Benzodiazepine Binding Site Occupancy by the Novel GABA<sub>A</sub> Receptor Subtype-Selective Drug 7-(1,1-Dimethylethyl)-6-(2-ethyl-2H-1,2,4-triazol-3-ylmethylx)-3-(2-fluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine (TPA023) in Rats, Primates, and Humans

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ABSTRACT

The GABA<sub>A</sub> receptor α2/α3 subtype-selective compound 7-(1,1-dimethylethyl)-6-(2-ethyl-2H-1,2,4-triazol-3-ylmethylx)-3-(2-fluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine (TPA023; also known as MK-0777) is a triazolopyridazine that has similar, subnanomolar affinity for the benzodiazepine binding site of α1-, α2-, α3- and α5-containing GABA<sub>A</sub> receptors and has partial agonist efficacy at the α2 and α3 but not the α1 or α5 subtypes. The purpose of the present study was to define the relationship between plasma TPA023 concentrations and benzodiazepine binding site occupancy across species measured using various methods. Thus, occupancy was measured using either in vivo [3H]flumazenil binding or [11C]flumazenil small-animal positron emission tomography (microPET) in rats, [123I]iomazenil γ-scintigraphy in rhesus monkeys, and [11C]flumazenil PET in baboons and humans. For each study, plasma-occupancy curves were derived, and the plasma concentration of TPA023 required to produce 50% occupancy (EC<sub>50</sub>) was calculated. The EC<sub>50</sub> values for rats, rhesus monkeys, and baboons were all similar and ranged from 19 to 30 ng/ml, although in humans, the EC<sub>50</sub> was slightly lower at 9 ng/ml. In humans, a single 2-mg dose of TPA023 produced in the region of 50 to 60% occupancy in the absence of overt sedative-like effects. Considering that nonselective full agonists are associated with sedation at occupancies of less than 30%, these data emphasize the relatively nonsedating nature of TPA023.

The GABA<sub>A</sub> receptor is a pentameric assembly of subunits derived from a family of genes of which there are 19 members (α1–6, β1–3, γ1–3, δ, ε, θ, π, and ρ1–3), with the predominant composition of native receptors being α, β, and γ subunits, in a 2:2:1 stoichiometry and an αββγ arrangement as viewed from the synapse (Sieghart and Sperk, 2002). Clinically used benzodiazepines, such as diazepam and lorazepam, exert their actions on GABA<sub>A</sub> receptors containing β and γ2 subunits along
with either an α1, α2, α3, or α5 subunit (Sieghart, 1995, 2006), the collective population of which comprises approximately 75% of total brain GABA\textsubscript{A} receptors (McKernan and Whiting, 1996; Sieghart and Sperk, 2002). The different GABA\textsubscript{A} receptors with which benzodiazepines interact are associated with specific aspects of the pharmacology of these drugs. For example, the α1 subtype is associated with sedation (Rudolph et al., 1999; McKernan et al., 2000), whereas the α2 and/or α3 subtypes mediate anxiolytic effects (Löw et al., 2000; Dias et al., 2005). Accordingly, it has been hypothesized that compounds that selectively modulate the α2 and/or α3 subtypes without effects on α1-containing receptors should prove to be nonsedating anxiolytics (Atack, 2005). This α2/α3 selectivity may be achieved either by compounds having higher affinity at the α2 and α3 subtypes compared with α1 and α5 subtypes, or, alternatively, by binding to all subtypes but only having modulatory effects (i.e., agonism) at the α2 and α3 subtypes (Atack, 2005). This latter approach has been used to identify a series of triazolopyrimidines (Carling et al., 2005) of which the prototypic compound is L-838417 (McKernan et al., 2000).

Although L-838417 is a useful pharmacological tool compound, pharmacokinetic issues precluded its further development. However, the structurally related compound TPA023 (also known at MK-0777) (Lewis et al., 2008) has more favorable pharmacokinetic properties, and the in vitro and in vivo properties of this compound have been described previously (Atack et al., 2006b). Thus, TPA023 has equivalent affinity properties of this compound have been described previously ablepharmacokinetic properties, and the in vitro and in vivo (also known at MK-0777) (Lewis et al., 2008) has more favorably, pharmacokinetic issues precluded its further development. In the course of identifying subtype-selective GABA\textsubscript{A} modulators, compounds were prioritized for subsequent behavioral analyses based upon their potencies in a rat [\(^{3}H\)flumazenil in vivo binding assay, with potency being defined in terms of the dose required to produce 50% occupancy (Occ\textsubscript{50}) as well as the corresponding plasma drug concentration (EC\textsubscript{50}). Since the plasma EC\textsubscript{50} was a key selection criterion, it was considered important to evaluate to what extent this parameter translates across species. Accordingly, the purpose of the present study was to measure the plasma EC\textsubscript{50} for TPA023 in several preclinical species (rats, rhesus monkeys, and baboons) using either [\(^{3}H\)flumazenil in vivo binding or [\(^{11}C\)flumazenil small-animal positron emission tomography (microPET) in rats, [\(^{12}I\)iomazenil γ-scintigraphy in rhesus monkeys, or [\(^{11}C\)flumazenil PET in baboons and to compare these plasma-occupancy relationships to that observed in humans measured using [\(^{11}C\)flumazenil PET.

### Materials and Methods

#### Drugs

TPA023 was synthesized as described previously (Carling et al., 2005). Flumazenil was obtained from Sigma-Aldrich (St. Louis, MO), and bretazenil was synthesized in-house (Merck, Harlow, UK). [\(^{3}H\)Flumazenil ([\(^{3}H\)Ro 15-1788; 70–87 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA), and [\(^{11}C\)Flumazenil was synthesized as described in more detail below.

#### Rat [\(^{3}H\)]Flumazenil In Vivo Binding Studies

Experiments measuring the inhibition of the in vivo binding of [\(^{3}H\)flumazenil by TPA023 (i.e., TPA023 occupancy) were carried out as described previously (Atack et al., 2006b). In brief, rats were dosed orally with either vehicle (0.5% methyl cellulose) or 1, 3, or 10 mg/kg TPA023, and then 3 min before killing at 0.75, 1.5, 3, 6, or 18 h after dosing, animals received a tail-vein injection of [\(^{3}H\)Flumazenil (1 ml/kg radioligand diluted 1:150 in isotonic saline). Three hours later, animals were killed by stunning and decapitation, the brain was rapidly removed and homogenized, and aliquots of homogenate were filtered and washed over Whatman GF/F glass fiber filters (Whatman, Maidstone, UK). Filters were then placed in vials, scintillant cocktail was added, and radioactivity was counted using an LS6500 scintillation counter (Beckman Coulter, High Wycombe, UK). To determine the level of nonspecific binding, a separate group of animals were given 5 mg/kg bretazenil (intraperitoneally in 100% PEG 300 with a treatment time of 30 min), injected with [\(^{3}H\)Flumazenil, and then killed. The brain was processed as described above. The occupancy of TPA023 was defined as the extent by which the specific binding in TPA023-treated animals was reduced relative to the vehicle-treated group.

#### Rat [\(^{11}C\)]Flumazenil MicroPET Studies

Rat microPET studies were performed as described in more detail previously (Atack et al., 2007). In brief, cannulated rats (right femoral artery for blood sampling, right femoral vein for compound administration and left femoral vein for [\(^{11}C\)flumazenil administration) were anesthetized with isoflurane, placed in a microPET P4 scanner (Concorde Microsystems Inc., Knoxville, TN), and maintained under isoflurane anesthesia for the duration of the experiment. [\(^{11}C\)Flumazenil (specific activity, ~6 Ci/μmol at the time of injection) was synthesized as described previously (Atack et al., 2007), and 60 to 90 MBq was injected over a 40-s period to initiate each study. Ten and 26 min after [\(^{11}C\)Flumazenil administration, rats were dosed with either vehicle (aqueous solutions of 2% dimethyl sulfoxide and 10% hydroxypropyl-β-cyclodextrin; n = 2) or low and high doses of TPA023 (0.05 and 0.2 mg/kg i.v., respectively; n = 3). Forty-six minutes after the study started, rats received an i.v. injection of 0.25 mg/kg flumazenil to determine nonspecific binding. The time-activity curve after flumazenil injection was extrapolated backward to estimate the level of nonspecific binding throughout the duration of the study. Having corrected for the estimated levels of nonspecific binding, the percentage of occupancy of TPA023 was calculated as the extent by which the TPA023 time-activity curves (n = 3) were reduced during the 15- to 25-min (low-dose) and 36- to 46-min (high-dose) periods relative to vehicle-treated animals (n = 2).

#### Rhesus Monkey [\(^{23}I\)]Iomazenil γ-Scintigraphy

The occupancy of rhesus monkey brain GABA\textsubscript{A} receptors by TPA023 was carried out as described in more detail previously (Atack et al., 2009a). In brief, four separate studies were performed in the same rhesus monkey. For each study, the monkey was anesthetized with ketamine (10 mg/kg i.m.) followed by propofol (2–5 mg/kg i.v. bolus followed by a constant infusion of 0.5 mg/kg/min i.v.). The
monkey was intubated and then ventilated with oxygen at ~50 ml per breath at a rate of 20 to 24 respirations/min. Body temperature was maintained with circulating water-heating pads, and temperature and heart rate were monitored for the duration of the study. The monkey was positioned on the camera head (Siemens Orbitber using a LEAP collimator; Siemens Medical Solutions, Hoffman Estates, IL) for lateral brain imaging.

[123I]iomazenil, which was synthesized by oxidative radiiodination of the trimethyl-tin precursor essentially as described previously (McBride et al., 1991; Atack et al., 2009), was injected as a bolus (~0.4 mL) followed by infusion (0.05–0.06 mL/h) to reach steady state. After 2 h, TPA023 was administered for an additional 3-h period using an ETOH/PEG 400/water [1:3:6 (v/v)] vehicle and a bolus plus infusion paradigm using one of four different doses of TPA023 (0.04 mg/kg over 5 min and then 0.01 mg/kg/h; 0.1 mg/kg over 5 min and then 0.025 mg/kg/h; 0.2 mg/kg over 5 min and then 0.05 mg/kg/h; 0.25 mg/kg over 5 min and then 0.125 mg/kg/h). Nonspecific binding was defined by the infusion of 1 mg/kg flumazenil (using the same vehicle as TPA023) at the end of the 0.125 mg/kg/h scan. Occupancy was defined as the percentage of inhibition of steady-state [123I]iomazenil brain radioactivity having subtracted nonspecific binding.

### Baboon [11C]Flumazenil PET

The baboon [11C]flumazenil PET studies were conducted as part of a series of experiments to evaluate the abuse potential of TPA023 (Ator et al., 2009). During the course of these PET scans, blood samples were collected and centrifuged, and plasma was removed and stored before subsequent bioanalysis of drug concentrations.

### Human PET Imaging Studies


[11C]Flumazenil was synthesized in the Laboratory of Nuclear Chemistry at Johns Hopkins Medical Institutions (Baltimore, MD) by N-alkylation of Ro 15-5528, the desmethyl precursor of flumazenil (ABX GmbH, Dresden, Germany). In brief, [11C]carbon dioxide was produced by the 14N(p, α)11C reaction using a nitrogen gas target containing oxygen. Irradiation with 17-MeV protons, produced by the General Electric PETTrace cyclotron at the Johns Hopkins PET Center (Baltimore, MD), gave 11C in the form of [11C]carbon dioxide. After bombardment, the [11C]carbon dioxide was swept at a flow rate of 100 mL/min under nitrogen gas pressure to a hot cell and collected on activated molecular sieves in a PETTrace Me1 MicroLab (General Electric Medical Systems, Uppsala, Sweden) and then immediately reduced in the presence of a nickel catalyst and hydrogen gas to [11C]methane. The [11C]methane was converted to [11C]methyl iodide by recirculation across heated molecular iodine collected on a Porapak Q column (Sigma-Aldrich, St. Louis, MO). After complete collection, the column was heated to release the [11C]methyl iodide under a stream of helium that was then trapped in a cooled (~78°C) solution of the precursor (Ro 15-5528) dissolved in dimethyl formamide whereupon aqueous tetrabutylammonium hydroxide (0.4 M) was added, and the reaction mixture was heated for 2 min. After dilution with high-performance liquid chromatography buffer, the solution was injected onto a semipreparative high-performance liquid chromatograph, and the appropriate fraction was collected and transferred remotely to a rotary evaporator to remove the solvent. The residue was redissolved in sterile normal saline (7 mL), and the solution passed through a 0.22-μm filter into a sterile evacuated vial. After the addition of 3 mL of sterile 8.4% sodium bicarbonate, a sample was taken for determination of identity, radiochemical and chemical purity, pH, and assessment of specific radioactivity. Radiochemical purity and specific activity for each batch was >90% and >1000 Ci/mmol, respectively.

#### Imaging Studies.

This was a double-blind, single oral dose, randomized, placebo-controlled study in six healthy subjects (five male, one female; average age, 34 years). The ethics committee of Johns Hopkins University approved the protocol, and each volunteer gave written informed consent. For each participant studies were conducted in one ~10-h day after fasting (except for water) from midnight of the preceding day. Subjects were cannulated under local anesthesia in a radial artery for blood sampling and also had a venous cannula inserted for [11C]flumazenil injection, and three scans were performed commencing 1 h before or 1.5 and 6 h after receiving a single oral dose of either placebo (n = 1), TPA023 (2 mg; n = 3), or lorazepam (2 mg; n = 2). Scans were performed on a GE Advance PET scanner (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and were initiated by the injection of [11C]flumazenil (~15 mL), with 24 scans being acquired over a 1-h period (4 × 0.25 min, 4 × 0.5 min, 3 × 1 min, 2 × 2 min, 5 × 4 min, and 6 × 5 min). The measurement of the arterial plasma concentration of [11C]flumazenil and its labeled metabolites during the scan allowed a metabolite-corrected arterial input function for the tracer to be generated (data on file at Johns Hopkins University PET Center). Venous plasma samples were collected at 0.5, 1.5, 2, 2.5, 3, 4, 5, 6, 7, and 7.5 h after dosing for determination of plasma levels of TPA023 using liquid chromatography-tandem mass spectrometry.

#### Human Brain Image Analysis

##### Generation of Regions of Interest.

Before the PET scans, standard noncontrast magnetic resonance images were obtained for all subjects, and these were aligned with [11C]flumazenil PET images from the first scan to allow the manual delineation of regions of interest on the occipital cortex, frontal cortex, cerebellum, and pons. Images from the second and third PET scans were aligned with the first study to obtain the same regions of interest in all studies and from these aligned images time-activity curves were generated.

##### Modeling and Occupancy Calculation.

The [11C]flumazenil time-activity curves were used together with the metabolite-corrected plasma curve obtained from blood sampling during the corresponding scan to calculate the in vivo ligand binding of [11C]flumazenil to the benzodiazepine site of GABA_A receptors using a one-tissue linear compartmental model (Koeppe et al., 1991). This permitted an estimation of the rate constant for forward capillary exchange (K_1), the volume of distribution of flumazenil (V_D), and the blood volume. V_D is proportional to the ratio of the number of benzodiazepine binding sites and the equilibrium dissociation constant, B_max/K_D (and can be estimated independent of any flow changes, which will be reflected in changes of K_1).

\[ V_D = \frac{K_1}{K_2} \left(1 + \frac{B_{max}}{K_D}\right) + \lambda \]  

where \(B_{max}\) is the density of benzodiazepine binding sites available for binding, \(K_D\) is the equilibrium dissociation rate constant, and \(\lambda\) corresponds to the nonreceptor-bound volume of distribution. The \(\lambda\) value for [11C]flumazenil may be considered as constant in all gray matter regions and also in different subjects.

Comparison of the \(V_D\) values obtained from the three [11C]flumazenil scans were used to estimate receptor occupancy at the studied times and percentage of receptor occupancy at \(T = 2\) and 6.5 h after drug administration where calculated from eq. 1 using \(\lambda = 0.8\) in eq. 2 as follows:

\[ \text{Occupancy}_{TV} = \frac{\text{DV}_{\text{baseline}} - \text{DV}_{TV}}{\text{DV}_{\text{baseline}} - \lambda} \times 100\% \]  

#### Results

**Rat [3H]Flumazenil In Vivo Binding and Plasma Pharmacokinetics of TPA023.** The occupancy by TPA023 of GABA_A receptor benzodiazepine binding sites in rat brain was clearly time- and dose-dependent (Fig. 1). Hence, at all three doses, maximum occupancy was observed 0.75 h after dosing, with respective occupancies at 1, 3, and 30 mg/kg being 74 ± 3, 89 ± 1, and 97 ± 0% and the extrapolated Occupancy...
Fig. 1. Occupancy of rat brain GABA<sub>A</sub> receptor benzodiazepine sites and corresponding plasma concentrations after oral dosing of TPA023. A, occupancy of rat brain GABA<sub>A</sub> receptor benzodiazepine binding sites was measured as the extent by which the in vivo binding of [3H]flumazenil was reduced by dosing of TPA023 (1, 3, or 10 mg/kg p.o. in 0.5% methyl cellulose vehicle). B, trunk blood was collected from animals used for the occupancy study, and plasma concentrations were determined. Values shown are mean ± S.E.M. (n = 5–10/group).

Fig. 2. Rat plasma TPA023 concentrations and cortex time-radioactivity curves measured using [11C]flumazenil microPET. A, mean (± S.E.M.; n = 3) plasma TPA023 concentration during the low-dose (10–26-min) and high-dose (26–52-min) phases of the study. B, cortex time-activity curves in a representative vehicle- and TPA023-treated rat. Arrows indicate injections of the low dose (0.05 mg/kg i.v.; t = 10 min) and high dose (0.2 mg/kg i.v.; t = 26 min) of TPA023 as well as a dose of flumazenil (bu.; 0.25 mg/kg i.v.) sufficient to block all binding sites and therefore define nonspecific binding. Nonspecific binding values before t = 52 min were estimated through backward exponential extrapolation. C, mean (± S.E.M.; n = 3) TPA023 occupancy derived from the 15- to 25-min and 36- to 46-min periods of the low- and high-dose periods of the experiment.

Fig. 3. Displacement by TPA023 of [123I]iomazenil radioactivity from rhesus monkey brain as measured using γ-scintigraphy. Two hours after the bolus/infusion of [123I]iomazenil, TPA023 was infused at rates of 0.01, 0.025, and 0.125 mg/kg/h [vehicle, EtOH/PEG 400/water in a ratio of 1:3:6 (v/v)]. The extent of the displacement of [123I]iomazenil by TPA023 (i.e., the occupancy of benzodiazepine binding sites) was measured, and plasma samples were removed once relative steady-state conditions had been achieved (i.e., after 3 h of TPA023 infusion). Nonspecific binding was estimated by injection of flumazenil (1 mg/kg in EtOH/PEG/water) at t = 5 h. Data shown are three separate experiments performed using the same monkey.

Being 0.29 mg/kg, thereforer, occupancy at 1- and 3-mg/kg doses dropped rapidly, being 34 ± 5 and 54 ± 2% 3 h after dosing. In contrast, occupancy at 10 mg/kg was more sustained such that at 6 h after dose, 72 ± 10% occupancy remained but this decreased to only 12 ± 7% after 18 h. Measurement of plasma TPA023 concentrations showed that there was rapid absorption of compound after oral dosing, with C<sub>max</sub> being achieved at 0.75 h at each dose. Moreover, peak plasma concentrations were roughly dose-proportional in that doses of 1, 3, and 10 mg/kg resulted in C<sub>max</sub> values of 48 ± 9, 131 ± 15, and 430 ± 41 ng/ml, respectively.

Rhesus Monkey [123I]Iomazenil γ-Scintigraphy. Figure 3 shows the displacement of [123I]iomazenil binding in the rhesus monkey brain produced by infusions of 0.01, 0.025, and 0.125 mg/kg/h TPA023. These doses produced respective occupancies of 20, 72, and 97%, respectively.
**Human PET.** Figure 4 shows the images of $[11C]$flumazenil binding in all subjects and illustrates the widespread distribution of benzodiazepine binding sites. The time-activity curves for single representative subjects given either placebo, lorazepam (2 mg; lorazepam subject 1), or TPA023 (2 mg; TPA023 subject 1) and receiving $[11C]$flumazenil injections before or 1.5 or 6 h after dosing (scans 1, 2, and 3, respectively) are shown in Fig. 5. The time-activity curves for the subject given placebo were very reproducible. Furthermore, subjects given lorazepam had time-activity curves 1.5 to 2.5 and 6 to 7 h after treatment (scans 2 and 3, respectively) that were very similar to the baseline scan before lorazepam treatment (scan 1), suggesting that lorazepam produced levels of occupancy that were below the reliable level of detection using PET ($<10\%$). In contrast, 2 mg of TPA023 produced a marked alteration in the time-activity curve, indicative of a reduction of $[11C]$flumazenil binding due to the occupancy of benzodiazepine binding sites by TPA023. When averaged across brain regions for the three subjects, the mean occupancies 2 and 6.5 h after oral administration of TPA023 were $58 \pm 6$ and $46 \pm 7\%$, respectively, with the estimated $\text{Occ}_{50}$ value ($t = 2$ h) being a total dose of 1.5 mg (or $\sim 0.02$ mg/kg).

**Plasma-Occupancy Relationships.** For each species, the occupancy data obtained were plotted as a function of plasma TPA023 concentrations and these data are presented in Fig. 6 and the plasma TPA023 concentrations required to produce 50% occupancy (the EC$_{50}$) values, and their associated 95% confidence intervals, are summarized in Table 1. The data used for the rat $[3H]$flumazenil in vivo binding analysis were derived from the occupancy time course experiment (Fig. 1). This permitted the plasma-occupancy relationship to be measured at various times after dosing, and but there was no tendency for the plasma-occupancy relationship to vary as a function of time with EC$_{50}$ values, 0.75, 1.5, 3, and 6 h after dose, being 27, 17, 25, and 23 ng/ml, respectively. Accordingly, all the data from this experiment were pooled for the analysis shown in Fig. 6A. These data clearly show that in preclinical species and despite the method of radioactivity measurement (ex vivo in brain homogenates or in vivo using PET or single photon emission computed tomography) or the choice of radioligand ($[3H]$flumazenil, $[11C]$flumazenil, or $[123I]$iomazenil), the EC$_{50}$ values are relatively comparable across species, ranging from 19 mg/ml in baboons to 30 ng/ml in rhesus monkeys, with the rat EC$_{50}$ value being, depending on the methodology, 21 to 25 ng/ml (Table 1).

**Discussion**

In rats, the plasma EC$_{50}$ value was essentially the same at different times between 0.75 and 6 h after dose and ranged from 17 to 27 ng/ml. These data suggest that TPA023 did not have a slow off-rate (i.e., did not have prolonged occupancy), in which case the EC$_{50}$ value would decrease as a function of time. In this regard, TPA023, with an affinity of 0.2 to 0.4

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**Fig. 4.** Pseudocolored images of horizontal sections of the brain of subjects receiving either placebo, lorazepam (2 mg), or TPA023 (2 mg), illustrating the uptake of $[11C]$flumazenil into the brain. Images were acquired over the duration of each 60-min scan. Figures associated with the 2- and 6.5-h scans represent the average reduction in cumulative radioactivity relative to each subject’s baseline scan.

**Fig. 5.** Representative time-activity curves for the uptake of $[11C]$flumazenil into occipital cortex of subjects treated orally with placebo, lorazepam (2 mg), and TPA023 (2 mg). Scans 1, 2, and 3 refer to the periods 1.5 to 0.5 h before dosing (baseline) and 1.5 to 2.5 and 6 to 7 h after dosing, respectively.
nM, is comparable with flumazenil itself, which, with a comparable affinity (0.5–1.5 nM) (Atack et al., 2005), clearly has a rapid rate of dissociation in vivo as evidenced by the fact that not only is flumazenil cleared rapidly from the rat brain with a half-life of 16 min (Lister et al., 1984) but also the displacement of the in vivo binding of [11C]flumazenil binding occurs rapidly in rats (Fig. 2) and also baboons and humans (Figs. 4 and 5). In addition, the GABA_A α5 subtype-selective triazolophthalazine 3-(5-methylisoxazol-3-yl)-6-[(1-methyl-1,2,3-triazol-4-yl)methoxy]-1,2,4-triazolo[3,4-a]phthalazine (α51A), which is structurally related to TPA023, also showed no tendency for a slow off-rate since the plasma EC_{50} was, like TPA023, time-independent (Atack et al., 2009a).

The similarity of the EC_{50} values obtained in rat using either [3H]flumazenil in vivo binding or [11C]flumazenil microPET (respective EC_{50} values = 25 and 21 ng/ml) suggests that despite the marked methodological differences between these techniques, they produce similar results, consistent with analogous data obtained for lorazepam (Atack et al., 2007). Furthermore, the comparable EC_{50} values for rhesus monkey and baboon (30 and 19 ng/ml) obtained by [123I]iomazenil γ-scintigraphy and [11C]flumazenil PET, respectively, indicate that these differing radioligands and imaging modalities produce similar results, although others have suggested that these ligands have different in vivo properties (Hosoi et al., 1999).

Studies using [11C]flumazenil PET in humans have shown that a dose of clonazepam that produces sleep and ataxia (0.03 mg/kg p.o.) occupies 15 to 23% BZ sites (Shinotoh et al., 1989), whereas sedative doses of diazepam (30 mg p.o.) and alprazolam (0.5 mg every 6 h) have respective occupancies of 24 and 16% (Pauli et al., 1991; Fujita et al., 1999). In addition, the usual clinical dose of zolpidem (10 mg) is predicted to correspond to 15% occupancy based on the fact that a dose of clonazepam that produces sleep and ataxia (0.3 mg/kg p.o.) occupies 21 ng/ml. Data shown are from three TPA023-treated subjects, each of which generated two data points (i.e., measurements 2 and 6.5 h after dose). Plasma drug concentrations were the average of those at the start and end of each 60-min acquisition period (1.5–2.5 and 6–7 h after dose). See Table 1 for a summary of plasma concentrations of TPA023 required for 50% occupancy in different preclinical species and humans.

**Table 1**

<table>
<thead>
<tr>
<th>Species</th>
<th>Assay</th>
<th>EC_{50} (Hill Slope)</th>
<th>95% CI</th>
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<tr>
<td>Rat</td>
<td>[3H]flumazenil</td>
<td>25 (1.2)</td>
<td>23–26</td>
</tr>
<tr>
<td>Rat</td>
<td>[11C]flumazenil</td>
<td>21 (2.1)</td>
<td>18–25</td>
</tr>
<tr>
<td>Rhesus</td>
<td>[123I]iomazenil γ-scintigraphy</td>
<td>30 (1.5)</td>
<td>17–53</td>
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<tr>
<td>Baboon</td>
<td>[11C]flumazenil PET</td>
<td>19 (1.6)</td>
<td>16–22</td>
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<tr>
<td>Human</td>
<td>[11C]flumazenil PET</td>
<td>9 (1.0)</td>
<td>7–12</td>
</tr>
</tbody>
</table>

* 95% CI, 95% confidence interval.

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**Fig. 6.** Plasma-occupancy relationship for TPA023 across species. A, occupancy in rat brain as measured using [3H]flumazenil in vivo binding. Data from each animal used in the time course study (see Fig. 1) was plotted as a function of the corresponding plasma TPA023 concentration. These data were fit with a single-site model that gave an EC_{50} value of 25 ng/ml (Hill slope, 1.17; n = 104). B, rat brain occupancy measured using [11C]flumazenil microPET. EC_{50} = 21 ng/ml. C, occupancy in rhesus monkey brain measured using [123I]iomazenil γ-scintigraphy. Plasma drug concentrations were measured once steady-state levels had been achieved (i.e., 3 h after initiation of TPA023 infusion). EC_{50} = 30 ng/ml. D, baboon brain occupancy as measured using [11C]flumazenil PET. EC_{50} = 19 ng/ml. E, plasma-occupancy curve for TPA023 in humans. Occupancy was measured as the extent to which uptake of [11C]flumazenil PET (calculated from the time-activity curves) was reduced relative to baseline 2 and 6.5 h after a single dose of TPA023 (2 mg). Plasma drug concentrations were the average of those at the start and end of each 60-min acquisition period (1.5–2.5 and 6–7 h after dose). Data shown are from three TPA023-treated subjects, each of which generated two data points (i.e., measurements 2 and 6.5 h after dose). F, comparison of plasma-occupancy curves across species.
respectively (Malizia et al., 1996). It is interesting to note that comparable data (7 and 39% occupancy at the low and high dose) were obtained using total head counts (Malizia et al., 1996), suggesting that nontomographic methods, which require lower levels of radioactivity compared with tomographic techniques, may be used to determine levels of occupancy in humans (Malizia et al., 1995). In a \([123I]\)lomazenil single photon emission computed tomography study, a sleep-inducing, steady-state infusion of midazolam (6 mg/h) gave cortical occupancy in the region of 20 to 30% (Videbaek et al., 1993), which is comparable with \([11C]\)flumazenil data (Malizia et al., 1996). Together taken, these data clearly demonstrate that for classical benzodiazepines (clonazepam, diazepam, alprazolam, and midazolam) as well as the \(\alpha_1\)-subtype preferring drug zolpidem, occupancies of >15 to 30% are associated with sedation or sleep. Pagoclone (0.4 mg) gives occupancy of 11 to 15% in humans with a reduced effect on saccadic eye movements relative to lorazepam (Lingford-Hughes et al., 2005), and although it is unclear to what extent this occupancy is related to parent compound or the pharmacologically active metabolite 5’-hydroxy pagoclone (Atack et al., 2006a), these data are consistent with an in vivo partial agonist pharmacological profile (Lingford-Hughes et al., 2005).

In contrast to the classical benzodiazepines, a 2-mg dose of TPA023 produced occupancy in the region of 50 to 60% yet showed no overt sedative-like effects, suggesting that it is the intrinsic efficacy of this compound rather than a lack of sufficient occupancy that accounts for the lack of sedation seen in humans. These data are consistent with the lack of efficacy of TPA023 at \(\alpha_1\)-containing receptors, the subtype of GABA\(_A\) receptor responsible for the sedative effects of diazepam (Rudolph et al., 1999; McKernan et al., 2000) and are in agreement with the pharmacodynamic effects of TPA023, which showed that doses of 0.5 and 1.5 mg did not produce the sedation-like effects (e.g., increased body sway, decreased alertness measured using a visual analog scale) observed with lorazepam (de Haas et al., 2007). Similarly, data with the \(\alpha_2/\alpha_3\)-selective compound TPA023B, which is an imidazotriazine follow-up compound to TPA023, showed that levels of occupancy in the region of 50% could be achieved in the absence of overt sedation (Van Laere et al., 2008; Atack et al., 2009b). However, in contrast to the relatively high levels of occupancy achieved in human TPA023 and TPA023B in the absence of overt sedation, MRK-409 (also known as MK-0343) (de Haas et al., 2008), produced sedation and somnolence at doses of 1.5 and 2 mg, with occupancy at the maximal tolerated dose of 1 mg being below the levels of detection (\(<10\%\)) (Atack et al., 2009c). The sedation produced by MRK-409 was attributed to the weak partial agonist efficacy that this compound had at the \(\alpha_1\) subtype and clearly demonstrates the value of the measurement of receptor occupancy in interpreting clinical data (Atack et al., 2009c).

The fact that a 2-mg dose of lorazepam produced occupancy that was below the limits of detection (i.e., \(<10\%\)) in the human PET study is consistent with previous studies showing that therapeutically relevant (anxiolytic) doses of lorazepam produce levels of occupancy of \(<3\%\) (Sybirska et al., 1993) or, depending on the brain region, 6 to 9% (1 mg) (Lingford-Hughes et al., 2005). The maximal plasma concentrations of lorazepam after a single 2-mg dose are in the region of 20 ng/ml (Greenblatt et al., 1988; de Haas et al., 2007), and a similar estimated concentration of TPA023, \(~20\ ng/ml\), is achieved after a 2-mg dose of TPA023 (calculated by extrapolating from plasma pharmacokinetics of single doses of 0.5 and 1.5 mg of TPA023 (respective \(C_{\text{max}}\) values \(\sim 5\) and \(\sim 15\ ng/ml\)) (de Haas et al., 2007). However, despite 2-mg doses of TPA023 and lorazepam achieving comparable plasma concentrations (20 ng/ml), TPA023 gave much higher levels of occupancy in humans compared with lorazepam (50–60% versus \(<10\%\)). This is consistent with the lorazepam plasma \(E_{\text{C50}}\) value in rats (90–130 ng/ml; lorazepam microPET data) being greater than for TPA023 (21–25 ng/ml). Since the binding of TPA023 and lorazepam to human plasma protein are comparable at around 90% (Greenblatt, 1981), the greater potency of TPA023 relative to lorazepam is presumably related to the higher affinity of TPA023 for the benzodiazepine binding site (0.2–0.4 nM) (Atack et al., 2006b) compared with lorazepam (2–6 nM; J. Atack, unpublished observations).

The high affinity of TPA023 also presumably accounts for why effects of TPA023 on saccadic eye movement peak velocity were seen at doses of 0.5 and 1.5 mg (de Haas et al., 2007) that were an order of magnitude less than the minimally effective dose of 25 mg required for the lower affinity, \(\alpha_2/\alpha_3\)-preferring compound SL65.1498 (de Haas et al., 2009). Hence, the affinity of TPA023 for the different subtypes of GABA\(_A\) receptors ranges from 0.2 to 0.4 nM, whereas SL65.1498 has affinity at the \(\alpha_1\), \(\alpha_2\), and \(\alpha_3\) subtypes, ranging from 17 to 80 nM, with even lower affinity (215 nM) at the \(\alpha_5\) subtype (Griebel et al., 2001). The \(C_{\text{max}}\) values for the 0.5- and 1.5-mg doses of TPA023 were 5 and 13 ng/ml, respectively (de Haas et al., 2007), whereas respective values for the 2.5-, 7.5-, and 25-mg doses of SL65.1498 were 37, 126, and 375 ng/ml, respectively (de Haas et al., 2009). Accordingly, the plasma drug concentrations required for effects on saccadic eye movement peak velocity for TPA023 and SL65.1498 were 5 and 375 ng/ml, respectively, again emphasizing the relative potency of TPA023. However, in the absence of receptor occupancy data, it is difficult to interpret the relatively modest pharmacodynamic effects of SL65.1498 seen at the highest dose tested (25 mg).

The plasma-occupancy relationship for TPA023 in humans is slightly more potent than in preclinical species (\(E_{\text{C50}}\) value in humans is 9 ng/ml versus 19–30 ng/ml in preclinical species). Likewise, the structurally related compound \(\alpha_51A\) was also more potent in humans compared with preclinical species (respective \(E_{\text{C50}}\) values of 10 ng/ml versus 52–57 ng/ml) (Atack et al., 2009a) as was the imidazotriazine TPA023B (\(E_{\text{C50}}\) values of 19, 10, and 5.8 ng/ml in rats, baboons, and humans, respectively) (Atack et al., 2009b). In contrast, lorazepam seems to have a similar potency in rats and humans, albeit that the low levels of occupancy achieved in humans limit the reliability of such a comparison. Nevertheless, the relatively low levels of occupancy of 6 to 9% produced by 1 mg of lorazepam (Lingford-Hughes et al., 2005) occur, assuming dose-proportional plasma drug concentrations in humans (Greenblatt et al., 1988; de Haas et al., 2007), at a plasma concentration of approximately 10 ng/ml, and this is comparable with the \(<10\%\) occupancy produced at a concentration of 10 ng/ml in rats (Atack et al., 2007). The greater potency of TPA023 in humans compared with lorazepam is also reflected in comparison with other benzo-
In summary, the occupancy of brain benzodiazepine binding sites was measured in several preclinical species and humans using a variety of methods and was related to plasma drug concentrations. Despite the different detection methods (ex vivo or in vivo PET or γ-scintigraphy) and radioligands ([3H]flumazenil, [11C]flumazenil, or [123I]iomazenil) used, the plasma TPA023 EC50 was relatively constant in preclinical species, ranging from 19 to 30 ng/ml, whereas TPA023 was slightly more potent in humans, with an EC50 value of 9 ng/ml. This latter EC50 value is much more potent in terms of receptor occupancy than existing benzodiazepine site ligands for which comparable data are available (lorazepam, zolpidem, and alprazolam) and suggests that despite TPA023 being an α2/3-selective GABA_A receptor partial agonist, the higher levels of occupancy required for such a compound to have putative clinical efficacy relative to non-selective full agonists should be readily achievable with comparatively small doses. More generally, the present study clearly demonstrates that receptor occupancy is a translational marker that can be applied across species and aids the interpretation not only of preclinical (Atack et al., 2006b; Ator et al., 2009) but also clinical data (Atack et al., 2009b,c) and that for TPA023 clearly emphasize the fact that GABA_A α2/3 subtype-selective compounds have novel pharmacological profiles.

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