 appears to penetrate the BBB rapidly, perhaps the achievement of higher brain-to-plasma ratios is required in order to result in in vivo activity.

Dose-normalized AUCbrain was significantly higher for the BZT analogs compared to cocaine. In addition, AUCbrain/AUCplasma ratio for cocaine was ~2.0, a value in accordance with previously reported values (Pan et al., 1998, Javaid and Davis 1993). AHN 2-003, was found to have the smallest AUC ratio of 2.1 in comparison to 2.0 for cocaine, although this compound had the highest Vdss. This points to the possibility of AHN 2-003 being extensively distributed to tissues other than the brain. The in vivo brain uptake results are in agreement with our in vitro permeability studies. The transport of AHN 2-003 across BBMECs was not significantly different from that of cocaine. Alternatively, AHN 2-005 was seen to have the highest brain uptake ratio (Ri = 6.6) compared to cocaine. Interestingly, it displayed the smallest Vdss (12.3 L/kg) of the analogs and had an in vitro permeability similar to cocaine. The brain uptake ratios for AHN 1-055 and JHW 007 were in accordance with their in vitro permeabilities, both compounds had significantly higher BBB permeability and brain uptake as compared to cocaine. It is important to point out that although our in vitro studies suggested a very high
efflux for JHW 007 across the BBMEC system, a very high brain uptake for this compound was observed in our *in vivo* studies.

The disposition of the BZT analogs are in accordance with the requirements of a substitute therapeutic agent. The elimination of BZT analogs was significantly slower as compared to cocaine as can be seen from their long half-life (4.12 – 16.49 hr) and slow clearance (1.3 – 3.2 L/hr/kg). The clearance for all analogs, except JHW 007, was significantly slower than that for cocaine. Cocaine has two ester groups that are highly susceptible to hydrolysis by plasma esterases. The BZT analogs have a 3α-diphenylmethoxy group, that is metabolically more stable that the ester groups of cocaine (He et al., 1995). As a result, the elimination t1/2β is longer for the BZT analogs than for cocaine. It should be noted that amongst the BZT analogs, clearance increased and elimination t1/2 decreased as the steric bulk of the N-substituent increased. AHN 2-003, the parent compound (NH), exhibited the lowest clearance (1.32 L/hr/kg). The longer elimination half-life for the analogs is supported by *in vivo* studies with AHN 1-055 that showed long-acting locomotor stimulation, as compared to cocaine (Katz et al., 1999).

In conclusion, the BZT analogs are highly permeable across the BBB, but are also transported by an efflux system out of the cells. The net transport of the BZTs, although slightly lower, was not significantly different from cocaine. The BZT analogs possess a pharmacokinetic profile different from cocaine, with a significantly higher distribution, and most importantly a longer elimination half-life. Entry of these compounds into the brain was rapid, with a T_{max} of 5 minutes. Thus a distinctive behavioral profile, as seen with the BZTs, may not be due to a slow entry into the brain but may rely on the achievement of high brain to blood ratios or some other as of yet determined pharmacodynamic factor. In light of the desirable pharmacokinetic properties for substitute therapeutic medications, the differences in distribution and elimination
properties of the BZTs and cocaine identify the BZT analogs as promising candidates for treatment of cocaine abuse. Furthermore, as some of the BZTs do not appear to produce cocaine-like behaviors in animal models (Katz et al., 1999, 2001, Woolverton et al., 2000, 2001) their abuse liability may be limited and this further supports their continued development toward a cocaine abuse medication. Since the frequency of craving associated with cocaine abuse is influenced by the rapid clearance of this drug and subsequent rapid disappearance of the elevated dopamine levels, a slow clearance and much longer half-life as seen with these BZT analogs may be beneficial in reducing repeated drug administration associated with cocaine abuse. Studies are underway to evaluate the relationship between the pharmacokinetics of the BZT analogs and their pharmacodynamics (i.e., dopamine vs. time profile) in relation to cocaine.
References


Acknowledgements

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Table 1. Structural substituents, physicochemical properties, DAT binding and DA Uptake Inhibition Data on BZT Analogs and Cocaine (Agoston et al., 1997).

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>M.W.</th>
<th>ClogP&lt;sup&gt;1&lt;/sup&gt;</th>
<th>[&lt;sup&gt;3&lt;/sup&gt;H] WIN Binding&lt;sup&gt;2&lt;/sup&gt; K&lt;sub&gt;i&lt;/sub&gt; (nM)</th>
<th>DA Uptake&lt;sup&gt;3&lt;/sup&gt; IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHN 2-003</td>
<td>H</td>
<td>365.85</td>
<td>3.10</td>
<td>11</td>
<td>9.7</td>
</tr>
<tr>
<td>AHN 1-055</td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>393.16</td>
<td>3.95</td>
<td>12</td>
<td>71</td>
</tr>
<tr>
<td>AHN 2-005</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;CH=CH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>405.92</td>
<td>4.52</td>
<td>30</td>
<td>14</td>
</tr>
<tr>
<td>JHW 007</td>
<td>(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>421.96</td>
<td>5.53</td>
<td>25</td>
<td>370</td>
</tr>
<tr>
<td>Cocaine</td>
<td></td>
<td>303.31</td>
<td>2.72</td>
<td>300-400</td>
<td>150-200</td>
</tr>
</tbody>
</table>

<sup>1</sup>Calculated log of the partition coefficient (cLogP); <sup>2</sup>Affinity for the dopamine transporter (DAT) by determination of the displacement of [<sup>3</sup>H] WIN 35,428, an analog of cocaine; <sup>3</sup>Inhibition of [<sup>3</sup>H] dopamine (DA) uptake.
Table 2.  Bidirectional apparent permeability coefficients (Papp) of BZT analogs and cocaine (1 x 10^{-4} M) and Apparent permeability coefficients (Papp) of BZT analogs with PSC-833 across BBMEC monolayers.

<table>
<thead>
<tr>
<th>Compound</th>
<th>A→B</th>
<th>B→A(^1)</th>
<th>B→A/A→B</th>
<th>A→B with PSC833(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocaine</td>
<td>1.63 (± 0.05)</td>
<td>6.88 (± 0.20)</td>
<td>4.2</td>
<td>2.12 (± 0.07)</td>
</tr>
<tr>
<td>AHN 2-003</td>
<td>1.54 (± 0.10)</td>
<td>8.51 (± 0.90)</td>
<td>5.5</td>
<td>1.84 (± 0.13)</td>
</tr>
<tr>
<td>AHN 1-055</td>
<td>2.15 (± 0.10)*</td>
<td>11.5 (± 1.40)</td>
<td>5.3</td>
<td>3.10 (± 0.10)</td>
</tr>
<tr>
<td>AHN 2-005</td>
<td>1.56 (± 0.03)</td>
<td>9.86 (± 1.08)</td>
<td>6.2</td>
<td>2.91 (± 0.15)</td>
</tr>
<tr>
<td>JHW 007</td>
<td>2.83 (± 0.10)*</td>
<td>22.8 (± 2.00)**</td>
<td>8.0</td>
<td>3.87 (± 0.02)</td>
</tr>
</tbody>
</table>

\(^1\)Significant difference (p < 0.05) for B→A vs A→B for all compounds, *significant difference (p < 0.05) for A→B, **significant difference (p < 0.05) for B→A and  \(^2\) Significant difference (p < 0.05) for A→B vs A→B with PSC833.

P_{app} listed as 10^{-4} cm/sec. Data listed as mean ± S.D at n = 3. P_{app} sucrose = 1 x 10^{-5} cm/sec, P_{app} propranolol = 15 x 10^{-5} cm/sec, TEER = 100-150 Ω.cm².
Table 3. Population pharmacokinetic parameters (mean ± SD) for the BZT analogs and cocaine after intravenous administration to male Sprague Dawley rats

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>COCAINE</th>
<th>AHN 2-003</th>
<th>AHN 1-055</th>
<th>AHN 2-005</th>
<th>JHW 007</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (mg.hr/L)</td>
<td>5932 (± 444)</td>
<td>30304 (± 8477)</td>
<td>21397 (± 6643)</td>
<td>14592 (± 3324)</td>
<td>11132 (± 1449)</td>
</tr>
<tr>
<td>Cmax (ng/ml)</td>
<td>64283 (± 2163)</td>
<td>1239 (± 349)</td>
<td>1477 (± 162)</td>
<td>3389 (± 725)</td>
<td>984 (± 95)</td>
</tr>
<tr>
<td>Vss (L/kg)</td>
<td>0.9 (± 0.1)</td>
<td>30.5 (± 11.9)*</td>
<td>18.7 (± 2.3)*</td>
<td>12.3 (± 3.2)*</td>
<td>23.2 (± 3.2)*</td>
</tr>
<tr>
<td>Cl (L/hr/kg)</td>
<td>3.1 (± 0.2)</td>
<td>1.3 (± 0.5)*</td>
<td>1.8 (± 0.5)*</td>
<td>2.6 (± 0.6)*</td>
<td>3.2 (± 0.4)</td>
</tr>
<tr>
<td>t1/2-α (hr)</td>
<td>0.01 (± 0.0003)</td>
<td>0.17 (± 0.001)*</td>
<td>0.09 (± 0.003)*</td>
<td>0.21 (± 0.005)*</td>
<td>0.31 (± 0.004)*</td>
</tr>
<tr>
<td>t1/2-β (hr)</td>
<td>0.49 (± 0.06)</td>
<td>16.49 (± 0.11)*</td>
<td>7.69 (± 1.76)*</td>
<td>4.12 (± 0.33)*</td>
<td>5.35 (± 0.48)*</td>
</tr>
<tr>
<td>AUC_{Br}/AUC_{plasma}</td>
<td>2.1</td>
<td>2.0</td>
<td>4.8</td>
<td>6.6</td>
<td>5.6</td>
</tr>
<tr>
<td>Brain-t1/2-λ_z (hr)</td>
<td>0.6</td>
<td>12.5</td>
<td>6.2</td>
<td>5.8</td>
<td>8.5</td>
</tr>
</tbody>
</table>

Cocaine dose = 5 mg/kg, BZT analog dose = 10 mg/kg. * significant difference P < 0.05
Figure Captions

Figure 1. Chemical Structures of (A) 3α[bis (4′fluorophenyl) methoxy] tropane BZT analogs; structural substituents (R-) presented in Table 1 and (B) cocaine.

Figure 2. BZT Analogs and Cocaine Plasma Concentration vs Time Profiles. (A) Naïve-pooled averaging based pharmacokinetic profiles for plasma concentration vs. time profiles of AHN 2-003 (10 mg/kg iv), AHN 1-055 (10 mg/kg iv), JHW 007 (10 mg/kg iv) and cocaine (5 mg/kg iv) in male Sprague Dawley rats (325 g) and (B) Observed and predicted plasma vs. time profiles for AHN 2-003 (10 mg/kg iv), AHN 1-055 (10 mg/kg iv), JHW 007 (10 mg/kg iv) and cocaine (5 mg/kg iv) in male Sprague Dawley rats (325 g) using Winnonlin™. Data listed as mean ± SD at n = 3.

Figure 3. Naïve-pooled averaging based representative brain concentration vs. time profiles of BZTs and cocaine (BZTs: n = 33-36, Cocaine: n = 24).