A recombinant humanized anti-cocaine monoclonal antibody inhibits the distribution of cocaine to the brain in rats.

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Running title: Efficacy of a humanized cocaine immunotherapeutic in rats

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Abbreviations: mAb, monoclonal antibody; BE, benzoylecgonine; EME, ecgonine methyl ester; ELISA, enzyme-linked immunosorbent assay; H, immunoglobulin heavy chain; L, immunoglobulin light chain; V_dss, volume of distribution at steady state; t_1/2, elimination half-life in a non-compartmental pharmacokinetic model; t_1/2 alpha, distribution half-life in a two-compartment pharmacokinetic model; t_1/2 beta, terminal elimination half-life in a two-compartment pharmacokinetic model; AUC_(0-t), area under the drug concentration-time curve from time zero to
the time of the last data point; Cl, plasma clearance; GC/MS, gas chromatography/mass spectrometry; LC/MS, liquid chromatography/mass spectrometry; BSA, bovine serum albumin; CHO, Chinese Hamster Ovary; AIC, Akaike Information Criterion; SBC, Schwartz Bayesian Criterion; RBA, relative binding affinity; PBS, phosphate-buffered saline; SCID, severe combined immunodeficiency.
Abstract

The monoclonal antibody (mAb), h2E2, is a humanized version of the chimeric human/murine anti-cocaine mAb 2E2. The recombinant h2E2 protein was produced \textit{in vitro} from a transfected mammalian cell line and retained high-affinity (4 nM $K_d$) and specificity for cocaine over its inactive metabolites benzoylecgonine (BE) and ecygone methyl ester. In rats, pharmacokinetic studies of h2E2 (120 mg/kg i.v.) showed a long terminal elimination half-life ($t_{1/2\beta}$) of 9.0 days and a low volume of distribution ($V_{dss}$) of 0.3 l/kg. Pre-treatment with h2E2 produced a dramatic 8.8-fold increase in the area under the plasma cocaine concentration-time curve (AUC) and in brain a concomitant decrease of 68\% of cocaine’s AUC following an i.v. injection of an equimolar cocaine dose. Sequestration of cocaine in plasma by h2E2, shown via reduction of cocaine’s $V_{dss}$, indicates potential clinical efficacy. Although the binding of cocaine to h2E2 in plasma should inhibit distribution and metabolism, the elimination of cocaine remained multi-compartmental and was still rapidly eliminated from plasma despite the presence of h2E2. BE was the major cocaine metabolite and brain BE concentrations were 6-fold higher than in plasma, indicating that cocaine is normally metabolized in the brain. In the presence of h2E2 brain BE concentrations were decreased and plasma BE was increased, consistent with the observed h2E2-induced changes in cocaine disposition. The inhibition of cocaine distribution to the brain confirms the humanized mAb, h2E2, as a lead candidate for development as an immunotherapy for cocaine abuse.
Introduction

Active immunization in humans with cocaine vaccines (Kosten et al., 2002) in some cases produced levels of polyclonal anti-cocaine antibodies that were associated with a decrease in the use of cocaine (Martell et al., 2005), which has demonstrated the potential efficacy of immunotherapy for cocaine abuse. In animals, active immunization with hapten-carrier conjugate vaccines consistently elicits sufficient polyclonal anti-cocaine antibodies to reduce the amount of cocaine entering the brain, and concomitantly decrease the behavioral effects of cocaine (Fox et al., 1996). The decrease in brain cocaine concentrations is likely the mechanism by which the vaccine-induced anti-cocaine antibodies decreased the use of cocaine observed in the clinical studies. Passive immunization with murine anti-cocaine mAbs has also been shown in rats to attenuate the behavioral effects of cocaine (Fox et al., 1996; Mets et al., 1998; Carrera et al., 1995; Carrera et al., 2000) and therefore represents a potential adjunct to active immunization (Kosten and Owens, 2005), or an emergency rescue treatment in instances of cocaine overdose. However, for optimal safety and efficacy in clinical use anti-cocaine mAbs should have a human sequence and structure (Redwan et al., 2003; Norman and Ball, 2012).

The anti-cocaine mAb 2E2 was generated by immunization with a hapten-carrier conjugate of a transgenic mouse strain engineered to produce human sequence γ1 heavy chain (H) and κ light chain (L) antibodies (Lonberg et al., 2005) replacing mouse IgGs. However, the murine λ light chain gene was not knocked out in this transgenic mouse strain and 2E2 is a mixed-chain or chimeric mAb consisting of a human γ1 H and murine λ L chain (Norman et al., 2007). This unusual mAb has a high affinity for cocaine and selectivity for cocaine over its inactive metabolites (Paula et al., 2004), and in vivo studies with mice have demonstrated that...
infused hybridoma-derived 2E2 dramatically increases plasma cocaine levels and decreases the concentration of cocaine reaching the brain (Norman et al., 2007). Furthermore, in rats trained to self-administer cocaine, 2E2 increased the concentration of cocaine required to reinstate this behavior (Norman, et al. 2009). Thus, 2E2, despite being a mixed chain/chimeric anti-cocaine mAb had properties that made it a lead candidate for pre-clinical development.

The mAb 2E2 was obtained from the mAb-producing murine hybridoma cell line grown in nude (SCID) mice and then purified from endogenous mouse immunoglobulins and serum proteins of the ascites fluid. The mAbs produced from murine-derived hybridoma cell lines cultured in mice are unsuitable for human use due to the potential presence of mouse proteins, endotoxins and infectious viruses that will compromise safety in humans. Furthermore, the low levels of 2E2 production and between-batch variations from this in vivo platform meant unacceptably high production costs. Here we report, as is typical for therapeutic mAbs, that 2E2 has been cloned from the murine hybridoma cell line and that constructed genes encoding the H and L chains were incorporated into the genome of Chinese Hamster Ovary (CHO) cell lines and expressed as a recombinant protein (Bleck, 2012). During the cloning of the 2E2 human H chain and the murine L chain, the murine \( \lambda_c \) L chain constant region was replaced with the \( \lambda_c \) human chain constant region while the variable region of the \( \lambda \) chain was unmodified. This humanized version of 2E2, designated h2E2, has been evaluated as our new lead candidate immunotherapeutic agent.

The major metabolites of cocaine in most mammalian species are benzoylecgonine (BE) and ecgonine methyl ester (EME), formed by enzymatic hydrolysis of cocaine’s methyl-ester and benzoyl-ester moieties, respectively (Warner and Norman, 2000). Therefore, it is critical for a therapeutic antibody to have lower affinity for the inactive metabolites of cocaine as these tend
to have longer half-lives than cocaine and can build up to higher concentrations that may compete for cocaine binding after multiple doses of cocaine are consumed. Although the mAb 2E2 has selectivity for cocaine over its inactive metabolites, it does have an appreciable affinity for BE as compared to the much lower affinity for EME and ecegonine. Therefore, we investigated whether h2E2 retained 2E2’s specificity for cocaine over its inactive metabolites. Previous measurements of the effect of 2E2 on the disposition of cocaine were in mice where the major metabolite is EME. In contrast, in rats the major metabolite is BE. The efficacy of h2E2 as measured by the effects on the disposition of cocaine was therefore tested in rats. We report herein our demonstration that the recombinant anti-cocaine mAb, h2E2, retains the same high affinity and specificity for cocaine over its major inactive metabolites, and significantly decreases the distribution of cocaine to the brain in rats.
Materials and Methods:

The generation and production of h2E2.

Here we report the generation of stably transfected CHO-S cells that have incorporated multiple gene copies of the human sequence $\gamma_1$ heavy chain of mAb 2E2 and a partially humanized version of its murine $\lambda$ light chain. This humanized version of 2E2, designated h2E2, has been transfected into CHO-S cells and selected cell lines with high levels of expression of the recombinant h2E2 have been isolated. Cultured in serum-free medium these cell lines secrete the recombinant mAb h2E2 at concentrations approaching one g/l and h2E2 is readily purified by protein A affinity chromatography with initial yields of 0.5 g/l. An initial 10 liter production run yielded 5.7 g of purified h2E2. This is the first time that sufficient quantities of a humanized anti-cocaine mAb have been available to allow for studies in animals larger than mice.

The partial amino acid sequences of the H and L chains of mAb 2E2 produced from the hybridoma cell line were initially determined by liquid chromatography-tandem mass spectroscopy (LC/MS) analysis of their trypsin-digested fragments. In addition, the predicted amino acid sequences of the H and L chains of 2E2 were obtained from the cDNAs generated from the full-length H and L chain mRNAs (Paula et al., 2004). The proprietary GPEx gene expression technology (Bleck, 2012) of Catalent Inc., (Madison, WI) was used to carry out transfections that generated stably transfected CHO-S cells containing multiple transgene copies inserted into transcriptionally active regions of the genome. The GPEx technology utilizes replication-defective retroviral vectors derived from Moloney murine leukemia virus to achieve the targeting and the insertion of the RNA coded genes for the h2E2 $\gamma_1$ H and the modified $\lambda$ L chain. The electronic DNA and protein H and L sequences for 2E2 as well as encoding plasmids...
were used to construct the retroviral vectors. The re-engineering of the murine \( \lambda \) L chain of the chimeric 2E2 was accomplished using standard molecular biology techniques to synthetically construct the L chain with the human \( \lambda_c \) constant regions sequence replacing the murine sequence while leaving the \( \lambda_v \) region unmodified. The construction of the new expression constructs was verified by DNA sequencing.

After five transfection rounds of viral infection with the H chain and four rounds with the L chain containing retrovectors, the identification by dilution cloning of 24 cell lines (average gene copy numbers/clone: H chain, 1.75; L chain, 4.5) with high mAb production levels was achieved without antibiotic or methotrexate dependent cell selection procedures. The expressed and secreted recombinant h2E2 mAb used for these studies was affinity purified from the serum-free culture media (PF CHO LS media) of a batch culture growth of the selected top 5-stably transfected CHO-S cloned lines by Protein A HPLC chromatography. The final concentration of h2E2 in phosphate-buffered saline (PBS) was 5 mg/ml. The amino acid sequences of the H and L chains of the purified h2E2 were confirmed by LC/MS analysis of the fragments from the trypsin digestion of H and L chains following separation via SDS-gel electrophoresis. Approximately 94% of the residue sequences were identified and all of these were consistent with those predicted from the DNA sequences.

\[^{3}H\]Cocaine binding studies.

The binding affinities of 2E2 and recombinant h2E2 for cocaine were measured by the equilibrium binding to \[^{3}H\]cocaine in PBS (pH 7.0) at room temperature and incubated for 6 hours before separation of bound and free radioligand. The separation was accomplished by ultrafiltration of the precipitated anti-human F\(_c\) region antibody-h2E2 or 2E2-cocaine complex
through glass fiber (GF/B) filters (Whatman Ltd., GE Healthcare, Fairfield, CT). The $K_d$ of 
$[^3]H$ cocaine for the binding to mAbs 2E2 and h2E2 was calculated from best fit of a hyperbolic 
function to the specific binding as a function of $[^3]H$ cocaine concentration.

**Specificity of h2E2 for cocaine.**

The relative binding affinities of cocaine and its major metabolites for h2E2 used a 
competition enzyme-linked immunosorbent assay (ELISA). The wells in the polyvinyl chloride 
plates were coated with the hapten BE coupled to 1,4-diaminobutane (putrescine) derivatized-
bovine serum albumin (BSA). The ligand recognition site of h2E2 was generated in response to 
a hapten-cationized-KLH (keyhole limpet haemocyanin) conjugate that has the amide, four 
carbon-linker derivative of BE as the hapten. Consequently, the mAb has a higher affinity for 
the ethyl and propyl ester derivatives of cocaine than for cocaine, but a lower affinity for BE. 
The binding of h2E2 to the conjugate was measured in the presence of a range of concentrations 
of cocaine or various metabolites. Hapten-bound h2E2 was measured using a biotinylated 
purified goat anti-human IgG polyclonal antibody described previously (Paula et al., 2004). The 
relative binding affinities of the metabolites was determined by the ratios of their $IC_{50}$ values to 
the $IC_{50}$ value for cocaine.

**Animals.**

Jugular vein-catheterized male Sprague-Dawley rats (250 g) were purchased from Harlan 
(Indianapolis, IN). Animals were housed individually with free access to food and water and 
kept on a 12 h light/dark cycle. Studies using animals were carried out in accordance with the
Guide for the Care and Use of Laboratory Animals under a protocol approved by the Institutional Animal Care and Use Committee at the College of Medicine, University of Cincinnati.

*mAb h2E2 pharmacokinetic studies.*

The *in vivo* concentrations of h2E2 following a single injection (120 mg/kg i.v.) were determined using an ELISA described previously (Paula et al., 2004; Norman et al., 2007) that compared the quantity of mAb in varying dilutions of the rat blood samples to that quantified in a standard curve using known dilutions of purified h2E2. The blood samples (10 µl each) were collected from a small incision at the tip of the tail and the samples were collected at various time points up to 7 weeks after the administration of h2E2.

*Cocaine pharmacokinetic studies in rat plasma.*

The antibody (120 mg/kg, which has equimolar cocaine binding sites with 0.56 mg/kg of cocaine as the HCl salt) in PBS or an equivalent volume of vehicle (PBS) was infused i.v. at a rate of approximately 0.35 ml/min for up to 2 minutes, with the animal held under mild restraint. One hour after completion of the infusion of mAb, cocaine HCl (0.56 mg/kg) plus heparin (400 units/kg) was injected i.v. through the same catheter at a volume of 4.0 ml/kg body weight. At most sampling times, sodium pentobarbital (45 mg/kg, i.p.) was administered three minutes prior to sacrificing the animal. For the 0.75 minute time point the cocaine was injected into anesthetized rats. At the designated times after the injection of cocaine anesthetized rats were sacrificed by decapitation and trunk blood (typically 3-5 ml) was collected. From this volume two 1 ml aliquots were retained and placed in polypropylene microcentrifuge tubes containing 11.2 µl heparin (1.0 unit/µl) to inhibit blood coagulation and NaF (16 mg/0.8 ml of blood) to
inhibit enzymatic hydrolysis of cocaine (Warner and Norman, 2000). The blood samples were centrifuged at 5,000 x g for 3 min, then the plasma (typically 0.4-0.8 ml) was carefully separated from packed red blood cells, placed into sterile 1.5 ml Eppendorf microcentrifuge tubes, rapidly frozen on dry ice, and stored at -80º C until analysis.

*Cocaine pharmacokinetics in rat brain.*

The whole brain was quickly removed from each decapitated animal, surface blood was blotted away, and the brain was placed in a polypropylene tube, rapidly frozen on dry ice, and stored at -80º C until analysis. For analysis, individual brains were weighed and cold deionized, distilled water was added to produce a total volume of 1 ml, then the solution was homogenized and centrifuged at 13,000 rpm for 45 min at 4º C. The resulting supernatants (0.4-0.6 ml) were collected into sterile polypropylene microcentrifuge tubes and an aliquot (0.05-0.40 ml) was processed for cocaine/metabolite analysis by GC/MS and hemoglobin content. Any remaining sample was stored at -80º C.

*Determination of blood and brain hemoglobin concentrations.*

At the same time as plasma sample collections, a separate sample of blood (approximately 100 µl) was collected from each animal and rapidly frozen on dry ice before storage at –80º C. The concentration of hemoglobin and, where appropriate h2E2, was measured in these samples.

The hemoglobin contents of brain and blood were quantified spectroscopically by combining the method reported by Choudhri et al. (1997) and a protocol provided by Pointe Scientific, Inc. (Canton, MI). This procedure was identical to that reported in a previous study.
(Norman et al., 2007). The mean ± SEM concentration of hemoglobin in whole blood and brain were 7.22 ± 0.46 g/dl and 0.18 ± 0.05 g/dl, respectively. The average hemoglobin content in brain tissue relative to that present in whole blood was, therefore, 2.5%.

**Solid phase extraction and quantification of cocaine and metabolites from plasma and brain.**

Cocaine, BE and EME concentrations in plasma and brain were measured using solid phase extraction followed by GC/MS according to the procedures detailed by Norman et al. (2007).

**Chemicals, reagents, and reference standards.**

[^3H]Cocaine (26 Ci/mmol) was purchased from Perkin-Elmer NEN Radiochemical, Boston, MA. Standard solutions of cocaine, BE and EME (each 1 mg/ml) were prepared in methanol or acetonitrile and served as stock solutions for preparing the reference standard curves for GC/MS quantification. The deuterium-labeled cocaine-D₃, BE-D₃ and EME-D₃ that were used as the internal standards (0.1 mg/ml each in methanol or acetonitrile) and the derivatizing reagent N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane were purchased from Cerilliant (Round Rock, TX). The high and low hemoglobin concentration standards were purchased from Pointe Scientific, Inc. (Canton, MI). All other chemicals and immunoreagents were purchased from Sigma-Aldrich (St. Louis, MO) or Pierce Chemicals (Rockford, IL). All reagents and organic solvents were of analytical or HPLC grade.

**Data analysis and statistics.**
Cocaine and h2E2 pharmacokinetic data and the h2E2 and 2E2 pharmacodynamic (radioligand binding) data were analyzed using the program PKSolver (Department of Pharmaceutics, China Pharmaceutical University, Nanjing, China). PKSolver (Zhang et al., 2010) is a Microsoft Excel add-in program that has been validated for pharmacokinetic/pharmacodynamic data analysis against WinNonLin (Certara USA, Inc., St. Louis, MO). The program provides Akaike Information Criterion (AIC) and Schwartz Bayesian Criterion (SBC) measures of model diagnostics of the data to the pharmacokinetic models that were used. Pharmacokinetic data was analyzed according to a two-compartment model based on previous evidence that a single compartment model gave a poor fit to the cocaine pharmacokinetic data (Norman et al., 2007). The two-compartment pharmacokinetic model used to fit the data assumed that cocaine distributed between a central and a peripheral compartment. The fit to the data using a two-compartment model yielded optimal AIC and SBC measures, lack of systematic deviation from the data points, and a concomitant reduction in the sum of squares residuals. Applying pharmacokinetic models that assumed that cocaine distributed between more than two compartments only slightly improved the fit to the observed data and this additional complexity was considered unnecessary.

The h2E2 and 2E2 data from [3H]cocaine binding studies were analyzed in PKSolver to calculate the $K_d$ through the best fit of a hyperbolic function to the specific binding as a function of [3H]cocaine concentration.

The formation of metabolites was analyzed using SigmaPlot (Systat Software Inc., San Jose, CA) global curve fit analyses. Because the formation of metabolites continued over the time course of the experiments and the elimination of the major metabolite, BE, extended beyond the time course of the study, a pharmacokinetic analysis of BE was deemed inappropriate.
Results

Binding properties of recombinant mAb h2E2.

As shown in Fig. 1, the re-engineered mAb h2E2 (K_d = 3.9 nM) retained a high affinity for [3H]cocaine similar to that of the mouse hybridoma cell-derived 2E2 (K_d = 4.4 nM) with the cocaine affinities of the two structural variants indistinguishable. In addition, h2E2 retained essentially the same specificity as 2E2 for cocaine over its major inactive metabolites, BE, EME and ecdgonine (Table 1). Furthermore, like 2E2, h2E2 had a higher affinity for the active metabolite cocaethylene than for cocaine and a moderately high affinity for the active metabolite norcocaine (Table 1).

The pharmacokinetics of mAb h2E2.

To determine the pharmacokinetics of mAb h2E2 in rats (n = 4), the first samples of tail vein blood were taken 1 hour after completion of the i.v. infusion of h2E2 (120 mg/kg) via the implanted jugular vein catheter. The initial mean blood concentration of mAb was determined to be 600 μg/ml. As shown in Fig. 2, the observed mean blood concentrations of h2E2 were highest at the earliest time points. There was an initial rapid decrease in h2E2 concentrations over the first 2 days followed by a slower exponential decrease over the 6 week duration of the study. The declining concentrations of h2E2 in blood was adequately described by a two-compartment pharmacokinetic model with a distribution half-life (t_1/2α) of 19 hours and an elimination half-life (t_1/2β) of 9.0 days (Fig. 2). This model gave a calculated V_dss for h2E2 of 0.3 l/kg.

The pharmacokinetics of cocaine in plasma and brain.
The pharmacokinetics of cocaine in rat plasma subsequent to its i.v. injection via the jugular vein was determined. The highest mean plasma concentration measured (380 ng/ml) was observed at the earliest sampling time, after which a rapid decline in cocaine concentrations was observed (Fig. 3A). The pharmacokinetic model generated parameter estimates for the cocaine $t_{1/2\alpha}$ and $t_{1/2\beta}$ of 0.4 and 32.9 min, respectively. The calculated cocaine $V_{dss}$ was 2.6 l/kg.

As shown in Fig. 3B, the cocaine concentration-time profile in rat brain differed substantially from that observed in the plasma (Fig. 3A). The initial mean brain cocaine concentrations (corrected for cocaine present in residual blood) at 45 sec (1,500 ng/g) after the injection was approximately 4-fold higher than that measured in the plasma at that time. The brain cocaine concentrations subsequently increased further with the highest mean measured concentrations observed at 1.5 min (1,800 ng/g), after which cocaine concentrations rapidly declined. A pharmacokinetic model that assumed a first-order cocaine input to the brain and a first-order elimination was used to describe the increase and subsequent decrease in brain cocaine concentrations. The estimated input $t_{1/2}$ was 0.7 min and the elimination $t_{1/2}$ was 25 min. The estimated $AUC_{(0-t)}$ for brain cocaine concentrations was 29,900 (ng/g)-min.

Effect of h2E2 on the pharmacokinetics of cocaine in plasma and brain.

As shown in Fig. 3A, in the presence of h2E2, the peak mean plasma concentrations (6,300 ng/ml) of cocaine were also observed at the earliest time point (45 sec) after h2E2 injection. This is similar to what was observed in the absence of h2E2. However, the peak plasma concentration in the presence of h2E2 was 16.5-fold higher. Furthermore, the bi-exponential decrease in cocaine concentrations observed in the absence of h2E2 held true in the presence of h2E2 as well. The distribution and elimination phases were described by a $t_{1/2\alpha}$ of
2.2 minutes and a $t_{1/2\beta}$ of 13.8 minutes (Table 2). h2E2 also produced a sustained increase in the plasma cocaine concentration that resulted in an 8.8-fold increase in the plasma AUC (Table 2). Consistent with these results, the calculated $V_{dss}$ of cocaine in the presence of h2E2 was 0.1 l/kg compared to 2.6 l/kg in the absence of h2E2 (Table 2). The calculated plasma clearance (Cl) of cocaine was dramatically reduced by 97% in the presence of h2E2 (Table 2).

In the presence of h2E2, the highest mean brain cocaine concentrations (450 ng/g) were observed at the earliest sample times with a subsequent decline over time (Fig. 3B). Importantly, h2E2 abolished the initial fast increase and peaking of the cocaine concentrations. In the presence of h2E2 the estimated cocaine AUC$_{(0-t)}$ was 9,460 (ng/g)-min, which represents an approximately 68% decrease from the AUC in the absence of h2E2 (Fig. 3B, Table 2).

The metabolism of cocaine and the effect of h2E2.

Concomitant with the determination of cocaine concentrations in the plasma and brain of rats, the concentrations of the inactive metabolites of cocaine, BE and EME, were measured. The major metabolite in both plasma and brain was BE, with EME detectable only in trace amounts. A maximum EME concentration of 9 ng/ml was detected in plasma and a higher concentration of 29 ng/g was detected in brain. These values were about 88% lower and 93% lower than the peak BE concentrations in plasma and brain, respectively. Therefore, the metabolism of cocaine to EME was considered negligible and EME concentration data are not shown. As shown in Fig. 4A, in the absence of h2E2 BE concentrations increased gradually in plasma reaching a peak of 73 ng/ml at the last time point measured. BE was also found in the brain with a mean peak concentration of 430 ng/g at approximately 5 min and then declined gradually. Thus, the pharmacokinetics of BE was different in plasma and brain and the peak
brain BE concentration was approximately 6-fold higher in brain than in plasma. In the presence of h2E2, the time course of BE formation in the plasma was similar to that observed in the absence of h2E2, but the mean peak BE concentration in plasma was increased approximately 2-fold (Fig. 4A). In the brain in the presence of h2E2, the highest BE concentration occurred at the earliest time point measured before declining to a stable concentration. While initial BE concentrations were similar whether h2E2 was present or absent, only in the absence of antibody did BE concentrations increase to a peak before declining (Fig. 4B). Unfortunately, the limited time course of these studies compromised the ability to more thoroughly estimate the pharmacokinetic parameters for BE.
Discussion

The recombinant humanized mAb, h2E2, retained the same high affinity and specificity for cocaine over cocaine’s major inactive metabolites, BE, EME, and ecgonine, as the hybridoma-derived chimeric human/murine mAb 2E2 (Paula et al., 2004). Thus, exchanging the murine $\lambda$ constant region for a human $\lambda$ sequence did not adversely alter the binding characteristics of the anti-cocaine mAb.

The pharmacokinetics of h2E2 and the effects of h2E2 on cocaine’s pharmacokinetics in rats were then investigated. The $V_{dss}$ of h2E2 in rats was similar to that observed in mice using 2E2 (Norman et al., 2007) and to that previously reported for human polyclonal IgG1 antibodies in rats (Bazin-Redureau et al., 1997). This low $V_{dss}$ is consistent with h2E2’s distribution being predominantly restricted to the blood and interstitial fluid volumes. Additionally, the elimination ($t_{1/2\beta}$) value for h2E2 was relatively long and similar to that reported for other human antibodies in rats (Bazin-Redureau et al., 1997), and indicates that h2E2’s effects in humans will potentially be long lasting. The initial more rapid decrease of plasma h2E2 in rats likely corresponds to the distribution of h2E2 from plasma to the interstitial spaces, and this is typical of human IgG1 antibodies in rodents.

Here we report for the first time the effects of h2E2 on cocaine pharmacokinetics in rats. The effect of h2E2 on cocaine pharmacokinetics is equivalent to an increase in plasma protein binding for cocaine. This results in the observed dramatic reduction in the total plasma clearance of cocaine, although the intrinsic clearance of cocaine may not be reduced to the same extent as for total plasma clearance. An initial distribution phase for cocaine was observed in the presence of h2E2 although the process was much slower and less pronounced than the distribution phase observed in the absence of h2E2. Although this initial decrease in plasma cocaine concentrations
in the presence of h2E2 is assumed to represent the distribution of cocaine from plasma to other tissues, it was not distributing to the brain. Therefore, the pharmacokinetics of cocaine in the presence of h2E2 may be more complex in rats than that of mice where no initial distribution phase was observed (in the presence of 2E2) (Norman et al., 2007), but the efficacy of h2E2 as defined as the prevention of cocaine entry into the brain was similar. The ability of recombinant h2E2 to inhibit cocaine entry into the brain in rats observed here is consistent with previous reports in rats that active immunization-induced anti-cocaine antibodies decreased cocaine levels in rat brain after i.v., intranasal, or i.p. cocaine administration (Fox et al., 1996, 1997; Carrera et al., 2000). The ability of h2E2 to decrease brain cocaine concentrations is also consistent with the previous reports of mAb-induced reductions in the brain concentrations of other psychoactive drugs such as phencyclidine (Valentine and Owens, 1996; Proksch et al., 2000), methamphetamine (Laurenzana et al., 2003), and nicotine (Keyler et al., 2005).

Consistent with previous reports (Booze et al., 1997; Warner and Norman, 2000), BE was the major metabolite of cocaine in rats. Indeed, the concentrations of EME in both plasma and brain were negligible under the conditions of this in vivo study. In contrast, in mice EME was the major metabolite (Norman et al., 2007). Despite the higher affinity of h2E2 for BE relative to that for EME, there appeared to be no attenuation of the magnitude of the effects of h2E2 on decreasing the brain concentrations of cocaine and increasing plasma concentrations in rats. Thus, the in vivo efficacy of h2E2 was independent of the route of cocaine metabolism. It should be noted that the current studies were conducted only after a single cocaine injection and after repeated cocaine injections that result in higher BE concentrations, there may be some loss of h2E2 efficacy. However, when cocaine was self-administered by rats over several hours, which presumably results in higher BE concentrations than those resulting from a single low dose
cocaine injection, the effects of the anti-cocaine mAb 2E2 were stable (Norman et al., 2009). In humans, BE and EME are both produced by the metabolism of cocaine at ratios that are between those of rats and mice and so there would be no expectation that the clinical efficacy of h2E2 should be diminished in the presence of differing routes of cocaine metabolism.

The finding of higher concentrations of BE in the brain relative to those in plasma in the control rats suggest that at least some of the cocaine that enters the brain is metabolized there to BE, which is consistent with previous reports in animals and humans (Karch, 1996). As the brain cocaine concentration rapidly declined due to cocaine’s metabolism and redistribution to plasma this resulted in no further increase in brain BE concentrations. The lower brain BE concentrations in the presence of h2E2 (in the plasma) reflects the lowered brain cocaine concentration, which is also consistent with the idea that cocaine is metabolized to BE in the brain. The relatively small decrease in brain BE concentrations in the presence of plasma h2E2 relative to the dramatic h2E2-induced reductions in brain cocaine concentrations may indicate that BE can enter the brain from the periphery. However, it has been reported that BE does not cross the blood-brain barrier (Misra et al., 1975) due to its physicochemical properties. Further studies on the formation and/or transport of BE across the blood-brain barrier will required to fully understand the implications of these findings.

The increased plasma BE concentrations in the presence of h2E2 is consistent with cocaine being sequestered and then available to be metabolized to BE in the peripheral circulation. Curiously, despite the greater than 16-fold increase in peak plasma cocaine concentrations in the presence of h2E2, plasma BE concentrations were increased less than three-fold. Whether the metabolism of cocaine to BE in the presence of h2E2 is reduced or whether there is a mAb-induced alteration in the routes of cocaine elimination from the periphery that
results in altered BE levels will require further studies. However, there was no evidence of an increase in EME formation.

In summary, the high affinity anti-cocaine mAb h2E2 retained the binding properties of 2E2 and in rats limited the distribution of cocaine to the plasma, thus decreasing the levels of cocaine reaching the brain without inhibiting the elimination of cocaine. In rats where cocaine is metabolized predominantly to BE, as opposed to mice where cocaine is metabolized predominantly to EME, the efficacy of h2E2 (defined as the ability to decrease brain cocaine concentrations) is similar. Therefore, the efficacy of h2E2 should be independent of species, animal size or route of cocaine metabolism. These findings further support the general concept of the usefulness of immunotherapy for the treatment of drug abuse and are consistent with mAb h2E2 being a lead candidate for development as a passive immunotherapy for cocaine abuse.
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Authorship Contributions

Participated in research design: Norman, Ball.

Conducted experiments: Norman, Gooden, Tabet,

Contributed new reagents or analytic tools: Not applicable.

Performed data analysis: Norman, Gooden, Tabet

Wrote or contributed to the writing of the manuscript: Norman, Gooden, Tabet, Ball.
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Footnotes. This work was supported by National Institutes of Health National Institute on Drug Abuse [grant DP1DA031386]. Send reprint requests to Andrew B. Norman, Ph.D., Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati, OH 45267-0575, USA. Email: andrew.norman@uc.edu.
Figure Legends

**Figure 1.** The binding affinities of 2E2 and h2E2 for [3H]cocaine. Antibodies and [3H]cocaine were incubated in PBS at pH 7.0 at room temperature for six hours. The $K_d$, defined as the concentration of [3H]cocaine at which 50% of $B_{max}$ occurred, was 4.4 nM for 2E2 and 3.9 nM for h2E2.

**Figure 2.** The pharmacokinetics of the anti-cocaine mAb h2E2 in rats. Animals received an i.v. infusion of 120 mg/kg of h2E2. Samples of blood (10 μl) were obtained from tail veins at the indicated times after completion of the mAb infusion. Concentrations of h2E2 in blood were determined using an ELISA. Data points represent the mean ± SEM from four rats. The $V_{dss}$ was 0.3 l/kg. A two-compartment model with a $t_{1/2\alpha}$ of 19 hours described the distribution phase and a $t_{1/2\beta}$ of 9.0 days described the elimination phase, represented by the best-fit regression line.

**Figure 3.** The effect of h2E2 on the pharmacokinetics of cocaine in plasma (A) and brain (B) in rats. Rats received an i.v. infusion of 120 mg/kg of h2E2. One hour later the rats received an i.v. injection of cocaine HCl (0.56 mg/kg). The animals were sacrificed at the indicated times and blood and the brain were collected. Cocaine concentrations were determined by GC/MS. Each data point represents the mean ± SEM from three rats. In the absence of h2E2 (open circles), the cocaine concentration-time profile in plasma (A) was described by a two-compartment pharmacokinetic model with a $t_{1/2\alpha}$ of 0.4 min and a $t_{1/2\beta}$ of 32.9 min. In the presence of h2E2 (closed circles), a two-compartment pharmacokinetic model indicated a $t_{1/2\alpha}$ of 2.2 min and a $t_{1/2\beta}$ of 13.8 min. h2E2 produced a 8.8-fold increase in the area under the plasma cocaine time-concentration curve (AUC). The $V_{dss}$ in the absence and presence of h2E2 was 2.6 and 0.1 l/kg.
respectively. In the brain (B) in the absence of h2E2 (open circles) a two-compartment pharmacokinetic model with an AUC of 29,900 (ng/g)-min described the cocaine concentration-time profile. In the presence of h2E2 (closed circles), a two-compartment pharmacokinetic model with an AUC of 9,460 (ng/g)-min described the cocaine concentration-time profile. h2E2 produced a 68% decrease in the brain cocaine AUC.

**Figure 4.** The effect of h2E2 on the formation of benzoylecgonine (BE) via cocaine metabolism in plasma (A) and brain (B) in rats. Concomitant to determination of cocaine concentrations using GC/MS, the concentrations of BE were determined. Each data point represents the mean ± SEM from three rats. In the absence of h2E2 (open circles), BE is formed at a relatively constant rate in plasma (A) but in the brain (B) it is present in higher concentrations that decline over time. In contrast, the presence of h2E2 (closed circles) causes an increase in BE levels in plasma (A) and in the brain (B) a corresponding decrease in BE formation.
Table 1. The relative binding affinities (RBAs) of mAbs 2E2 and h2E2 for cocaine and its metabolites.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>2E2</th>
<th>h2E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocaine</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cocaethylene</td>
<td>0.5</td>
<td>0.6</td>
</tr>
<tr>
<td>Norcocaine</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Benzoylecgonine</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>Ecgonine methyl ester</td>
<td>1,800</td>
<td>1,900</td>
</tr>
<tr>
<td>Ecgonine</td>
<td>23,000</td>
<td>18,000</td>
</tr>
</tbody>
</table>

The RBAs were measured using a competition ELISA as described in the methods section. The measured IC₅₀ values for each metabolite were compared to that of cocaine, which was designated an RBA of 1. Values higher or lower than 1 indicate, respectively, a lower or higher affinity for h2E2 than that of cocaine.
Table 2. Pharmacokinetic parameter estimates for cocaine in the presence and absence of h2E2

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Plasma</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>h2E2</td>
</tr>
<tr>
<td>AUC(0-t) ((ng/ml) or (ng/g))*min</td>
<td>6,370</td>
<td>56,200</td>
</tr>
<tr>
<td>AUC(0-∞) ((ng/ml) or (ng/g))*min</td>
<td>8,710</td>
<td>58,200</td>
</tr>
<tr>
<td>t1/2α (min)</td>
<td>0.4</td>
<td>2.2</td>
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<tr>
<td>t1/2β (min)</td>
<td>32.9</td>
<td>13.8</td>
</tr>
<tr>
<td>Cl (l/min)</td>
<td>0.7</td>
<td>0.02</td>
</tr>
<tr>
<td>Vdss (l/kg)</td>
<td>2.6</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The parameter estimates were generated using the program PKSolver from the models shown in Figures 3A and 3B. + represents the fold increase from the control values and – represents the percent decrease from control values.