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The Novel *N*-Substituted Benztropine Analog GA2-50 Possesses Pharmacokinetic and Pharmacodynamic Profiles Favorable for a Candidate Substitute Medication for Cocaine Abuse

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Abstract

GA2-50 is a novel *N*-substituted benztropine analog with improved potency and selectivity for the dopamine transporter. The pharmacokinetic and pharmacodynamic properties of GA2-50 were characterized as a part of its preclinical evaluation as a substitute medication for cocaine abuse. *In vitro* transport and metabolism studies as well as pharmacokinetic studies in rats were conducted. Effect of GA2-50 on the extracellular nucleus accumbens (NAc) dopamine levels and on cocaine's induced dopamine elevation was evaluated using intracerebral microdialysis. GA2-50 showed high transcellular permeability despite being a P-glycoprotein substrate. GA2-50 was a substrate of human CYP2D6, CYP2C19, CYP2E1, rat CYP2C11, CYP2D1, CYP3A1, and CYP1A2; with low intrinsic clearance values. *In vivo*, GA2-50 showed high brain uptake ($R_i \sim 10$), large volume of distribution ($V_{ss} = 37$ L/kg), and long elimination half-life ($t_{1/2} = 19$ h). GA2-50 resulted in 1.6- and 2.7-fold dopamine elevation at the 5 and 10 mg/kg i.v. doses. Dopamine elevation induced by GA2-50 was significantly reduced, slower and longer lasting than previously observed for cocaine. GA2-50 had no significant effect on cocaine's induced dopamine elevation upon simultaneous administration. Results from the present study indicate that GA2-50 possesses several attributes sought after for a substitute medication for cocaine abuse.

Keywords

cocaine abuse; substitution therapy; pharmacokinetics; pharmacodynamics; microdialysis

INTRODUCTION

Cocaine abuse continues to represent a serious health problem in the United States. Recent estimates (2005) indicated that 2.4 million Americans aged 12 or older were current cocaine users. In addition, approximately 2,400 Americans initiated cocaine use daily.¹ Currently, there are no approved pharmacotherapies for treatment of cocaine abuse² which further aggravates the problem.

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The substitute approach for treatment of drug abuse is based on replacing the illicit drug with a safer legal maintenance medication, that shares mechanisms of action and produces some effects in common with the illicit drug, in order to stabilize the patient.³ This approach has shown success for treatment of opioid and nicotine dependence.^{4,5} In addition, several preclinical and clinical reports^{6–8} emphasize the validity and signal the possible successful outcomes of pursuing the substitute approach for treatment of cocaine abuse.

Cocaine inhibits the reuptake of dopamine (DA), serotonin, and norepinephrine via blocking their corresponding transporters.^{9,10} However, the addictive and euphorogenic effects of cocaine are attributed mainly to the blockade of the DA transporter (DAT) and the resultant potentiation of dopaminergic neurotransmission.^{11–13} Therefore, much research has focused on the DA uptake inhibitors as candidate substitute medications for cocaine abuse.^{2,14} However, in order to be considered as a promising candidate, the DA uptake inhibitor should have high selectivity for DAT; so as to minimize the side effects that may result from actions at other sites. More importantly, it should result in a slow onset and significantly reduced magnitude of DA elevation in comparison with cocaine. In addition, the DA uptake inhibitor should be eliminated slowly from the body and possess long duration of action. These characteristics are believed to reduce the abuse potential^{15–18} which is a considerable safety concern for a medication targeted to such a vulnerable patient population.

The analogs of benztropine (BZT) are potent DA uptake inhibitors that have been synthesized as potential substitute therapeutics for cocaine abuse. The design of these analogs included several structural modifications in order to improve the affinity and selectivity to DAT (for review, see^{19,20}). When tested in animal models of drug abuse, many BZT analogs demonstrated reduced cocaine-like behavioral effects and low preclinical abuse potential.^{21, 22} In addition, recent studies have indicated that certain compounds from this series may even block the stimulant effects of cocaine *in vivo*.²³ These attributes suggest that a successful cocaine medication may come from this class of compounds. Pharmacokinetic and pharmacodynamic evaluation of several BZT analogs in our laboratory have indicated that these compounds have slower clearance and result in slower and prolonged DA elevation in comparison with cocaine.^{24,25} However, recent studies indicated that the pharmacokinetics and pharmacodynamics of the BZT analogs were highly sensitive to structural modifications, resulting in significant differences in the rate of elimination, apparent distribution characteristics, as well as in onset, extent and duration of the extracellular DA elevation induced by these compounds in experimental rats.^{26,27}

GA2-50 is a novel BZT analog with high affinity and selectivity to the DAT as well as high potency as a DA uptake inhibitor (Tab. 1). The structural modifications in the design of GA2-50 included *para*-substitution of a fluoro-group on each of the pendant phenyl rings of BZT. In addition, the *N*-methyl group of BZT was replaced with an (*R*)-2''-amino-3''-methyl-*n*-butyl group (Fig. 1). It has been previously shown that the di-fluoro substitution enhanced the affinity and selectivity of the BZT analogs to DAT versus the serotonin and norepinephrine transporters (NET). In addition, a bulky *N*-substitution significantly reduced the affinity to the muscarinic M₁ receptors.^{19,28} Stereoselectivity was originally reported for DAT binding with GA2-50,²⁸ however, subsequent testing showed no stereoselectivity at DAT and only a twofold difference in affinity at muscarinic M₁ receptors.¹⁹ Preliminary behavioral evaluation of GA2-50²⁹ suggested that this dopamine uptake inhibitor does not demonstrate a cocaine-like behavioral profile and may have potential for development as a cocaine abuse medication.

The present study was designed to evaluate the impact of the structural modifications of GA2-50 on its *in vitro* transport, *P*-glycoprotein mediated efflux, metabolism, as well as on its *in vivo* pharmacokinetics, and effect on extracellular brain DA levels in Sprague–Dawley rats. In addition, the study was designed to determine whether GA2-50 will competitively block the

effect of cocaine on the brain DA levels upon simultaneous administration. The results from this study are essential to further explore the potential of this compound as a candidate medication for cocaine abuse. In addition, they highlight some of the mechanisms that may be underlying the low abuse potential of GA2-50 despite its high *in vitro* potency as a DA uptake inhibitor.

MATERIALS AND METHODS

Materials

Caco-2 cells were purchased from American Type Culture Collection (Manassas, VA). MDCK-MDR1 cells were a gift from Dr. Peter Swaan (University of Maryland Baltimore, Baltimore, MD). Cell culture supplies [Dulbecco's modified Eagle's medium, phosphate-buffered saline with Ca^{2+} and Mg^{2+} (PBS), 100× L-glutamine, nonessential amino acids, fetal bovine serum, 0.25% trypsin-1 mM EDTA, and penicillin G-streptomycin sulfate antibiotic mixture] were purchased from Invitrogen (Carlsbad, CA). Transwell clusters were purchased from Corning Life Sciences (Acton, MA). Microsomes from baculovirus-infected insect cells expressing human CYP1A2, 2A6, 2B6, 2C8, 2C9*1(Arg144), 2C19, 2D6*1, 2E1, and 3A4 and rat CYP1A2, 2B1, 2C11, 2D1, 2E1, and 3A1 (Supersomes) as well as insect cell control and rat P450 reductase insect cell control Supersomes were purchased from BD Gentest (Woburn, MA). The P450s were coexpressed with their corresponding human or rat cytochrome P450 reductase, in addition, human CYP2A6, 2B6, 2C8, 2C9*1, 2C19, 2E1, and 3A4 and rat CYP2B1, 2C11, 2D1, 2E1, and 3A1 were coexpressed with human cytochrome b5. Pooled human liver microsomes, pooled male Sprague-Dawley rat liver microsomes, and NADPH-regenerating systems were also purchased from BD Gentest. Potassium monobasic phosphate and potassium dibasic phosphates were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Sodium phosphate dibasic, verapamil HCl, cocaine HCl, xylazine, dopamine HCl, disodium ethylenediaminetetra-acetic acid (EDTA), octyl sulfate sodium salt, (2-Hydroxypropyl)- β -cyclodextrin, and sodium phosphate dibasic were purchased from Sigma-Aldrich (St. Louis, MO). Mono chloroacetic acid was purchased from J.T. Baker (Phillipsburg, NJ). Sodium hydroxide was purchased from American Bioanalytical (Natick, MA). Artificial cerebrospinal fluid (aCSF) containing 150 mM Na, 3.0 mM K, 1.4 mM Ca, 0.8 mM Mg, 1.0 mM P, and 155 mM Cl were purchased from Harvard Bioscience (Holliston, MA). Ketamine HCl injection was purchased from Bedford Laboratories (Bedford, OH). Methohexital (Brevital sodium) was purchased from Henry Schein® (Port Washington, NY). All chemicals and solvents were high-performance liquid chromatography (HPLC) grade or American Chemical Society analytical grade. The synthesis of GA2-50, JHW 025, and JHW 007 was conducted in the Medicinal Chemistry Section of the National Institute on Drug Abuse Intramural Research Program as previously described.^{28,30}

Cell Culture

Cells were grown at 37°C, 95% relative humidity, and 5% CO₂ atmosphere on 12-well Costar inserts (Transwell; 0.4- μm pore polycarbonate filter, 1 cm² in diameter). Caco-2 cells (passages 41 and 42) were seeded at a density of 80000 cells/cm². The cells were grown for 26–27 days in 1× Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum, 2% glutamine, 1% nonessential amino acids, 1% penicillin-streptomycin, with the medium changed every other day. MDCK-MDR1 cells were seeded at a density of 425000 cells/cm² and grown for 3–4 days in a medium similar to that used for Caco-2 cells with a daily medium change.

Characterization of Caco-2 and MDCK-MDR1 Cell Monolayers

The transport and inhibition studies were conducted in parallel with previously reported studies for the chloro-BZT analogs and the characteristics of the monolayers used in these experiments were previously reported.²⁶ Across MDCK-MDR1 monolayers, the apparent permeability

coefficient (P_{app}) for mannitol (a marker for paracellular transport) ranged from 3.89 to 4.46×10^{-6} cm/s, and the transepithelial electrical resistance values (TEER; another measure of monolayer integrity) were $>600 \Omega \text{ cm}^2$. The permeability of propranolol (a marker for transcellular transport) was 24.33×10^{-6} cm/s, and the efflux ratio of the P-gp substrate paclitaxel was 101, indicating high level of P-gp expression. Across Caco-2 monolayers, mannitol P_{app} ranged from 0.22 to 0.28×10^{-6} cm/s, and the TEER values ranged from 450 to $500 \Omega \text{ cm}^2$; indicating high monolayer integrity. Propranolol permeability was 13.34×10^{-6} cm/s, indicating higher transcellular resistance compared with MDCK-MDR1 cells. Paclitaxel efflux ratio ranged from 40 to 43 across Caco-2 cells.

GA2-50 Bidirectional Transport and Inhibition Studies

These studies were conducted to characterize the *in vitro* permeability and the P-gp interaction of GA2-50. JHW 025, a di-fluoro BZT analog that possesses a quaternary amine group and consequently was expected to have low *in vitro* permeability was used as another control for this study. The transport experiments were conducted following a previously described protocol²⁶ in both the apical-to-basolateral (A-B) and the basolateral-to-apical (B-A) directions across Caco-2 and MDCK-MDR1 monolayers in the presence and absence of the P-gp inhibitor verapamil. At the time of experiment, the culture medium was removed from both the apical and basolateral sides of the monolayers and washed twice with PBS. The monolayers ($n = 3/\text{group}$) were incubated with either 200 μM verapamil in PBS or PBS for 30 min. Following the preincubation period, mixtures of GA2-50 or JHW 025 (0.1 mM) with either verapamil (200 μM) or with PBS were added to the donor compartments. The receiver compartments solution consisted of either 200 μM verapamil in PBS (transport in presence of verapamil) or PBS (transport in absence of verapamil). For the A-B study, the inserts were moved to new Transwells[®] containing 1.5 mL of the corresponding receiver compartment solution at 30, 60, 90, and 120 min. For the B-A study, samples were drawn from the apical chamber at the same time points and replaced with equivalent volumes of fresh receiver compartment solution. Transport experiments were performed at 37°C with continuous agitation on a plate shaker (75 cycles/min), and samples were stored at -80°C until the time of analysis.

Characterization of Human and Rat Cytochrome P450 Enzymes Involved in the Metabolism of GA2-50

These studies were conducted to characterize the *in vitro* metabolism of GA2-50 and to determine whether the sterically bulky substituent at the tropane nitrogen will significantly affect its substrate status toward the different metabolizing enzymes. The metabolism studies for GA2-50 were conducted in parallel with previously reported metabolism studies for 4',4"-diCl BZT²⁶ using the same microsomal preparations. GA2-50 was incubated with human CYP1A2, 2A6, 2B6, 2C8, 2C9*1(Arg144), 2C19, 2D6*1, 2E1, and 3A4 as well as rat CYP1A2, 2B1, 2C11, 2D1, 2E1, and 3A1 Supersomes for 60 min. For each enzyme tested, the reaction mixture consisted of 50 pmol/mL P450, NADPH-regenerating system (1.3 mM NADP⁺, 3.3 mM glucose 6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, and 3.3 mM magnesium chloride), and 10 μM GA2-50 in 100 mM potassium phosphate buffer, pH 7.4 (final volume 500 μL). The reactions were initiated by adding ice-cold Supersomes to the prewarmed mixture of buffer, substrate, and cofactors. After a 60-min incubation period at 37°C, the reactions were stopped by the addition of 250 μL of acetonitrile and centrifuged at 10000g for 5 min. Two hundred microliters of the supernatants was injected onto the HPLC for determination of unchanged GA2-50 concentrations. Similar incubations with insect cell control and rat P450 reductase insect cell control Supersomes were performed to control for the native activities and non-P450-specific effects. Metabolism incubations were performed in triplicates.

Determination of the Time Course of GA2-50 Metabolism

The time course of GA2-50 metabolism (4 μ M final concentration; $n = 3$) by pooled human liver microsomes, pooled male rat liver microsomes, and the human and rat P450s that significantly reduced the concentration of GA2-50 in the initial screening experiments was determined following a previously described method.²⁶ The used GA2-50 concentration (4 μ M) was more than six-fold lower than the K_m value estimated using rat CYP 2C11 in a pilot study (data not shown). The microsomes were used at a concentration of 0.8 mg/mL. The P450 isoforms, cofactor, and buffer concentrations were similar as described in the screening study with a final reaction volume of 1500 μ L. The reactions were initiated by adding GA2-50 to the prewarmed reaction mixture. After 0, 5, 10, 20, 30, 40, and 60 min of incubation at 37°C, 200 μ L of the reaction mixture was sampled, immediately vortexed with 100 μ L of acetonitrile to terminate the reaction, and centrifuged at 10000g for 5 min. Aliquots of the supernatant were then collected for HPLC analysis.

Pharmacokinetic and Pharmacodynamic Studies

Animals—Adult male Sprague–Dawley rats (250–350 g) were used in the pharmacokinetic and microdialysis studies and 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 $72 \pm 2^\circ\text{F}$. 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—GA2-50 HBr and cocaine HCl were dissolved in 20% (2-hydroxypropyl)- β -cyclodextrin in 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.

GA2-50 Pharmacokinetic Study—This study was conducted to characterize the pharmacokinetics and brain uptake of GA2-50 in male Sprague–Dawley rats at two dose levels. The rats were administered GA2-50 at an i.v. dose of 5 or 10 mg/kg. At the tested doses, GA2-50 did not show any signs of toxicity. 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, 600, and 1440 min. Blood was collected by heart puncture using heparinized syringes and centrifuged for 10 min at 3000 rpm using Beckman TJ-6 Table-Top Centrifuge (Beckman, Inc., Fullerton, CA). The plasma was separated and stored at -80°C until the analysis. Brain tissues were immediately removed, blotted on a filter paper, weighted, and stored at -80°C until the time of analysis. The brain samples were used without perfusion since the contribution of the blood in the cerebral vasculature (approximately 2–5%) to the levels of the drug in the brain is believed to be negligible with compounds of high brain penetration.

Pharmacodynamic Studies—These studies were conducted to characterize the effect of GA2-50 on the extracellular levels of DA in the nucleus accumbens (NAc) of Sprague–Dawley rats as well as the possible effect of GA2-50 on the DA elevation induced by cocaine.

Surgical Procedures—The surgical and microdialysis procedures followed previously published protocols.^{27,31} In brief, rats were anaesthetized with i.p. administration of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg). Catheters made of Silastic Medical Grade tubing (Dow Corning, Midland, MI) were then implanted in the right external jugular vein of the anaesthetized rats. Subsequently, rats were placed in a stereotaxic apparatus where craniotomy was performed followed by implantation of plastic guide canulae (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, dorsal–ventral –6.0 mm from dura with a flat skull.^{27,32} The guide cannulae were cemented in place with three stainless-steel screws and dental acrylic. Following the surgery, the rats were placed in separate cages and allowed free access to food and water for a minimum of 5 days of postsurgical recovery.

In Vivo Microdialysis—On the night prior to the experiment, CMA/12 microdialysis probes (2 mm × 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 μ L/min pumped using CMA 400 syringe pump (CMA Microdialysis). The probes were perfused with aCSF while immersed initially in ethanol then aCSF (10 min each) in order to clean them from any manufacturing contaminants. After the *in vitro* probe perfusion, rats were lightly anaesthetized with the ultra short acting barbiturate, methohexital (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 plexiglass 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 afterwards throughout the experiment at a flow rate of 1 μ L/min. The overnight perfusion allows for establishment of stable basal neurotransmitter levels after the initial neurotransmitter elevation resulting from the unavoidable damage associated with insertion of the microdialysis probe (for detailed discussion, see³³). 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 either vehicle (1 mL/kg), GA2-50 (5 or 10 mg/kg) or cocaine +GA2-50 (5 mg/kg of each) via the jugular vein catheter. The dialysate samples were collected at 10, 20, 30, 40, 50, 60, 90, 120, 180, 240, and 360 min. Dialysate 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 sampling times mentioned above allowed for adequate characterization of the time course of the effect of GA2-50 on the NAc DA levels. 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 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 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.³² Rats with incorrect probe placement were excluded from the data analysis.

Bioanalytical Methods

Analysis of the Transport, Metabolism, and Pharmacokinetic Samples—A previously published valid and specific UV-HPLC method with few modifications was used for analysis of the transport, metabolism and pharmacokinetic samples of GA2-50 and the transport samples of JHW 025.³⁴ The chromatographic conditions consisted of a Symmetry C₁₈ column (150 mm × 4.6 mm; 5 μ m), 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, 80:20 (v/v) (B)], and a flow rate of 1 mL/min pumped using a gradient profile optimized for the analog and type of sample. The transport and metabolism samples were analyzed directly without further processing. For the pharmacokinetic study, 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. JHW 007 was used as internal standard for GA2-50 without any interference. The calibration curves were linear ($r^2 > 0.993$) in the range of 25–10000, 200–10000, 50–5000, and 100–20000 ng/mL for the transport, metabolism, plasma, and brain matrices, respectively, with intra and interday variability and error of $\leq 15\%$.

Analysis of the Dialysate Samples—The dialysate samples were analyzed for dopamine content by HPLC with electrochemical detection as previously described.^{27,31} The mobile phase consisted of 150 mM monochloroacetic acid, 1.5 mM sodium octane sulphonic acid, 145 mM sodium hydroxide, 215 μ M 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 μ L/min. The HPLC system consisted of an ISCO 260 D syringe pump (ISCO, Lincoln, NE), BAS Model LC-4C amperometric detector (Bioanalytical Systems, Inc., West Lafayette, IN), and BAS UniJet™ microbore analytical column (C18, 100 mm $\times$ 1 mm, 5 μ M, Bioanalytical Systems, Inc.). 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 μ L. The calibration curves for dopamine were linear in the range of 0.1–5 ng/mL ($r^2 \geq 0.996$) without any interfering peaks and with variability and error of $\leq 15\%$.

Data Analysis

Transport Data Analysis—The apparent permeability coefficients (in presence and absence of the P-gp inhibitor verapamil) were determined at sink conditions using the following equation:

$$P_{app} = \frac{dQ/dt}{AC_0} \quad (1)$$

where dQ/dt is equal to the linear appearance rate of mass in the receiver solution, A is the cross sectional area of the insert filters, and C_0 is the donor concentration at time zero. All values are represented as mean and standard deviation from three transwell inserts. Efflux ratios across the monolayers were calculated using the equation:

$$\text{Efflux Ratio} = \frac{P_{app}(B-A)}{P_{app}(A-B)} \quad (2)$$

where $P_{app}(B-A)$ is the permeability from the basolateral to the apical direction (secretory transport) and $P_{app}(A-B)$ the permeability from the apical to basolateral direction (absorptive transport). Enhancement ratio in the apical to basolateral transport induced by verapamil was calculated according to the equation:

$$\text{Enhancement Ratio} = \frac{P_{app}(A-B)_{ver}}{P_{app}(A-B)} \quad (3)$$

The statistical significance of effect of verapamil on the permeability of GA2-50 and JHW 025 was determined with two sample Student's t -test at $\alpha = 0.05$ using Microsoft® Excel software. An F -test was performed to check for the homogeneity of the treatment group variances and the appropriate t -test was selected accordingly. The delta method was used to calculate the standard error of the ratios and statistical significance was declared when the 95% confidence interval of two ratios did not overlap.

Metabolism Data Analysis

Identification of the P450s Involved in Metabolism—The human and rat P450 isoforms involved in the metabolism of GA2-50 were identified by analyzing the differences in mean substrate concentrations remaining after 60-min incubations by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons of P450 incubations versus control

incubations. The percentages of the mean control concentration remaining after 60-min incubation were also calculated according to the following equation:

$$\begin{aligned} & \% \text{ of substrate remaining after 60 min} \\ &= \frac{C_{\text{CYP}, 60 \text{ min}}}{C_{\text{average ctrl, 60 min}}} \times 100 \end{aligned} \quad (4)$$

where $C_{\text{CYP}, 60 \text{ min}}$ is the substrate concentration from the r th replicate after 60 min of incubation with a particular P450 Supersome, and $C_{\text{average ctrl, 60 min}}$ is the average ($n=3$) substrate concentration after 60 min incubation with insect cell control Supersomes (for human P450s) or rat P450 reductase insect cell control SupersomesTM (for rat P450s). The percentages are represented as mean and standard deviation from triplicate reactions.

Intrinsic Clearance Calculation—The intrinsic clearance values were calculated based on the substrate disappearance rate as previously described³⁵ and previously applied for the study of the metabolism of the BZT analog 4',4''-diCl BZT.²⁶ Assuming first order disappearance of substrate, the disappearance rate constant (K_e) was calculated from the slope of $\log C_t$ versus time profile based on the equation:

$$\log C_t = \log C_0 - \frac{K_e t}{2.303} \quad (5)$$

where C_t is the concentration of the substrate at the different time points, C_0 is the substrate concentration at time zero. The initial metabolic rate (V_0) (pmol/min/pmol P450 or mg microsomal protein) was calculated from the equation:

$$V_0 = \frac{K_e C_0}{P_{\text{MS}}} \quad (6)$$

where P_{MS} is CYP concentration (pmol/mL) or microsomal protein concentration (mg/mL). V_0 can also be described using Michaelis–Menton equation as follows:

$$V_0 = \frac{V_{\text{max}} C_0}{K_m + C_0} \quad (7)$$

Assuming that $C_0 \ll K_m$, Eq. (7) can be written as

$$V_0 = \frac{V_{\text{max}} C_0}{K_m} \quad (8)$$

Accordingly, the intrinsic clearance was calculated based on the formula

$$\text{CL}_{\text{int}} = \frac{V_{\text{max}}}{K_m} = \frac{V_0}{C_0} \quad (9)$$

The intrinsic clearance values were calculated separately from each of the replicates and compared statistically using one-way ANOVA followed by Duncan multiple range test. CL_{int} values are presented as mean $\pm$ SD from the three replicates performed for each reaction.

Pharmacokinetic Data Analysis—The destructive sampling data obtained from the pharmacokinetic study of GA2-50 were initially analyzed by the naïve 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, Cary, NC). Various compartmental models were evaluated to determine the most appropriate model. Subsequently, nonlinear mixed effect modeling was then conducted using NONMEM version 5, level 1.1 (GloboMax LLC, Hanover, MD). This approach has been previously shown to result in less biased estimates of the structural model parameters than the naïve pooling of the data in destructive sampling designs.³⁶ The data from the two dose levels (data from 54 animals) were compiled and analyzed. The final parameter estimates from the naïve averaging analysis were used as initial estimates for the population analysis. Based on the results from the naïve averaging analysis, the population analysis was conducted using a two compartment structural model. Interanimal variability in the pharmacokinetic parameters was estimated using an exponential error model as follows:

$$P_i = TVP \exp(\eta_i) \quad (10)$$

where η_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 is assumed to be normally distributed with a mean of 0 and a variance of ω^2 . The residual error (which includes model misspecification, intra-animal variability as well as errors in dosing, sampling times, and sample analysis) was described using a proportional error model as follows:

$$Y_{obs} = Y_{pred}(1 + \varepsilon) \quad (11)$$

where Y_{obs} is the observed plasma concentration, Y_{pred} is the model predicted plasma concentration and ε is a normally distributed parameter with a mean of 0 and variance of σ^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. The brain to plasma partition coefficient (R_i) was calculated as a measure of brain uptake according to the formula

$$R_i = \frac{AUC_{0-1440 \text{ min (brain)}}}{AUC_{0-1440 \text{ min (plasma)}}} \quad (12)$$

where $AUC_{0-1440 \text{ min (brain)}}$ and $AUC_{0-1440 \text{ min (plasma)}}$ are the area under the naïve-averaged observed brain and plasma concentrations versus time curves, respectively, from time 0 to 1440 min postdose. The $AUC_{0-1440 \text{ min}}$ was used instead of $AUC_{0-\infty}$ because the elimination phase from the brain was not properly characterized for the 5 mg/kg dose. The $AUC_{0-1440 \text{ min}}$ were calculated using WinNonlin noncompartmental analysis by applying the linear trapezoidal rule for the ascending portions of the concentration versus time profiles and the log linear trapezoidal rule for the descending portions.

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 ANOVA with repeated measures over time followed by Dunnett's *post hoc* analysis with time zero as control ($\alpha = 0.05$). In addition, the data for GA2-50 + cocaine interaction study were compared to previously published data for cocaine administration alone²⁷ using a two factor (treatment and time) ANOVA with repeated measures over time. All statistical analyses were conducted using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).

RESULTS

GA2-50 *In Vitro* Permeability and P-gp Mediated Efflux

The bidirectional permeability values of GA2-50 in comparison with the quaternary ammonium BZT analog, JHW 025 (control) across MDCK-MDR1 and Caco-2 monolayers are reported in Table 2. GA2-50 has shown relatively high permeability values that were around 6.8-fold and >4-fold those of JHW 025 across MDCK-MDR1 and Caco-2 monolayers, respectively. The permeability of GA2-50 across MDCK-MDR1 monolayers (Tab. 2) was even higher than that of propranolol (propranolol $P_{app} = 24.33 \times 10^{-6}$ cm/s). Conversely, and as expected based on its lipophobicity, the permeability of JHW 025 across both cell lines (Tab. 2) was low and almost comparable to the permeability of the paracellular marker mannitol (mannitol MDCK-MDR1 and Caco-2 $P_{app} = 3.89\text{--}4.46$ and $0.22\text{--}0.28 \times 10^{-6}$ cm/s, respectively). The exact apical to basolateral permeability of JHW 025 across Caco-2 monolayers was not determined because the concentrations in the receiver compartments at all the time points were below the limit of quantification.

GA2-50 has shown polarized transport across both cell lines with higher B-A permeability than A-B permeability (Tab. 2). The efflux ratio of GA2-50 across Caco-2 monolayers was higher than that observed across MDCK-MDR1 monolayers which contributed to the lower permeability of GA2-50 across Caco-2 cells when compared with MDCK-MDR1 cells. JHW 025 showed polarized transport as well, with lower efflux ratio when compared with GA2-50 (Tab. 2).

The P-gp inhibitor verapamil was used to verify the involvement of P-glycoprotein in mediating the observed efflux of GA2-50 and JHW 025. Verapamil significantly ($p < 0.05$) increased the A-B permeability of GA2-50 and JHW 025 (Fig. 2A). The enhancement ratio of GA2-50 A-B transport was 2.3 and 3.4 across MDCK-MDR1 and Caco-2 monolayers, respectively. The enhancement ratio of JHW 025 A-B transport was 1.2 across MDCK-MDR1 monolayers and could not be determined across Caco-2 monolayers; again because of the low permeability. Verapamil significantly reduced the efflux ratio of GA2-50 across both cell lines ($p < 0.05$), whereas the effect of verapamil on the efflux ratio of JHW 025 did not reach statistical significance (Fig. 2B).

Characterization of Human and Rat Cytochrome P-450 Enzymes Involved in the Metabolism of GA2-50

The objective of these studies was to screen for the enzymes involved in the metabolism of GA2-50 to determine whether or not the bulky *N*-substitution will significantly affect the metabolism of this class of compounds. Among the cytochrome P450 isoforms tested, human CYP2D6, CYP2C19, CYP2E1, rat CYP1A2, CYP2C11, CYP2D1, and CYP3A1 resulted in

statistically significant disappearance of GA2-50 after 60 min incubation when compared to the control ($p < 0.05$) (Fig. 3A and B).

Intrinsic Clearance of GA2-50 in Human and Rat Recombinant P450s and Pooled Human and Male Rat Liver Microsomes

Under the experimental conditions, GA2-50 metabolism followed first-order reaction with the concentration of the substrate declining monoexponentially with time. The intrinsic clearance values of GA2-50 are reported in Table 3. The preference for metabolizing GA2-50 was as follows: CYP2D6 > CYP2C19 > CYP 2E1 for human P450s and CYP2C11 > CYP2D1 > CYP3A1 > CYP1A2 for rat P450s. The intrinsic clearance of GA2-50 was 1.9-fold higher in pooled male rat liver microsomes than in human liver microsomes (Tab. 3).

Pharmacokinetics of GA2-50

Figure 4A represents the observed and NONMEM predicted plasma concentrations of GA2-50 upon i.v. administration to male Sprague–Dawley rats at two dose levels (5 and 10 mg/kg). Figure 4B represents the corresponding brain concentrations versus time profiles. The brain concentrations were higher than the plasma concentrations at each time point. The pharmacokinetics of GA2-50 followed bi-exponential pattern. The population model for the pharmacokinetics of GA2-50 consisted of a two-compartmental structural model parameterized in terms of clearance (CL), volume of the central compartment (V_c), volume of the peripheral compartment (V_p), and intercompartmental clearance (Q) (ADVAN3 TRANS4 NONMEM subroutines) with the interanimal variability estimated on all the parameters. Figure 5 represents the relevant diagnostic plots for the population analysis. In general, there was no systematic bias in the fit to the data except for the highest concentration of the 10 mg/kg dose where the model under-predicted these concentrations (based on the population mean). 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 4. The pharmacokinetic parameters were all estimated with good precision and the interanimal variability on these parameters was estimated with adequate precision. Due to the destructive sampling design, precise estimation of the residual random error was not possible. Consequently, its value was fixed to 10% based on a sensitivity analysis and the value selected was the one resulting in the lowest objective function. The 10% residual random error was comparable to the estimated or fixed values for previously studied BZT analogs using identical experimental technique.^{26,27} The elimination half life ($t_{1/2}$) and the steady state volume of distribution (V_{ss}) were calculated as secondary parameters (along with their associated variability) from the NONMEM empirical Bayes estimates of the individual animal-specific pharmacokinetic parameters and are reported in Table 4. GA2-50 has a long elimination half-life and large volume of distribution. Based on the noncompartmental analysis, $AUC_{0-1440 \text{ min}}$ (plasma) was equal to 141.8 and 258.0 $\mu\text{g min/mL}$ for the 5 and 10 mg/kg doses, respectively, indicating almost linear dose-exposure relationship in the range studied. The brain-to-plasma partition coefficient (R_i) was 10.1 and 9.6 for the 5 and 10 mg/kg doses, respectively, indicating high brain distribution.

Effect of GA2-50 on the NAc DA Levels

Figure 6 shows the effects of i.v. administration of GA2-50 (5 or 10 mg/kg) on the levels of DA in the dialysate samples from the NAc of Sprague–Dawley rats. Administration of GA2-50 resulted in a significant elevation of the DA levels at each dose (ANOVA, $p < 0.0001$). The maximal increase of DA was 159% and 274% of the basal values at the 5 and 10 mg/kg doses, respectively, and was observed 20 min postdose for the 5 mg/kg dose and 30 min postdose for the 10 mg/kg dose. After reaching the peak, the DA levels declined slowly and remained significantly elevated above the basal values for up to 1 h for the 5 mg/kg dose and during the

6 h of the experiment for the 10 mg/kg dose (*post hoc*, $p < 0.05$). When the effects of the two doses of GA2-50 were compared using a two-way ANOVA, there was a significant effect of dose ($F_{1,140} = 6.45$, $p = 0.029$), time ($F_{14,140} = 14.05$, $p < 0.0001$), and dose $\times$ time interaction ($F_{14,140} = 4.68$, $p < 0.0001$). Administration of the vehicle had no effect on the DA levels and the levels remained stable throughout the time course of the experiment (not shown).

Effect of GA2-50 on Cocaine-Induced DA Elevation

This study was conducted to determine whether GA2-50 will competitively block the DA elevation induced by cocaine upon concurrent administration to Sprague–Dawley rats. Figure 7 shows the effects of i.v. administration of GA2-50 + cocaine (5 mg/kg of each) on the levels of DA in the dialysate samples from the NAc of Sprague–Dawley rats in comparison with the effects of cocaine administration alone (5 mg/kg) and GA2-50 administration alone (5 mg/kg). When administered at the same time, GA2-50 did not have significant effect on the DA elevation induced by cocaine which indicates that GA2-50, at the tested dose, does not significantly block the DA elevation induced by cocaine.

DISCUSSION

GA2-50 demonstrated high binding selectivity to DAT over the serotonin (SERT) and NET as well as the muscarinic M₁ and histamine H₁ receptors (Tab. 1). Moreover, preliminary behavioral evaluation of GA2-50 has indicated that this compound, despite its high *in vitro* potency as a DA uptake inhibitor, demonstrated significantly reduced cocaine-like activity in animal models of drug abuse.²⁹ These characteristics warranted further investigation of the potential of GA2-50 as a cocaine substitute therapeutic. Consequently, the present study was conducted to characterize the pharmacokinetic and pharmacodynamic properties of GA2-50 as a part of its preclinical evaluation. In addition, such characterization is required for understanding some of the mechanisms underlying the behavioral differences between GA2-50 and cocaine.

A prerequisite for the efficacy of a substitute therapeutic agent is to be able to penetrate the blood brain barrier to achieve brain concentrations high enough to inhibit the reuptake of DA.³⁷ In the present study, the A-B permeability values of GA2-50 were several fold higher than those of the charged BZT analog JHW 025 (Tab. 2). In addition, the permeability of GA2-50 across MDCK-MDR1 monolayers was even higher than the permeability of the transcellular marker propranolol. These results indicated GA2-50 partitioned across the plasma membrane at a high rate resulting in high transcellular permeability.

The transport of GA2-50 was polarized across the P-gp overexpressing cell line MDCK-MDR1 as well as across Caco-2 cells; indicating that GA2-50 was a substrate for the efflux transporter P-gp. The efflux ratio of GA2-50 was higher across Caco-2 cells than across the MDCK-MDR1 cells, which resulted, along with the higher resistance of Caco-2 monolayers, in lower Caco-2 permeability. The P-gp inhibitor verapamil enhanced the absorptive transport and reduced the efflux ratios of GA2-50 (Fig. 2) which further emphasized that GA2-50 was a good P-gp substrate. These results are consistent with our previous studies which indicated that several BZT analogs were substrates for the efflux transporter P-gp with variable *in vitro* efflux ratios.^{24,26}

In vivo, GA2-50 showed high brain uptake demonstrated by a brain-to-plasma partition coefficient of ~ 10 in Sprague–Dawley rats. The high brain uptake, despite the *in vitro* P-gp efflux, was previously observed for the BZT analogs JHW 007²⁴ and 4',4"-diCl BZT,²⁶ and is consistent with the conclusion that P-gp does not significantly limit the brain exposure of the analogs of BZT.²⁶

The present metabolism study indicated that GA2-50 has high metabolic stability *in vitro* as evident from its low intrinsic clearance values (Tab. 3). GA2-50 is a substrate of human CYP2D6, CYP2C19 as well as rat CYP2C11 and CYP3A1. These results are consistent with those from a previously reported metabolism study with the BZT analog 4',4''-diCl BZT.²⁶ The highest extent of GA2-50 metabolism was observed, similar to 4',4''-diCl BZT, with rat CYP2C11. However, the intrinsic clearance values of GA2-50 in rat CYP2C11 as well as in pooled human and male rat liver microsomes were respectively 6.3-, 4.0-, and 3.6-fold lower than the previously reported values for 4',4''-diCl BZT. This may indicate that the sterically bulky *N*-substitution of GA2-50 (Fig. 1) enhances its metabolic stability relative to the *N*-methyl group of 4',4''-diCl BZT toward some metabolizing enzymes such as CYP2C11; most probably by decreasing the rates of the *N*-dealkylation and *N*-oxidation that were previously identified among the major metabolic routes for the parent compound BZT.³⁸ In addition to the previous enzymes, GA2-50 was a substrate for human CYP 2E1, rat CYP1A2 and CYP 2D1, for which 4',4''-diCl BZT was not a substrate. Therefore it is plausible to assume that these enzymes may be involved in the metabolism of the *N*-((*R*)-2''-amino-3''-methyl-*n*-butyl) group of GA2-50 or that the fluoro substituents on the phenyl rings of GA2-50 may be more favorable for interaction with the catalytic sites of these enzymes relative to the chloro substituents of 4',4''-diCl BZT. Further studies to evaluate the possible contribution of Phase II enzymes in the metabolism of GA2-50 and to identify the metabolites of this compound are still needed.

The pharmacokinetic study indicated that GA2-50 was characterized by the most extensive distribution ($V_{ss}=37$ L/kg), which was paralleled by the highest brain uptake ($R_1 \sim 10$), amongst all the BZT analogs tested so far.^{24,26,39} In addition, GA2-50 has shown the longest elimination half-life ($t_{1/2} \sim 19.2$ h) when compared to both the first generation²⁴ and the more selective second generation of the *N*-substituted analogs of BZT.³⁹ The long $t_{1/2}$ is a favorable attribute for a substitute medication as it will allow for reasonable dosing schedules.¹⁴ Moreover, the long $t_{1/2}$ of GA2-50 may contribute to reducing its risk of abuse in the target population. Previous studies with the chloro-BZT analogs suggested that the short $t_{1/2}$ of the BZT analogs 3'-Cl BZT ($t_{1/2} \sim 1.9$ h) and 4'-Cl BZT ($t_{1/2} \sim 3.5$ h) might be contributing to their observed higher preclinical abuse potential relative to the slowly eliminated analogs such as 4',4''-diCl BZT ($t_{1/2} \sim 21$ h).^{26,27} This was supported by several studies which indicated that the rate of elimination of a drug from the body affects its reinforcing properties and abuse potential,^{17,18} and that the rapid elimination of cocaine, due to its low metabolic stability ($t_{1/2} \sim 30$ min), is responsible for its high rates of self-administration.¹⁵ It is noteworthy that even though GA2-50 demonstrated higher *in vitro* metabolic stability than 4',4''-diCl BZT in pooled liver microsomes, the $t_{1/2}$ for GA2-50 was slightly shorter than 4',4''-diCl BZT *in vivo*. This may indicate that the elimination of the BZT analogs is probably governed by several factors besides phase I metabolism. These factors may include possible interaction with non-P450 metabolic enzymes, the extent of plasma protein and tissue binding, tissue distribution, fraction metabolized versus excreted unchanged, renal filtration and reabsorption as well as affinity to the different secretory transporters expressed in kidney and liver.

It was previously hypothesized that the substitute therapeutic agent should act as a partial rather than a full agonist *in vivo*.³⁷ Therefore, a DA uptake blocker should inhibit the reuptake of DA less effectively than cocaine, resulting in only modest DA elevations. In theory then, the substitute medication would have reduced abuse potential on its own. Yet, it might be able to correct the hypodopaminergic state experienced during cocaine withdrawal,⁴⁰ and as a result reduce cocaine craving and improve patient compliance. In the present study, administration of GA2-50 resulted in a dose-dependent elevation of the extracellular DA levels in the NAc, the brain reward center mediating the reinforcing effects for drugs of abuse. However, the maximal DA elevation induced by GA2-50 was only 1.6- and 2.7-fold the basal DA values at the 5 and 10 mg/kg i.v. doses, respectively. In our hands, and using identical stereotaxic

coordinates for microdialysis, cocaine resulted in 10-fold DA elevation at the 5 mg/kg i.v. dose.²⁷ Other studies by Baumann et al.⁴¹ using a similar microdialysis technique showed a six-fold DA increase for a 3 mg/kg i.v. dose of cocaine. The significantly reduced DA elevation induced by GA2-50 relative to cocaine (Fig. 7) is concordant with its reduced cocaine-like behavioral activity.²⁹

In the current study, the free unbound concentrations of GA2-50 at the striatum were not measured. However, the pharmacokinetic study indicated that the maximal total brain concentration of GA2-50 at the 10 mg/kg dose was approximately 5 µg/g, which corresponds to concentration well above the measured *in vitro* IC₅₀ and K_i values for GA2-50 at DAT (approximately 580- and 210-fold higher *in vivo* concentration than the *in vitro* IC₅₀ and K_i values, respectively, see Tab. 1).

The present study also indicated that GA2-50 resulted in slower DA elevation relative to cocaine (Fig. 7). Significant evidence indicated that the DA uptake inhibitors that result in slower rates of DA elevation may be associated with less reinforcing properties and lower abuse potential relative to those that induce faster rates of DA elevation.^{16,18,42} After the initial DA elevation, GA2-50 resulted in significantly slower and less abrupt backward DA decline (Fig. 6) than that observed with cocaine (Fig. 7). Moreover, the backward DA decline observed with GA2-50 was slower than that observed with the previously studied second generation *N*-substituted di-fluoro BZT analogs.³⁹ This pattern agrees with the slower elimination of GA2-50 from the body (Fig. 4). Studies with the DA uptake inhibitor methylphenidate indicated that the steady state and stable DA increases induced by methylphenidate upon oral dosing seemed to be associated with its therapeutic effects, while the fast DA fluctuations induced by methylphenidate upon i.v. dosing seemed to be associated with its abuse.⁴² As such, the relatively smooth dopaminergic pattern observed with GA2-50 in the current study, even with i.v. dosing, underscores the potential therapeutic utility of this compound.

It was previously suggested that the anticholinergic activity at the muscarinic M₁ receptors might be responsible for reducing the cocaine-like activity of the BZT analogs,⁴³ probably through reducing the dopaminergic transmission.⁴⁴ However, recent microdialysis studies with selective M₁ antagonists did not provide any supporting evidence for this hypothesis.⁴⁵ In the present study, the low extent of DA elevation by GA2-50, a DAT selective BZT analog with very low muscarinic affinity (Tab. 1), may further indicate that the reduced agonist activity of the BZT analogs is independent of their antimuscarinic activity.

Since GA2-50 showed high brain uptake and high affinity to DAT while at the same time seemed to have low intrinsic activity *in vivo*. One might assume that GA2-50 might be able to significantly reduce the binding of cocaine to the available DATs and consequently block cocaine's effects on the extracellular DA levels. Accordingly, the microdialysis interaction study was conducted to investigate this possibility. The results from this study indicated that there was no statistically significant difference ($p > 0.05$) between the DA elevation induced by cocaine alone (5 mg/kg) and cocaine administered simultaneously with GA2-50 (5 mg/kg of each); even though the combination seemed to keep DA elevated for a longer period of time (Fig. 7). Due to the small magnitude of the effect of GA2-50 relative to the observed variability in cocaine's response, the present study cannot detect an additive effect or a low grade of antagonism. However, the results from this study clearly indicate that GA2-50 does not significantly blunt the effect of cocaine on the NAc DA levels upon simultaneous administration. Recent studies indicated that the BZT analogs bind to DAT at slower rates than cocaine.^{23,46} Therefore, it is possible that the lack of antagonism in the current study may be a result of the simultaneous administration, and that GA2-50 may antagonize the effects of cocaine if the administration of cocaine is delayed relative to the administration of GA2-50 to

account for the difference in the rate of DAT association. Further studies to investigate this possibility are warranted.

CONCLUSIONS

In the present study, GA2-50 showed high brain uptake, high metabolic stability and slow elimination from the body. GA2-50 resulted in relatively slow and long lasting DA elevation with no abrupt DA fluctuations upon i.v. dosing. Taken together with its high selectivity and low preclinical abuse potential, the results from the present study indicate that GA2-50 possesses several favorable attributes sought after for a substitute therapeutic medication for treatment of cocaine abuse.

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Abbreviations

DA	dopamine
DAT	dopamine transporter
BZT	benztropine
GA2-50	<i>N</i> -((<i>R</i>)-2"-amino-3"-methyl- <i>n</i> -butyl)-3 α -[(bis-4'-fluorophenyl)methoxy]tropane
JHW 025	<i>N</i> -(dimethyl)-3 α -[(bis-4'-fluorophenyl)methoxy]tropane
JHW 007	<i>N</i> -(<i>n</i> -butyl)-3 α -[(bis-4'-fluorophenyl)methoxy]tropane
NAc	nucleus accumbens
aCSF	artificial cerebrospinal fluid
AUC	area under the curve
R_i	brain-to-plasma partition coefficient
CL	clearance
V_c	volume of the central compartment

V_p	volume of the peripheral compartment
Q	inter-compartmental clearance
IAV	interanimal variability
V_{ss}	steady-state volume of distribution

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Figure 1. Chemical structure of GA2-50 and benztropine. The substitutions (*R* and *R'*) are presented in Table 1.

Figure 2.

Effect of verapamil (200 μ M) on permeability and P-gp mediated efflux of GA2-50 in comparison with the lipophobic BZT analog, JHW 025 across MDCK-MDR1 and Caco-2 monolayers. (A) Apical to basolateral permeability in absence and in presence of verapamil across MDCK-MDR1 and Caco-2 monolayers respectively (data represented as mean $\pm$ SD). (B) Efflux ratios across MDCK-MDR1 and Caco-2 monolayers in absence and in presence of verapamil (data represented as the ratio of the mean permeability values $\pm$ SEM calculated using delta method). * $p < 0.05$. Black bar, in absence of verapamil. Gray bar, in presence of verapamil. ND, not determined.

Figure 3.

Metabolism of GA2-50 by the human recombinant P450s (A) and the rat recombinant P450s (B). GA2-50 (10 μ M) was incubated with the supersomes (50 pmol/mL) and cofactors for 60 min followed by termination of reactions and determination of unchanged substrate. Data are presented as mean percentage of the average control $\pm$ SD from triplicate reactions. * $p < 0.05$ based on Dunnett's test.

Figure 4.

Plasma and brain pharmacokinetic profiles of GA2-50 at two dose levels (5 and 10 mg/kg i.v.) in male Sprague-Dawley Rats ($n = 3/\text{time point/dose}$). (A) Observed (mean $\pm$ SD) and population predicted plasma concentration versus time profiles. (B) Observed brain concentrations (mean $\pm$ SD) versus time profiles.

Figure 5.

Diagnostic plots for the population analysis of the plasma concentrations of GA2-50 ($n = 54$ rats) conducted using NONMEM. The data from the two dose levels tested (5 and 10 mg/kg i.v.) were combined prior to the analysis. (A) Population predicted versus observed plasma concentrations. (B) Individual animal predicted versus observed plasma concentrations. (C) Weighted residuals versus time plot. The dashed lines in the weighted residual plot represent ± 3 SD.

Figure 6.

Time course of the effect of i.v. administration of GA2-50 (5 or 10 mg/kg) on extracellular levels of DA in the dialysate samples from the NAc of Sprague-Dawley rats. The arrow indicates the time of drug injection. Data are presented as percentages of the average of four baseline samples collected before drug treatment, and values are means with the vertical bars representing SEM ($n = 6$ rats/treatment group). When no error bar is visible, the deviation was within the size of the symbols. * $p < 0.05$ compared to the pretreatment basal DA values.

Figure 7.

Effect of i.v. administration of cocaine +GA2-50 (5 mg/kg of each) on extracellular levels of DA in the dialysate samples from the NAc of Sprague–Dawley rats in comparison with effect of cocaine alone (5 mg/kg) and GA2-50 alone (5 mg/kg). The arrow indicates the time of injection. Data are presented as percentages of the average of four baseline samples collected before drug treatment, and values are means with the vertical bars representing SEM ($n = 6$ rats/treatment group). When no error bar is visible, the deviation was within the size of the symbols. The data for cocaine alone are previously reported.²⁷ There was no statistically significant difference ($p > 0.05$) between dopamine elevation when rats were treated with cocaine alone and when rats were treated with cocaine + GA2-50.

Table 1
In Vitro Binding and Functional Data for GA 2–50 in Comparison with Benztropine (BZT)*

Compound	N-Subst./R'	DAT K_i (nM)	SERT K_i (nM)	NET K_i (nM)	M ₁ K_i (nM)	H ₁ K_i (nM)	DA Uptake IC ₅₀
GA2-50	R-2''-amino-3''-methyl-n-butyl/F	56.4 ± 9.6	3870 ± 135	2130 ± 160	4020 ± 592	218 ± 15.5	20.7 ± 2.85
BZT	CH3/H	118 ± 10.6	>10000	1390 ± 134	2.1 ± 0.29	15.7 ± 2.13	403 ± 115

* Data reported are from Newman and Katz¹⁹ and presented as mean ± SEM.

Table 2
Bidirectional Permeability and Efflux Ratios of GA2-50 Across MDCK-MDR1 and Caco-2 Monolayers in Comparison With the Lipophobic BZT Analog, JHW 025, at a Conc of 0.1 mM

Compound	MDCK-MDR1			Caco-2		
	P_{app} ($\times 10^{-6}$ cm/s)		Efflux Ratio	P_{app} ($\times 10^{-6}$ cm/s)		Efflux Ratio
	A-B	B-A		A-B	B-A	
GA2-50	34.01 $\pm$ 2.78	116.97 $\pm$ 5.23	3.4	1.37 $\pm$ 0.08	11.36 $\pm$ 1.41	8.3
JHW 025	5.00 $\pm$ 0.31	7.39 $\pm$ 1.17	1.5	<0.34 ^a	1.8 $\pm$ 0.25	ND

P_{app} values are expressed as mean $\pm$ SD ($n = 3$).

ND, not determined.

^aCalculation based on the assay LLOQ.

Table 3

Intrinsic Clearance Values for GA2-50 (4 μ M) in Human and Male Sprague–Dawley Rat Pooled Liver Microsomes (0.8 mg/mL) and in Human and Rat Recombinant P450s (50 pmol/mL)

Microsomal Preparation	Intrinsic Clearance ^a
Pooled HLM	3.17 $\pm$ 1.18
Pooled male RLM	5.96 $\pm$ 0.56
Human CYP 2C19	0.10 $\pm$ 0.01 (c)
Human CYP 2D6	0.15 $\pm$ 0.02 (b)
Human CYP 2E1	0.09 $\pm$ 0.02 (c,d)
Rat CYP 1A2	0.06 $\pm$ 0.01 (d)
Rat CYP 2C11	0.23 $\pm$ 0.01 (a)
Rat CYP 2D1	0.11 $\pm$ 0.02 (c)
Rat CYP 3A1	0.09 $\pm$ 0.02 (c,d)

^a Intrinsic clearance is reported as microliters per minute per milligram of protein for human liver microsomes (HLM) and rat liver microsomes (RLM) and as microliters per minute per picomole of P450 for the recombinant P450s ($n = 3$ /reaction). The intrinsic clearance values in the recombinant P450s that share the same lower case letters are not significantly different at $\alpha = 0.05$.

Table 4

Population Pharmacokinetic Parameters for GA2-50 ($n = 54$) Along With Their Associated Interanimal Variability Upon i.v. Administration to Sprague–Dawley Rats

Parameter	Estimate	% IAV ^a
CL (L/h/kg)	1.34 (9.3)	11.3 (69.3)
V_c (L/kg)	8.09 (13.6)	57.8 (38.3)
V_p (L/kg)	27.9 (10.5)	38.3 (79.6)
Q (L/h/kg)	7.8 (19.9)	34.1 (43.4)
% Residual error	10 (Fixed)	NE
$t_{1/2}$ (h) ^b	19.2	15.1
V_{ss} (L/h/kg) ^b	37.3	16.3

Values in parentheses are % CV.

CL, clearance; V_c , volume of the central compartment; V_p , volume of the peripheral compartment; Q , intercompartmental clearance; V_{ss} , steady state volume of distribution; NE, not estimated.

^aThe IAV estimates should be interpreted with caution given the difficulty in fully resolving the IAV from the residual random error with the destructive sampling design.

^b $t_{1/2}$ and V_{ss} were calculated as secondary parameters from the NONMEM empirical Bayes estimates of the individual animal-specific pharmacokinetic parameters.