Applicability of the Dopamine and Rate Hypotheses in Explaining the Differences in Behavioral Pharmacology of the Chloro-Benztropine Analogs: Studies Conducted Using Intracerebral Microdialysis and Population Pharmacodynamic Modeling

Ahmed A. Othman, Amy Hauck Newman, and Natalie D. Eddington

Pharmacokinetics-Biopharmaceutics Laboratory, Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, Baltimore, Maryland (A.A.O., N.D.E.); and Medicinal Chemistry Section, National Institute on Drug Abuse-Intramural Research Program, National Institutes of Health, Baltimore, Maryland (A.H.N.)

Received March 29, 2007; accepted May 21, 2007

ABSTRACT

Previous studies indicated that the chloro-benztropine analogs differed significantly in their cocaine-like activity, which was not expected based on the similarity in their in vitro binding affinity and functional potency at the dopamine transporter (DAT). The present study was designed to extend the understanding of the involvement of both pharmacokinetic and pharmacodynamic factors in mediating the behavioral differences among these analogs. The pharmacokinetics of 3'-chloro-3,4'-dichloro-3-(diphenylmethoxy)tropane (3'-Cl BZT), the analog showing a cocaine-like behavioral profile in rodents, was compared with previously reported pharmacokinetic characteristics of cocaine and 4',4'-diCl BZT, respectively. 4',4'-diCl BZT increased the DA levels at a slower rate and to a significantly lower extent relative to 3'-Cl BZT that were, in turn, lower than cocaine. The duration of dopamine elevation was as follows: 4',4'-diCl BZT > 3'-Cl BZT > cocaine. The model indicated faster association and dissociation with DAT for 3'-Cl BZT relative to 4',4'-diCl BZT. The present results indicate that behavioral differences among the chloro-analogs may be explainable based on both the dopamine and rate hypotheses of drug abuse.

Substantial evidence has accumulated over the years in favor of the dopamine (DA) hypothesis of cocaine abuse (Ritz et al., 1987; Kuhar et al., 1991; Volkow et al., 1997). According to this hypothesis, the ability of cocaine to block the dopamine transporter (DAT) and to inhibit DA uptake, which results in increased dopaminergic neurotransmission in the mesolimbic and mesocortical systems, is the critical element in mediating the reinforcing and psychostimulant effects of cocaine.

The benztropine (BZT) analogs are potent and selective DA uptake inhibitors (Newman et al., 1994, 1995; Katz et al., 1999). However, they are generally weak psychostimulants and weak reinforcers relative to cocaine in animal models of drug abuse (Katz et al., 1999, 2001; Woolverton et al., 2001). In addition, the BZT analogs have shown gradations in their cocaine-like activity, which was not expected based on the similarity in their in vitro binding affinity and functional potency at the dopamine transporter (DAT). The present results indicate that behavioral differences among the chloro-analogs may be explainable based on both the dopamine and rate hypotheses of drug abuse.

ABBREVIATIONS: DA, dopamine; DAT, dopamine transporter; BZT, benztropine; 3'-Cl BZT, 3'-chloro-3,4'-dichloro-3-(diphenylmethoxy)tropane; 4'-Cl BZT, 4'-chloro-3,4'-dichloro-3-(diphenylmethoxy)tropane; 4',4'-diCl BZT, 4',4'-dichloro-3,4'-dichloro-3-(diphenylmethoxy)tropane; NAc, nucleus accumbens; aCSF, artificial cerebrospinal fluid; HPLC, high-performance liquid chromatography; AUC, area under the curve; ANOVA, analysis of variance; AHN 1-055, 3α-[bis(4'-fluorophenyl)methoxy]tropane; AHN 2-005, N-allyl-3α-[bis(4'-fluorophenyl)methoxy]tropane; FDAT, free fraction of dopamine transporter; ODAT, fraction of the DA transporters occupied or blocked by the BZT analogs; CL, clearance; Vp, volume of the peripheral compartment; IAV, interanimal variability; Pb, brain-to-plasma partition coefficient; CV, coefficient of variation.
gradations in their in vitro affinity to DAT or in their potency as DA uptake inhibitors. For example, two studies indicated that 3'-chloro-3a-(diphenylmethoxy)tropane (3'-Cl BZT) significantly substituted for cocaine in rats trained to discriminate cocaine from saline (Kline et al., 1997; Katz et al., 2001). Conversely, neither 4'-chloro-3a-(diphenylmethoxy)tropane (4'-Cl BZT) nor 4',4'-dichloro-3a-(diphenylmethoxy)tropane (4',4'-diCl BZT) produced any cocaine substitution greater than saline (Kline et al., 1997; Katz et al., 2001). In addition, the chloro-analogs demonstrated reduced locomotor stimulant activity in mice relative to cocaine. However, 4'-Cl and 3'-Cl BZT were the closest to cocaine in terms of maximal stimulation during the 1st hour of administration followed by 3',4'-dichloro-3a-(diphenylmethoxy)tropane (3',4'-Cl BZT) followed by 4',4'-diCl BZT, which minimally stimulated the locomotor activity in mice (Katz et al., 2001). Moreover, the chloro-analogs differed significantly in their reinforcing efficacies in self-administration studies in rhesus monkeys, with the following rank order relative to cocaine: cocaine > 3'-Cl BZT = 4'-Cl BZT >> 3',4'-diCl BZT (Woolverton et al., 2001). The behavioral differences of the chloro-BZT analogs (compared with one another and with cocaine) seems to challenge the dopamine hypothesis given that these analogs (Fig. 1) possess comparable affinities and potencies at DAT that are higher than cocaine (DAT Kᵣ range, 20–32.5 versus 187 nM for cocaine; and DA uptake inhibition IC₅₀ range, 12.3–23.4 versus 236 nM for cocaine) (Katz et al., 2001). Recently, several studies focused on characterizing the mechanisms underlying the behavioral differences of the BZT analogs (Campbell et al., 2005; Desai et al., 2005a; Tanda et al., 2005; Othman et al., 2007). Such characterization could have important implications for successful design of substitute pharmacotherapies for treatment of cocaine abuse that should share the mechanism of action of cocaine without sharing its high abuse potential.

The rate and extent of brain entry, rate of occupancy of DAT, and/or rate of elevation of extracellular DA level, as well as extent and duration of DA elevation, are all critical factors in determining the abuse liability of DA uptake inhibitors (Volkow et al., 1995; Quinn et al., 1997; Gorelick, 1998, 2000, 2003). Previous pharmacokinetic studies from our laboratory in rats for cocaine, 4'-Cl, and 4',4'-diCl BZT (Raje et al., 2003; Othman et al., 2007) indicated that 1) the brain uptake of the chloro-analogs was comparable and higher than that of cocaine excluding the extent of brain exposure as a factor mediating the behavioral differences; 2) there was no time delay in brain entry of 4'-Cl BZT or 4',4'-diCl BZT in comparison with cocaine; and 3) the rank order for the plasma and brain elimination half-lives was the reverse of the differences in abuse liability, i.e., 4',4'-diCl BZT >> 4'-Cl BZT > cocaine.

The present study was designed to extend our understanding of the involvement of the pharmacokinetic and pharmacodynamic factors in mediating the differences in the behavioral pharmacology of the chloro-BZT analogs. Specifically, we wanted to 1) evaluate the pharmacokinetics and brain uptake of 3'-Cl BZT, the analog that has more consistently demonstrated cocaine-like effects in rodents and that may have higher abuse potential than the other chloro-BZT analogs; and 2) evaluate whether 3'-Cl and 4',4'-diCl BZT, two analogs representative of the extremes of the behavioral spectrum of the chloro-series, differ qualitatively and/or quantitatively in their effect on nucleus accumbens (NAc) DA level in terms of rate, extent, and duration of elevation. In addition, this study was designed to characterize the quantitative relationship between the plasma concentrations of the chloro-analogs and their effects on NAc DA level using a mechanistic population pharmacodynamic modeling approach that models the effect on the NAc DA level as a function of the binding kinetics to DAT.

**Materials and Methods**

**Materials**

Cocaine HCl, xylazine, dopamine HCl, EDTA, octyl sulfate sodium salt, (2-hydroxypropyl)-β-cycloexetrin, and sodium phosphate dibasic were purchased from Sigma-Aldrich (St. Louis, MO). Monochloroacetic acid was purchased from J. T. Baker (Phillipsburg, NJ). Sodium hydroxide was purchased from American Bioanalytical (Natick, MA). Potassium monobasic phosphate and potassium dibasic phosphates were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Artificial cerebrospinal fluid (aCSF) containing 150 mM sodium, 3.0 mM potassium, 1.4 mM calcium, 0.8 mM magnesium, 1.0 mM phosphorus, and 155 mM chloride were purchased from Harvard Apparatus Inc. (Holliston, MA). Ketamine HCl injection was purchased from Bedford Laboratories (Bedford, OH). Methohexital (brevital sodium) was purchased from Henry Schein (Port Washington, NY). 3'-Cl BZT HCl, 4'-Cl BZT HCl, and 4',4'-diCl BZT HBr were synthesized in the Medicinal Chemistry Section (National Institute on Drug Abuse-Intramural Research Program, Baltimore, MD) as described previously (Newman et al., 1994, 1995; Kline et al., 1997). All chemicals and solvents were high-performance liquid chromatography (HPLC) grade or American Chemical Society analytical grade.

**Animals**

Adult male Sprague-Dawley rats (250–350 g) were used in the pharmacokinetic and microdialysis studies. They were purchased from Harlan (Indianapolis, IN). The study protocols were approved by the Institutional Animal Care and Use Committee of the School of Pharmacy (University of Maryland, Baltimore). Rats were housed in the animal facility at a room temperature of 23 ± 1°C. They were allowed free access to food (Purina 5001 Rodent Chow; Purina, St. Louis, MO) and water ad libitum, and they were maintained on a 12-h light/dark cycle (light on from 7:00 AM to 7:00 PM).
Dosing Solutions

3'-Cl BZT HCl, 4',4'-diCl BZT HBr, and cocaine HCl were dissolved in 20% (2-hydroxypropyl)-β-cyclodextrin with sterile water for injection. The dosing for the pharmacokinetic and microdialysis studies was conducted at a volume of 1 ml/kg and at a dose of the free base equivalent to 5 or 10 mg/kg of the hydrochloride salt.

3'-Cl BZT Pharmacokinetic Study

The rats were administered 3'-Cl BZT at an i.v. dose of 5 or 10 mg/kg. A destructive sampling design was adapted where groups of three animals were sacrificed by CO2 asphyxiation predose and postdose at 5, 30, 60, 120, 240, 360, 480, and 600 min. Blood was collected by heart puncture using heparinized syringes, and the blood was centrifuged at 10 min at 3000 rpm. The plasma was separated and stored at −80°C until the analysis. Brain tissues were immediately removed, blotted on a filter paper, weighed, and stored at −80°C until the time of analysis.

Analysis of the Pharmacokinetic Samples

A previously published UV-HPLC method with few modifications was used for analysis of 3'-Cl BZT (Raje et al., 2002). The chromatographic conditions consisted of a Symmetry C18 column (150 × 4.6 mm; 5 μm; Waters, Milford, MA), UV detector (λ = 220 nm), mobile phases [methanol/0.05 M Na2HPO4 pH 3.0, 40:60 (v/v) (A) and methanol/0.05 M Na2HPO4 pH 3.0, 50:50 (v/v) (B)], and a flow rate of 1 ml/min pumped using an isotropic profile (55% solvent A and 45% solvent B). The plasma samples were extracted with hexane followed by evaporation and reconstitution in the mobile phase. The brain samples were double extracted with hexane following homogenization with distilled water. 4'-Cl BZT was used as an internal standard for analysis of 3'-Cl BZT without any interference. The calibration curves were linear (r² = 0.999) in the range of 25 to 5000 ng/ml and 100 to 20,000 ng/ml for the plasma and brain samples, respectively.

Pharmacodynamic Studies

Surgical Procedures. The surgical and microdialysis procedures followed a previously published protocol with minor modifications (Baumann et al., 2005). In brief, rats were anesthetized with i.p. administration of a mixture of 100 mg/kg ketamine and 20 mg/kg xylazine. Catheters made of Silastic Medical Grade tubing (Dow Corning, Midland, MI) were then implanted in the right external jugular vein of the anesthetized rats. Subsequently, rats were placed in a stereotactic apparatus where craniotomy was performed followed by implantation of plastic guide cannulae (CMA 12; CMA Microdialysis, Acton, MA) above the NAc at the following coordinates: anterior-posterior, +1.6 mm and medial-lateral, −1.7 mm from bregma; and dorsal-ventral, −6.0 mm from dura with a flat skull (Paxinos and Watson, 1986). The guide cannulae were cemented in place with three stainless steel screws and dental acrylic. After the surgery, the rats were placed in separate cages, and they were allowed free access to food and water for a minimum of 5 days of postsurgical recovery.

In Vivo Microdialysis. On the night before the experiment, CMA/12 microdialysis probes (2 × 0.5-mm exchange surface; CMA Microdialysis) were attached to the microdialysis inflow and outflow tubing and perfused with aCSF at a flow rate of 1 μl/min, pumped using CMA 400 syringe pump (CMA Microdialysis). After a short period of in vitro probe perfusion, rats were lightly anesthetized with the ultrashort-acting barbiturate methohexital at 5 mg/kg i.v., and a probe was inserted into the guide cannula of each rat. A plastic collar was placed around the neck of each rat and the animal was then placed in a Flexiglas chamber (Coulbourn Instruments, Allentown, PA). The animals were allowed free movement in the chambers while connected through tethering systems attached to their plastic collars. After insertion of the dialysis probes, perfusion with the aCSF continued overnight and afterward throughout the experiment at a flow rate of 1 μl/min. On the next morning, four dialysate samples were collected for each animal to determine the baseline dopamine level and to ensure its stability. The rats were then administered vehicle [20% (2-hydroxypropyl)-β-cyclodextrin; 1 ml/kg], 5 mg/kg cocaine, 5 or 10 mg/kg 3'-Cl BZT, or 5 or 10 mg/kg 4',4'-diCl BZT via the jugular vein catheter. The selected doses were in agreement with the studied doses in the pharmacokinetic studies as well as the behaviorally active doses of these drugs, while taking into account the route of administration. The dialysate samples were collected at 10, 20, 30, 40, 50, 60, 90, 120, 180, 240, 360, 540, and 720 min or until dopamine returned to its basal value. Less frequent sampling scheme that covered the whole study duration was adapted for the vehicle study (10, 30, 60, 120, 240, 360, 540, and 740 min). Dialysis collection took place throughout the 10-min preceding each sampling time and the reported sampling times are corrected for the lag caused by the outlet tubing and probe dead volumes. The dialysate samples were immediately analyzed for their dopamine content using HPLC with electrochemical detection. At the end of the microdialysis experiments, animals were returned to their housing, and they were allowed free access to food and water until euthanized by CO2 asphyxiation. Once the animals were euthanized, the brains were removed from the skulls and stored in 10% formalin. The brains were sectioned on a cryostat, and the sections were mounted on glass slides followed by verification of the probe tip placement within the NAc by visual inspection according to the atlas of Paxinos and Watson (1986). Rats with incorrect probe placement were excluded from the data analysis.

HPLC Analysis of the Dialysate Samples. Samples were analyzed for dopamine content by HPLC with electrochemical detection as described previously (Baumann et al., 2005) with minor modifications. The mobile phase consisted of 150 mM monochloroacetic acid, 1.5 mM sodium octane sulfonic acid, 145 mM sodium hydroxide, 215 μM disodium EDTA, 6% (v/v) methanol and 6% (v/v) acetonitrile (final pH 5.3). The mobile phase was pumped at a flow rate of 60 μl/min. The HPLC system consisted of an ISCO 260 D syringe pump (ISCO, Lincoln, NE), BAS model LC-4C amperometric detector (BAS Bioanalytical Systems, West Lafayette, IN), and BAS UniJet microbore analytical column (C18, 100-× 1 mm, 5 μM; BAS Bioanalytical Systems). The working glassy carbon electrode was set at +650 mV relative to Ag/AgCl reference electrode. The signal filter and the range were set to 0.1 Hz and 0.5 nA, respectively. The injection volume was 8 μl. The calibration curves for dopamine were linear in the range of 0.1 to 5 ng/ml (r² = 0.996) without any interfering peaks.

Data Analysis

Pharmacokinetic Data Analysis. The destructive sampling data obtained from the pharmacokinetic study of 3'-Cl BZT were initially analyzed by the naive averaging method to determine the appropriate model and to derive initial parameter estimates for the population analysis. The plasma concentrations from the three animals at each time point for each dose level were averaged. The average concentrations versus time data were then used for compartmental modeling using WinNonlin version 4.1 software (Pharsight, Mountain View, CA). Various compartmental models were evaluated to determine the most appropriate model. Subsequently, nonlinear mixed effect modeling was conducted using NONMEM version 5, level 1.1 (GlobosMax LLC, Hanover, MD). This approach has been shown previously to result in less biased estimates of the structural model parameters than the naive pooling of the data in destructive sampling designs (Hing et al., 2001). The data from the two dose levels (data from 42 animals) were compiled and analyzed. The final parameter estimates from the naive averaging analysis were used as initial estimates for the population analysis. Based on the results from the naive averaging analysis, the population analysis started with a two-compartment structural model. Interanimal variability in the pharmacokinetic parameters was estimated using an exponential error model as follows:

\[ P_i = TVP_{exp}(\eta_i) \]
where $\eta_i$ is the proportional difference between the hypothetical true parameter estimate of the $i$th animal ($P_i$) and the typical population parameter value (TVP), and $\eta_i$ is assumed to be normally distributed with a mean of zero and a variance of $\sigma^2$. The residual error (which includes model mis-specification, intra-animal variability as well as errors in dosing, sampling times, and sample analysis) was described using a proportional error model as follows:

$$Y_{\text{obs}} = Y_{\text{pred}} \times (1 + \epsilon)$$

where $Y_{\text{obs}}$ is the observed plasma concentration, $Y_{\text{pred}}$ is the model predicted plasma concentration, and $\epsilon$ is a normally distributed parameter with a mean of zero and variance of $\sigma^2$. The analysis was performed using the first-order estimation method. The final model was determined based on inspection of goodness of fit plots, precision of parameter estimates, and the value of the objective function. In addition, the likelihood ratio test ($\alpha = 0.05$) was used as a selection criterion between rival hierarchical models (Sheiner and Beal, 1981).

The brain-to-plasma partition coefficient ($R_i$) was calculated as a measure of brain uptake according to the following formula:

$$R_i = \frac{\text{AUC}_{0-\text{inf}}(\text{brain})}{\text{AUC}_{0-\text{inf}}(\text{plasma})}$$

where $\text{AUC}_{0-\text{inf}}(\text{brain})$ and $\text{AUC}_{0-\text{inf}}(\text{plasma})$ are the area under the curve for brain and plasma concentrations, respectively. The $\text{AUC}_{0-\text{inf}}$ was calculated using WinNonlin noncompartmental analysis by applying the log-linear trapezoidal rule.

**Pharmacodynamic Data Analysis.** For the microdialysis experiments, the brain dialysate dopamine level in each sample (uncorrected for probe recovery) was expressed as a percentage of the mean basal dopamine values. The mean basal dopamine value for each rat was calculated as the mean of dopamine concentrations in the four samples immediately preceding the drug or vehicle injections. For each treatment group, brain dialysate DA levels at the different times points were compared by one-way analysis of variance (ANOVA) with repeated measures over time followed by Dunnett's post hoc analysis with time 0 as control ($\alpha = 0.05$). In addition, the two doses of each of the chloro-analogs were compared using a two-factor (drug dose and time) ANOVA with repeated measures over time.

**Pharmacodynamic Modeling.** The concentrations of 3'-Cl BZT and 4',4'-diCl BZT were not determined at the site of action (the nucleus accumbens); therefore, the plasma concentrations were used for the pharmacodynamic modeling. The effect-time profiles of 3'-Cl BZT and 4',4'-diCl BZT were delayed relative to their plasma profiles. Three pharmacodynamic models were considered to account for this time delay: 1) effect compartment model, which assumes slow equilibration between the plasma and the biophase (Sheiner et al., 1979); 2) indirect response model, with inhibition of output as a secondary parameter from the ratio between brain and plasma concentrations versus time curves, respectively. The $\text{AUC}_{0-\text{inf}}$ was calculated using WinNonlin noncompartmental analysis by applying the log-linear trapezoidal rule.

**Results**

**Pharmacokinetics of 3'-Cl BZT.** Figure 2A represents the observed and the population predicted plasma concentrations of 3'-Cl BZT upon i.v. administration to male Sprague-Dawley rats at two dose levels (5 and 10 mg/kg). Figure 2B
represents the corresponding brain concentrations versus time profiles. The final population pharmacokinetic model for 3'-Cl BZT consisted of a two-compartmental structural model parameterized in terms of clearance (CL), volume of the central compartment, volume of the peripheral compartment (Vp) and intercompartmental clearance (ADVAN3 TRANS4 NONMEM Subroutines). The interanimal variability in CL and Vp were not significantly different from zero based on the likelihood ratio test (p > 0.05), and they were omitted from the model. Due to the destructive sampling design, precise estimation of the residual random error was not possible. Consequently, the residual error was fixed to 10.7% based on a sensitivity analysis, and the final selected value was the value resulting in the lowest objective function and the highest precision in estimation of interanimal variability. The 10.7% residual random error was comparable with the estimated or fixed values for previously studied BZT analogs using identical experimental technique (Othman et al., 2007). Figure 3 represents the relevant diagnostic plots for the final population pharmacokinetic model for 3'-Cl BZT, and it indicates that the model described the pharmacokinetics of 3'-Cl BZT without any systematic bias. The population pharmacokinetic parameters along with their associated interanimal variability and the precision of the parameter estimates (expressed as coefficient of variation) are reported in Table 1. The secondary pharmacokinetic parameters of interest along with their associated standard deviation were calculated from empirical Bayes estimates of the animal-specific parameters provided by NONMEM. These secondary parameters are reported in Table 2 in comparison with the previously reported parameters for 4'-Cl BZT, 4',4''-diCl BZT, and cocaine (Othman et al., 2007). The calculated pharmacokinetic parameters for 3'-Cl BZT based on the population analysis were in good agreement with results of the assumption-free noncompartmental analysis (data not shown). The dose-exposure relationship for 3'-Cl BZT was linear in plasma over the dose range studied (Fig. 2A; Table 2; data from noncompartmental analysis). However, the brain exposure observed at the 10-mg/kg dose was about 3.4-fold that of the 5-mg/kg dose (Fig. 2B). This resulted in a higher brain-to-plasma partition coefficient for the 10-mg/kg dose compared with the 5-mg/kg dose of 3'-Cl BZT (Table 2). The significance of this difference could not be assessed given the destructive sampling design and the naive averaging of the brain data. 3'-Cl BZT had the shortest plasma and brain elimination half-lives (t1/2) among all the chloro-BZT analogs (Table 2).

Effect of Cocaine, 3'-Cl BZT, and 4',4''-diCl BZT on the NAc DA Levels. Figure 4 shows the effects of i.v. administration of the vehicle [20% (2-hydroxypropyl)-β-cyclo-
In this model, the drug effects are governed by the drug concentration as well as by the association and dissociation kinetics with DAT. The relevant diagnostic plots for the final pharmacodynamic models for 3'-Cl BZT and 4',4''-diCl BZT are presented in Fig. 6. The scatter plots of the observed versus population and individual predicted dialysate dopamine levels (Fig. 6, A, B, D, and E) and the dose stratified observed and mean population predicted versus time profiles (Fig. 7) indicated that the models adequately described the pharmacodynamics of 3'-Cl BZT and 4',4''-diCl BZT. In addition, the scatter plots of the weighted residuals versus time did not show any significant pattern, indicating the lack of significant bias in the fit to the data (Fig. 6, C and F). In addition, the majority of weighted residuals were within ±3 S.D. of the mean (Fig. 6, C and F). The population pharmacodynamic parameters along with their associated interanimal variability and the precision of the parameter estimates (expressed as coefficient of variation) are reported in Table 3. The pharmacodynamic parameters were estimated with adequate precision. Precise estimation of the baseline for 4',4''-diCl BZT was not possible, most probably because the DA levels remained elevated after drug administration during the 12-h observation period. Therefore, the baseline was fixed in the model to its known value (i.e., 100%). The interanimal variability in the pharmacodynamic parameters was best described with an exponential pharmacostatistical model. The estimation of interanimal variability in the pharmacodynamic parameters was relatively imprecise; most probably due to the relatively small sample sizes. As indicated previously, the mean population pharmacokinetic parameters were used for the pharmacodynamic analysis because the pharmacokinetic and pharmacodynamic experiments were not conducted in the same set of animals. Therefore, the estimates for interanimal variability in the pharmacodynamic parameters should be interpreted with caution, because they may be confounded with the pharmacokinetic variability.

The model estimates indicated significant difference between 3'-Cl BZT and 4',4''-diCl BZT in terms of interaction with DAT at 3'-Cl BZT having significantly higher apparent association and dissociation rate constants. In addition, 3'-Cl BZT had lower apparent $K_d$ value ($K_{off}/K_{on}$ ratio) compared with that of 4',4''-diCl BZT (Table 3).

**Discussion**

Behavioral studies of the chloro-BZT analogs revealed significant differences in their cocaine-like activity, and consequently, in their preclinical abuse liability. These differences seemed to contradict the dopamine hypothesis of psychostimulant abuse; given the similarity among these analogs in their in vitro affinities to DAT, and in their in vitro potencies as DA uptake inhibitors. Because of the shared main mechanism of action between the chloro-BZT analogs and cocaine,
TABLE 2
Secondary pharmacokinetic parameters (calculated from the NONMEM empirical Bayes estimates of the individual animal-specific pharmacokinetic parameters) and brain-to-plasma partition coefficient and brain t (based on the noncompartmental analysis performed with WinNonlin) for 3′-Cl BZT in comparison with previously published data for 4′-diCl BZT, 4′,4′-diCl BZT, and cocaine (Othman et al., 2007)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>3′-Cl BZT</th>
<th>4′-Cl BZT</th>
<th>4′,4′-diCl BZT</th>
<th>Cocaine</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mg/kg</td>
<td>10 mg/kg</td>
<td>5 mg/kg</td>
<td>10 mg/kg</td>
<td>5 mg/kg</td>
</tr>
<tr>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>AUC (µg - min/ml)</td>
<td>n = 18</td>
<td>n = 24</td>
<td>n = 18</td>
<td>n = 24</td>
</tr>
<tr>
<td>Vss (l/kg)</td>
<td>80.5 ± 1.9</td>
<td>161.0 ± 1.0</td>
<td>63.3 ± 2.6</td>
<td>126.6 ± 4.4</td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>10.4 ± 1.9</td>
<td>10.3 ± 1.1</td>
<td>24.1 ± 3.6</td>
<td>21.6 ± 4.4</td>
</tr>
<tr>
<td>K1</td>
<td>1.9 ± 0.4</td>
<td>1.9 ± 0.2</td>
<td>3.52 ± 0.5</td>
<td>3.16 ± 0.7</td>
</tr>
<tr>
<td>Brain t1/2 (h)</td>
<td>5.3</td>
<td>9.7</td>
<td>4.6</td>
<td>3.1</td>
</tr>
</tbody>
</table>

Vss, steady-state volume of distribution.

Fig. 4. Time course of the effects of i.v. administration of the vehicle [20% (2-hydroxypropyl)-β-cyclodextrin; 1 ml/kg], 5 mg/kg cocaine (A), 5 or 10 mg/kg 3′-Cl BZT (B), and 5 or 10 mg/kg 4′,4′-diCl BZT (C) on extracellular levels of DA in the dialysate samples from the nucleus accumbens of Sprague-Dawley rats. Arrows indicate the time of drug injection. Data are presented as a percentage of the average of four baseline samples collected before drug treatment, and values are means with the vertical bars representing S.E.M. (n = 6 rats for each of the treatment groups except for vehicle and 10-mg dose of 3′-Cl BZT where n = 4 rats). When no error bar is visible, the deviation was within the size of the symbols. Mean baseline level of DA in the dialysate samples was 2.17 ± 0.17 nM (n = 32 rats). *, p < 0.05 compared with the pretreatment basal DA values.

Fig. 5. Mean observed plasma concentration and effect (NAc dialysate DA levels) versus time plots for 10 mg/kg cocaine (A), 5 or 10 mg/kg i.v. 3′-Cl BZT and 10 mg/kg i.v. 4′,4′-diCl BZT upon administration to Sprague-Dawley rats. The figure shows the time delay in the effect of the chloro-BZT analogs. The plasma concentrations for 4′,4′-diCl BZT are from a previously reported study (Othman et al., 2007).

it was of considerable interest to delineate the causes of the behavioral differences of this class of compounds (Tolliver et al., 1999; Woolverton et al., 2000, 2001; Katz et al., 2001), because it may improve the overall understanding of the determinants of abuse potential of DA uptake inhibitors, which is essential for informed design of low-risk substitute therapeutics for treatment of cocaine abuse.

Several reports indicated that 3′-Cl BZT possessed some behavioral effects similar to cocaine (Kline et al., 1997; Woolverton et al., 2000, 2001; Katz et al., 2001). Conversely, 4′,4′-diCl BZT was almost totally devoid of these cocaine-like behavioral patterns (Katz et al., 1999, 2001). The results of the present study show remarkable quantitative and qualitative differences among 3′-Cl BZT, 4′,4′-diCl BZT, and cocaine in their effects on the extracellular DA level in the NAc, the reward center responsible for the reinforcing effects of most drugs of abuse.

3′-Cl BZT resulted in a rapid DA elevation (DA Tmax = 20–30 min) that although slower than that of cocaine (DA Tmax = 10 min), still it was much faster than the rate of DA elevation observed with 4′,4′-diCl BZT (DA Tmax = 120–240 min) (Fig. 4). These differences in onset of DA elevation are in agreement with the rate hypothesis of psychostimulant abuse, which postulates that drugs of slower onset of action are less likely to be abused than drugs of faster onset (Gorelick, 1998). In addition, the present results agree with the evidence that the rate at which DA increases is an essential variable for determining the reinforcing effects of most drugs of abuse (Volkow, 2006). The differences in the rate of DA elevation observed in the present study cannot be accounted for by difference in the rate at which the drugs reached the site of action for two reasons. First, the microdialysis studies were conducted using the i.v. route, which excludes any possible difference in the absorption rate from
Fig. 6. Diagnostic plots for the population analysis of the NAc dialysate dopamine levels after i.v. administration of 5 or 10 mg/kg 3′-Cl BZT (n = 10) or 5 or 10 mg/kg 4′, 4′-diCl BZT (n = 12) to Sprague-Dawley rats. The analysis was conducted with NONMEM using a kinetic binding model. A and D, population predicted versus observed dialysate dopamine levels. B and E, individual animal predicted versus observed dialysate dopamine levels. C and F, weighted residuals versus time plots. The dashed lines in the weighted residual plots represent ± 3 S.D.

Fig. 7. Observed (closed circles) and mean population predicted (solid lines) time courses of the effect of the 3′-Cl BZT and 4′,4′-diCl BZT on the NAc DA levels stratified by dose.
the site of administration. Second, the brain uptake studies for cocaine (Raje et al., 2003), 4',4'-diCl BZT (Othman et al., 2007), and 3'-Cl BZT indicated that the three drugs were detected in the brain at their highest concentrations within 2 to 5 min of administration, which excludes the difference in brain entry rate as a cause for the difference in rate of DA elevation. This conclusion is further supported by previous microdialysis studies that indicated that even direct delivery of 4'-Cl BZT into the NAc by local infusion resulted in delayed onset of DA elevation relative to local infusion of cocaine (Tolliver et al., 1999). Based on that observation, Tolliver et al. (1999) suggested that the BZT analogs bind to DAT at slower rate than cocaine, a hypothesis that was later supported by in vivo DAT occupancy studies with a series of fluoro-BZT analogs (Desai et al., 2005a,b). In one of these studies, the two fluoro-BZT analogs AHN 1-055 and AHN 2-005 showed comparable and slower rates of association with DAT relative to cocaine (Desai et al., 2005a). When these two analogs were compared using microdialysis in our laboratory, AHN 1-055 and AHN 2-005 demonstrated almost superimposable DA profiles (Raje et al., 2005). By analogy, if the rates of association of 3'-Cl BZT and 4',4'-diCl BZT were not different, administration of these analogs would be expected to result in similar DA elevation patterns. Therefore, it seems that the rate of association with DAT differs significantly among the chloro-BZT analogs, with 4',4'-diCl BZT most probably having much slower rate of DAT association relative to 3'-Cl BZT.

The extent of DA elevation was also consistently different across the three drugs. At an i.v. dose of 5 mg/kg, cocaine resulted in about 10-fold increase in extracellular DA; in agreement with a report by Baumann et al. (1994), which showed, using a similar microdialysis technique, that 3 mg/kg i.v. cocaine resulted in a 6-fold increase in the DA levels. In contrast to cocaine, 3'-Cl and 4',4'-diCl BZT at the 5-mg/kg dose resulted in 4.4- and 2-fold increase in DA, respectively. At this dose, the brain uptake, as measured by the brain-to-plasma partition coefficients, for cocaine, 3'-Cl and 4',4'-diCl BZT was 2.1, 5.3, and 4.5, respectively. These results indicate that the differences in the extent of DA elevation by the three drugs most probably stem from differences in their in vivo potency or intrinsic activity rather than from differences in their brain exposure. The fact that even by doubling the dose, the DA elevation observed with 3'-Cl and 4',4'-diCl BZT (6.4- and 2.8-fold, respectively) was significantly lower than the 10-fold observed with cocaine at half the dose further supports this conclusion.

In the present study, 3'-Cl BZT has shown plasma and brain elimination half-lives around 1.9 and 1.5 h, respectively. These values were the shortest and closest to cocaine among all the BZT analogs tested so far (Raje et al., 2003; Othman et al., 2007; unpublished data from our laboratory). Moreover, the relatively short half-lives of 3'-Cl BZT contrast markedly with the long plasma and brain elimination half-lives of 4',4'-diCl BZT (Table 2). In agreement with the difference in the rate of elimination from the body, the rate of decline of DA after the peak was different across the three drugs, with cocaine showing very fast DA decline followed by 3'-Cl BZT, which maintained elevated levels for about 6 h. In contrast, 4',4'-diCl BZT showed sustained DA elevation during the 12-h time course of the experiment (Fig. 4). Several studies of psychostimulant drugs indicated that the timing of drug-seeking responses in experimental animals is dependent on the fluctuations in the extracellular DA concentrations; with higher probability of drug seeking when the DA concentrations fall near certain trigger points (Wise et al., 1995; Ranaldi et al., 1999). Therefore, it seems that the differences in the patterns of decline in DA might also explain, in part, the observed differences among cocaine and the chloro-compounds in the rates of self-administration in rhesus monkeys (Woolverton et al., 2000, 2001). It is noteworthy that even though the rank order for duration of DA elevation (cocaine < 3'-Cl BZT < 4',4'-diCl BZT) matches the pattern of their difference in the plasma and brain elimination half-lives (Table 2), it still seems that the dissociation of 4',4'-diCl BZT from DAT was slower than 3'-Cl BZT, which resulted in sustained DA elevation for 4',4'-diCl BZT even though its systemic concentrations were decreasing with time (Fig. 5).

The effect-time profiles of 3'-Cl BZT and 4',4'-diCl BZT were delayed relative to their plasma profiles. A kinetic binding model that assumes slow equilibration between the free drug, free DAT, and drug-DAT complex was used to describe the pharmacodynamics of 3'-Cl BZT and 4',4'-diCl BZT. The parameter estimates from the model indicated that 3'-Cl BZT binds with DAT approximately 5 times faster than 4',4'-diCl BZT, resulting in the difference in onset of DA elevation. In addition, the dissociation of 3'-Cl BZT was ~3-fold faster relative to 4',4'-diCl BZT, resulting in observed difference in duration of DA elevation and rate of DA decline. Moreover, the $K_d$ values ($K_{off}/K_{on}$) indicated that 4',4'-diCl BZT seems to have lower apparent in vivo affinity to DAT than 3'-Cl BZT, which may be responsible for the difference between these two analogs in terms of maximal DA elevation.

In conclusion, the present data make it clear that the behavioral profiles of the chloro-BZT analogs are supportive rather than contradictory to the dopamine hypothesis, and that these analogs differ significantly in their in vivo dopa-

### Table 3

Population pharmacodynamic parameters for 3'-Cl BZT ($n = 10$) and 4',4'-diCl BZT ($n = 12$) along with their associated interanimal variability using the DAT binding model

The analysis was conducted using NONMEM V software. Values in parentheses are % CV.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>3'-Cl BZT</th>
<th>4',4'-diCl BZT</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{on}$ (ml/ng · h)</td>
<td>0.010 (17.9)</td>
<td>0.002 (25.7)</td>
</tr>
<tr>
<td>$K_{off}$ (h$^{-1}$)</td>
<td>1.73 (8.1)</td>
<td>0.599 (12.8)</td>
</tr>
<tr>
<td>$K_{d}$ (ng/ml)*</td>
<td>173</td>
<td>300</td>
</tr>
<tr>
<td>Baseline</td>
<td>116 (7.7)</td>
<td>100 (Fixed)</td>
</tr>
<tr>
<td>% Residual error</td>
<td>21.9 (14.7)</td>
<td>15.6 (20.9)</td>
</tr>
</tbody>
</table>

*N.E., not estimated.

* Secondary parameter ($K_{off}/K_{on}$).
minergic patterns, which account for the differences in abuse liability in a way that corroborates the rate hypothesis of drug abuse. A substitute therapeutic agent that possesses a pharmacodynamic profile similar to 4’,4’-diCl BZT may be helpful for patients during withdrawal from cocaine use by correcting the hydropaminergic state underlying the dysphoria and anhedonia experienced during this phase (Volkow et al., 2002). At the same time, due to its slow onset and long duration of action, this substitute agent will have minimal risk of abuse on its own. An attractive feature of some BZT analogs, such as 4’,4’-diCl BZT, is their minimal abuse potential even when administered through the i.v. route, unlike methylenidate, for example, that has shown low abuse liability upon oral administration, whereas it is abused using the i.v. route (Parran and Jasinski, 1991). A drug that is devoid of abuse potential regardless of the route of administration and dosage form will definitely be favored taking in account the vulnerable nature of the target population.

Acknowledgments

We acknowledge Dr. Santosh Kulkarni and J. Cao (Medicinal Chemistry Section, National Institute on Drug Abuse-Intramural Research Program) for synthesizing multigram quantities of the BZT analogs used in this study; Dr. Michael Baumann for the technical assistance with microdialysis and probe placement verification; Dr. Angela Abéló for the technical assistance with some aspects of the pharmacodynamic modeling; and Dr. Pravin Jadhav for the thoughtful discussions.

References


Other references are available on the website: J Pharmacol Exp Ther 299:344–350.


Address correspondence to: Dr. Natalie D. Eddington, Department of Pharmacology, New York University School of Medicine, 550 Fifth Avenue, New York, NY 10017.