Original Paper

Preclinical and clinical pharmacology of TPA023B, a $GABA_A$ receptor α2/α3 subtype-selective partial agonist

Psychopharm

Journal of Psychopharmacology
25(3) 329–344

DOI: 10.1177/0269881109354928
jop.sagepub.com
SAGE

JR Atack[1], DJ Hallett[1], S Tye[1], KA Wafford[1], C Ryan[2], SM Sanabria-Bohórquez[2], Wai-si Eng[2], RE Gibson[2], HD Burns[2], GR Dawson[1], RW Carling[1], LJ Street[1], A Pike[1], I De Lepeleire[3], K Van Laere[4], G Bormans[4], JN de Hoon[5], A Van Hecken[5], RM McKernan[1], MG Murphy[6] and RJ Hargreaves[2]

Abstract

In the accompanying paper we describe how MRK-409 unexpectedly produced sedation in man at relatively low levels of $GABA_A$ receptor occupancy (~10%). Since it was not clear whether this sedation was mediated via the α2/α3 or α1 $GABA_A$ subtype(s), we characterized the properties of TPA023B, a high-affinity imidazotriazine which, like MRK-409, has partial agonist efficacy at the α2 and α3 subtype but is an antagonist at the α1 subtype, at which MRK-409 has weak partial agonism. TPA023B gave dose- and time-dependent occupancy of rat brain $GABA_A$ receptors as measured using an in vivo [^{3}H]flumazenil binding assay, with 50% occupancy corresponding to a respective dose and plasma drug concentration of 0.09 mg/kg and 19 ng/mL, the latter of which was similar to that observed in mice (25 ng/mL) and comparable to values obtained in baboon and man using [^{11}C]flumazenil PET (10 and 5.8 ng/mL, respectively). TPA023B was anxiolytic in rodent and primate (squirrel monkey) models of anxiety (elevated plus maze, fear-potentiated startle, conditioned suppression of drinking, conditioned emotional response) yet had no significant effects in rodent or primate assays of ataxia and/or myorelaxation (rotarod, chain-pulling, lever pressing), up to doses (10 mg/kg) corresponding to occupancy of greater than 99%. In man, TPA023B was well tolerated at a dose (1.5 mg) that produced occupancy of >50%, suggesting that the sedation previously seen with MRK-409 is due to the partial agonist efficacy of that compound at the α1 subtype, and highlighting the importance of antagonist efficacy at this particular $GABA_A$ receptor population for avoiding sedation in man.

Keywords

benzodiazepine, $GABA_A$ receptor, non-sedating anxiolytic, subtype-selective

Introduction

The $GABA_A$ receptor is a pentamer made up of different subunits from the $GABA_A$ receptor gene family (α1-6, β1-3, γ1-3, δ, ε, θ, π) (Barnard et al., 1998; Simon et al., 2004). The most common composition of native $GABA_A$ receptors is the αβγ configuration, with a stoichiometry of 2:2:1 arranged in a αβαβγ sequence as viewed from the synapse (Sieghart and Sperk, 2002; Sieghart, 2006). In addition to GABA recognition sites, the $GABA_A$ receptor also possesses binding sites for a number of distinct classes of compounds such as ethanol, certain volatile anaesthetics, neurosteroids, barbiturates and benzodiazepines (Sieghart, 1995, 2006). Of these, the benzodiazepines have attracted most attention based upon their clinical utility as anxiolytic, hypnotic, anticonvulsant (although not for prophylactic use), myorelaxant and cognition-impairing agents. Thus, the benzodiazepine binding site is well described as occurring at the interface of the α1, α2, α3 or α5 (but not α4 or α6) and γ2 subunits (Sigel, 2002; Wieland et al., 1992). Since $GABA_A$ receptors expressing these different α subunits have distinct neuroanatomical distributions (Pirker et al., 2000), it has been proposed that particular subtypes of the $GABA_A$ receptor are responsible for specific physiological functions as well as distinct components of benzodiazepine pharmacology (Möhler, 2007; Möhler et al., 2002). For example, and based upon the use of subtype-selective compounds as well as molecular genetic approaches,

[1]Neuroscience Research Centre, Merck Sharp & Dohme Research Laboratories, Harlow, UK.
[2]Imaging Research and Pharmacology, Merck Research Laboratories, West Point, PA, USA.
[3]Clinical Pharmacology, Merck Sharp & Dohme Research Laboratories, Brussels, Belgium.
[4]Department of Nuclear Medicine and Radiopharmacy, Katholieke Universiteit Leuven, Leuven, Belgium.
[5]Department of Clinical Pharmacology, Katholieke Universiteit Leuven, Leuven, Belgium.
[6]Clinical Pharmacology, Merck Research Laboratories, Bluebell, PA, USA.

Corresponding author:
Dr John R Atack, Neuroscience, Johnson & Johnson Pharmaceutical Research and Development, Turnhoutseweg 30, B-2340 Beerse, Belgium
Email: JAtack1@its.jnj.com

it appears that the α1 subtype is responsible for the sedative properties of benzodiazepines (McKernan et al., 2000; Rudolph et al., 1999; Sanger, 2004), whereas features of the cognition-modulating effects of not only benzodiazepine site ligands but also the anaesthetic etomidate are probably mediated via the α5 subtype (Ballard et al., 2009; Cheng et al., 2006; Crestani et al., 2002; Dawson et al., 2006). On the other hand, the anxiolytic properties of benzodiazepines appear to be associated with α2- and/or α3-containing receptors (Atack et al., 2005, 2006; Dias et al., 2005; Löw et al., 2000; Morris et al., 2006).

The observations that the α1-containing receptors are the 'sedative' subtype whereas the α2/α3 receptors are the 'anxiolytic' subtypes provides the conceptual framework for the hypothesis that a compound which possesses agonist efficacy at the α2 and/or α3 subtypes, yet has less efficacy at the α1 subtype, should prove to be a non-sedating anxiolytic (Atack, 2005). We have previously described compounds from the triazolopyridazine series, L-838417, TPA023 and MRK-409, as compounds that are non-sedating anxiolytics in preclinical species (Atack et al., 2006, 2010; McKernan et al., 2000). However, in man, TPA023 had minimal sedating effects at relatively high (up to 65%) receptor occupancy, whereas MRK-409 (also known as MK-0343) produced sedating effects at relatively low levels of occupancy (Atack, 2009; de Haas et al., 2007, 2008). Aside from the fact that the sedating effects of MRK-409 were not predicted in preclinical species, it remains uncertain as to whether MRK-409 causes sedation in man due to its higher α2/α3 efficacy compared with TPA023 or rather that the low level of α1 agonism in MRK-409 is, nevertheless, sufficient to produce sedation in man (cf. TPA023, which has no efficacy at the α1 subtype; Atack et al., 2006). In the present studies, we describe the properties of a compound that was developed to address this issue. Thus, TPA023B is an imidazotriazine (6,2′-difluoro-5′-[3-(1-hydroxy-1-methylethyl)imidazo[1,2-*b*][1,2,4]triazin-7-yl]biphenyl-2-carbonitrile; Figure 1), which has an efficacy profile that is a hybrid of TPA023 and MRK-409. More specifically (and like TPA023), TPA023B has no efficacy (i.e. it is an antagonist) at the benzodiazepine site of α1-containing $GABA_A$ receptors but has a partial agonist efficacy at the α2/α3 subtypes that is similar to MRK-409. In preclinical species, the non-sedating anxiolytic-like properties of TPA023B were characterized, and in man the safety, tolerability, pharmacokinetics and receptor occupancy were assessed. These collective data are then discussed in comparison with TPA023 and MRK-409.

Materials and methods

Animal procedures were all performed in accordance with the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines. Baboon positron emission tomography (PET) studies were conducted under the guiding principles of the American Physiological Society and the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health.

Drugs

TPA023B was synthesized as described elsewhere (Compound 11; Russell et al., 2006) with supplies for the clinical studies being made using a modified, large-scale synthetic route (Gauthier et al., 2005). Diazepam and chlordiazepoxide were purchased from Sigma-Aldrich (Gillingham, UK) and bretazenil was synthesized at Merck Research Laboratories (Harlow, UK). [^{3}H]Flumazenil ([^{3}H]Ro 15-1788) and [^{3}H]Ro 15-4513 were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA).

In vitro affinity and efficacy

The affinity and efficacy of TPA023B at the benzodiazepine site of human recombinant $GABA_A$ receptors stably expressed in mouse fibroblast L(tk-) cells were measured as described in more detail previously (Atack et al., 2006). In brief, the affinity of TPA023B for $GABA_A$ receptors containing a β3, a γ2 and either an α1, α2, α3 or α5 subunit was determined using a [^{3}H]flumazenil binding assay, whereas the affinity for corresponding receptors containing an α4 or α6 subunit was assessed using [^{3}H]Ro 15-4513. Non-specific binding was defined using 10 μM flunitrazepam and 10 μM Ro 15-4513 for the [^{3}H]flumazenil and [^{3}H]Ro 15-4513 assays, respectively (Atack et al., 2006).

The intrinsic efficacy of TPA023B was measured using whole cell patch-clamping of α1, α2, α3 and α5-containing human recombinant $GABA_A$ receptors stably expressed in the same system as used for the radioligand binding assays (Atack et al., 2006). Hence, the ability of TPA023B (pre-applied for 30 sec) to alter the current produced by a 5 sec

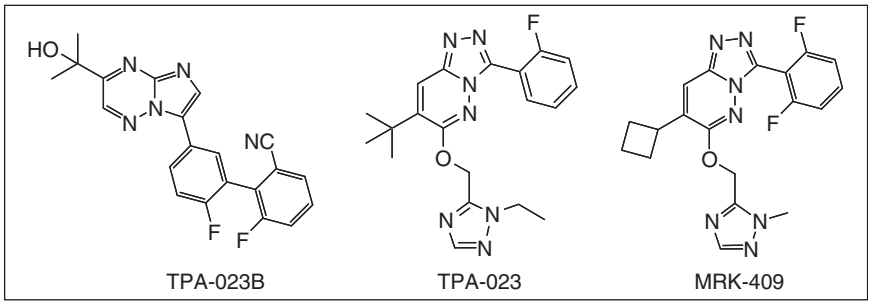

Figure 1. Comparison of the structures of TPA023B (6,2′-difluoro-5′-[3-(1-hydroxy-1-methylethyl)imidazo[1,2-*b*][1,2,4]triazin-7-yl]biphenyl-2-carbonitrile; Compound 11, Russell et al., 2006), TPA023 (Atack et al., 2006) and MRK-409 (Atack et al., 2010).

application of a GABA concentration that produced an effect that was 20% of the maximal (a GABA EC_{20} concentration) was measured. The peak current amplitude was quantified, with an enhancement of the GABA EC_{20} peak amplitude representing an agonist effect at the benzodiazepine binding site, whereas a reduction in the GABA EC_{20} current represents inverse agonism and no effect on the EC_{20} was classified as antagonist efficacy.

Similar patch clamp methodology was used to assess the efficacy of TPA023B at recombinant rat $GABA_A$ receptors expressed in human embryonic kidney (HEK) 293 cells transiently transfected with rat β3 and γ2 plus either α1 or α3 cDNA (6 μg of cDNA total per cover-slip) or native rat $GABA_A$ receptors in primary cultures of rat dorsal root ganglion neurons with recordings being made from the large-diameter neurons that contain primarily α2 and α3 subunit-containing $GABA_A$ receptors. Data analyses were performed as described above.

Finally, the efficacy of TPA023B was measured at human recombinant $GABA_A$ receptors expressed in the same cell lines as described above using a ^{36}Cl flux assay (Smith et al., 2001). In this 96-well plate assay, the enhancement of a GABA EC_{20}-induced uptake of ^{36}Cl into whole cells is measured and expressed relative to the enhancement produced by the non-selective full agonist chlordiazepoxide.

TPA023B $GABA_A$ receptor occupancy

The occupancy of the benzodiazepine binding site of rat (male 250–330 g Sprague-Dawley; B&K Universal, Hull, UK) and mouse (male 26–30 g Swiss-Webster; B&K Universal) brain $GABA_A$ receptors following p.o. dosing with TPA023B was measured using a [^{3}H]flumazenil in vivo binding assay (Atack et al., 2006). Rat occupancy studies were performed following p.o. dosing (1 mL/kg in 0.5% methyl cellulose vehicle) with different doses of TPA023B given with a pretreatment time of 0.75 h (0.1, 0.3, 1 or 3 mg/kg) or 8 h (0.03, 0.1, 0.3 mg/kg) or alternatively, fixed dose (0.3 mg/kg) and different pretreatment times (0.5, 1, 2, 4, 8 and 24 h). In addition, occupancy was measured following completion of the elevated plus maze trial (0.1, 0.3 and 1 mg/kg, pretreatment time prior to the 5 min plus maze trial = 0.5 h). Mouse brain occupancy was determined immediately after rotarod experiments using oral doses of 0.3, 1, 3 or 10 mg/kg TPA023B (10 mL/kg in 0.5% methyl cellulose with 0.5 h pretreatment time), in both the absence and the presence of co-administered ethanol (see below). Occupancy was also measured in animals used as positive controls in the rat elevated plus maze (chlordiazepoxide, 5 mg/kg i.p.) and mouse rotarod (diazepam, 3 or 10 mg/kg p.o.) and in animals pretreated for 0.5 h with bretazenil (5 mg/kg i.p. in 100% polyethylene glycol) in order to define the extent of non-specific in vivo binding of [^{3}H]flumazenil.

Animals pretreated with either vehicle or drug were given an i.v. injection of [^{3}H]flumazenil (70–88 Ci/mmol diluted 1:150 with saline; 5 μL/g for mice, 1 μL/g for rats) via a tail vein and 3 min later were killed by stunning and decapitation. Brains were rapidly removed and homogenized and 300 μL aliquots filtered and washed over Whatman GF/B filters. Washed filters were placed in scintillation vials, scintillation fluid added and radioactivity counted. In rat and mouse studies, trunk blood was collected into heparinized tubes immediately after decapitation. The plasma was separated by centrifugation, removed and stored at −20°C for subsequent measurement of drug concentrations using HPLC with tandem mass spectrometry detection.

The occupancy was defined as the extent to which the in vivo binding of [^{3}H]flumazenil was reduced by prior treatment with drug. Occupancy data from the different experiments was plotted as a function of either dose or plasma drug concentration, and from these graphs the dose and plasma drug concentrations corresponding to 50% occupancy (Occ_{50} and EC_{50}, respectively) were calculated using curve-fitting (GraphPad Prism).

Rat anxiolysis assays

Three different assays (the elevated plus maze, conditioned suppression of drinking and fear-potentiated startle) were used to measure the anxiolytic-like activity of TPA023B in rats; more detailed methods for these are presented elsewhere (Atack et al., 2006).

Briefly, male Sprague Dawley rats (260–310 g; B&K Universal) were used for the elevated plus maze and fear-potentiated startle experiments, whereas male hooded-Lister rats (200–250 g; B&K Universal) were used for the conditioned suppression of drinking. In all three assays dosing was p.o. with a 0.5% methyl cellulose vehicle (dose volume = 1 mL/kg), with TPA023B being formulated as a suspension, and a pre-treatment time of 0.75 h was used prior to testing.

Doses of TPA023B used for the elevated plus maze and fear-potentiated startle experiments were 0.1, 0.3 and 1 mg/kg, whereas for the conditioned suppression of drinking doses used were 1, 3 and 10 mg/kg, since it was hypothesized that the doses required to overcome the fear-related anxiety produced by the conditioning stimulus would be greater than those required to overcome the milder anxiety presumed to occur in the elevated plus maze and fear-potentiated startle. Following the rat elevated plus maze assay, occupancy was measured using [^{3}H]flumazenil as described above.

Rodent sedation assays

Rat chain-pulling: In the rat chain-pulling assay, male P.V.G. rats (270–350g; B&K Universal) trained to pull a chain to obtain a food pellet reward were dosed with either vehicle (0.5% methyl cellulose; 1 mL/kg p.o.), TPA023B (1, 3 or 10 mg/kg p.o.) or diazepam (10 mg/kg p.o.). Immediately thereafter, they were placed in the testing chamber and their chain-pulling activity was recorded over the subsequent 1 h period.

Mouse rotarod assay: Male BKTO mice (26–30 g, $n = 7$–8/group; B&K Universal) were trained to successfully complete a 2 min trial on a rotarod (Ugo basile, Comeno, Italy) revolving at a speed of 15 rpm. Mice were given either vehicle (0.5% methyl cellulose; 10 mL/kg p.o.), TPA023B (0.3, 1, 3 or 10 mg/kg p.o.) or diazepam (10 mg/kg p.o.) 0.5 h before being placed on the rotarod. The time to fall from the rotarod was recorded, or, if the mouse successfully completed the

2 min trial, the latency was recorded as 120 sec. Immediately following the rotarod trial, mice were given an i.v. injection of [^{3}H]flumazenil via a tail vein and receptor occupancy was measured as described above.

In order to assess the possible interaction of TPA023B with ethanol, the experiment described above was repeated in mice given a subthreshold dose of ethanol (1.5 g/kg i.p.) 0.5 h prior to initiation of the rotarod trial (i.e. immediately prior to receiving either vehicle, TPA023B or diazepam).

Primate anxiolysis and sedation assays

Conditioned emotional response: Eleven male squirrel monkeys (*Saimiri sciureus*, 0.7–1.2 kg) were trained to press a lever to obtain a fruit juice reward using a fixed reinforcement schedule that maintained their response rate at >10/min. In the conditioned emotional response anxiety assay, the presentation of a red cue light (illuminated at a time chosen at random between 5 and 35 min after the session started) was associated with a 10% chance of a mild electric tail-shock (1–7 mA for 0.5 sec) being delivered (Atack et al., 2006). On drug-testing days, animals received vehicle (0.5% methyl cellulose; 2 mL/kg) or TPA023B (0.1, 0.3, 1 or 3 mg/kg p.o.) 0.5 h prior to testing using a repeated-measures experimental design in which each animal received vehicle and each dose of TPA023B. On these days, the schedule was as above except that presentation of the cue (illumination of the red light) was not accompanied by tail-shocks (i.e. there is no potential analgesic confound). The response to presentation of the cue light was expressed as the ratio of responding after compared with before illumination of the light, with a significant increase in the suppression ratio relative to vehicle-treated animals being defined as anxiolytic-like activity.

Squirrel monkey lever pressing: The primate lever pressing assay is comparable to the rat chain pulling assay in that it assesses the ability of a drug to reduce the rate of responding to obtain a fruit juice reward, whether that be as a consequence of sedation, myorelaxation or ataxia. These studies were performed using the monkeys employed for the conditioned emotional response studies (n = 8–9/group), with the difference that no conditioning step was involved. Hence, in animals treated with vehicle or TPA023B (1, 3 or 10 mg/kg p.o.), the rate of responding 30 min after drug treatment was compared with that in the preceding, drug-free day.

Baboon PET studies

Baboon PET studies were performed using the ECAT EXACT HR + (CTI/Siemens, Knoxville, Tennessee) in 3D mode. Three male baboons (18–27 kg) were anaesthetized with ketamine (10 mg/kg i.m., followed by propofol (2 mg/kg i.v. bolus followed by a constant infusion of 0.4 mg/kg/min i.v.), intubated and then ventilated by using medical grade compressed air at ~100 mL per breath at a rate of 23 respirations per minute. Body temperature was maintained with circulating water heating pads, and temperature, SpO_2, and end tidal CO_2 were monitored for the duration of the study. Approximately 5 mCi of [^{11}C]flumazenil was injected i.v. over 15 sec and dynamic PET imaging was initiated at the time of injection. Forty minutes later, 0.0032 ($n = 2$ animals), 0.032 ($n = 3$) or 0.32 mg/kg ($n = 2$) TPA023B was administered (i.v. in 50% PEG400, 50% saline) over a 90 sec interval, and heparinized blood samples were obtained at 0, 5, 15, 25, 35, 45 and 55 min for drug concentration determinations. Additional scans were performed to estimate [^{11}C]flumazenil specific binding under baseline conditions and test–retest variability.

Regions of interest (ROI) were defined on the baseline images in frontal, parietal, temporal and occipital cortices, cerebellum and pons. For all studies, the regional [^{11}C]flumazenil specific binding (SB) was calculated using the area under the time-activity curves (TACs) from 85 to 95 min after tracer injection. The non-specific binding was estimated from the pons region after administration of 0.32 mg/kg of TPA023B ($n = 2$) in the same time interval, and specific binding was defined as

$$SB = (SUV\ ROI_{(85-95\ min)}/SUV\ Pons_{(85-95\ min;0.32\ mg/kg)}) - 1$$

For each region, the percentage occupancy by TPA023B was calculated as: $100 \times (1 - SB_{BL}/SB_{TPA023B})$, where SB_{BL} corresponds to the average SB calculated under baseline conditions and $SB_{TPA023B}$ to the specific binding calculated for different doses of TPA023.

Human safety and tolerability and pharmacokinetics

The safety and tolerability studies were performed according to protocols approved by the relevant Institutional Review Board and with appropriate signed informed consent forms.

In order to establish the tolerability of TPA023B, fasted, healthy young (18–45-year-old) male volunteers were given single doses of drug (ranging from 0.05 to 3 mg administered as dry-filled capsules) and then monitored clinically according to standard procedures. Blood samples were obtained at various times (1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, 48, 72, 96, 120 and 144 h) after dosing for subsequent analyses of plasma drug concentrations.

Results

In vitro affinity and efficacy of TPA023B

TPA023B had high affinity ($K_i = 0.7–2.0$ nM) for subtypes of human recombinant $GABA_A$ receptor containing either an α1, α2, α3 or α5 subunit, but over 1500-fold lower for the α4- and α6-containing subtypes ($K_i > 1000$ nM). The compound also had a comparable affinity for native rat $GABA_A$ receptors in different regions of the CNS (K_i values = 0.32–0.99 nM in cerebellum, spinal cord and frontal cortex) (Table 1).

The maximum potentiation of the GABA EC_{20}-induced currents measured using whole cell patch clamping in cell lines stably expressing human recombinant $GABA_A$ receptors containing β3, γ2 plus either α1, α2, α3 or α5 subunits (Figure 2A) were 4, 43, 67 and 45%, respectively (Table 2). When expressed relative to the potentiation produced by the non-selective full agonist chlordiazepoxide, the α1, α2, α3 and α5 subtypes had respective relative efficacy values of 0.03, 0.38, 0.50 and 0.37 (i.e., for example, efficacy at the α3 subtype was

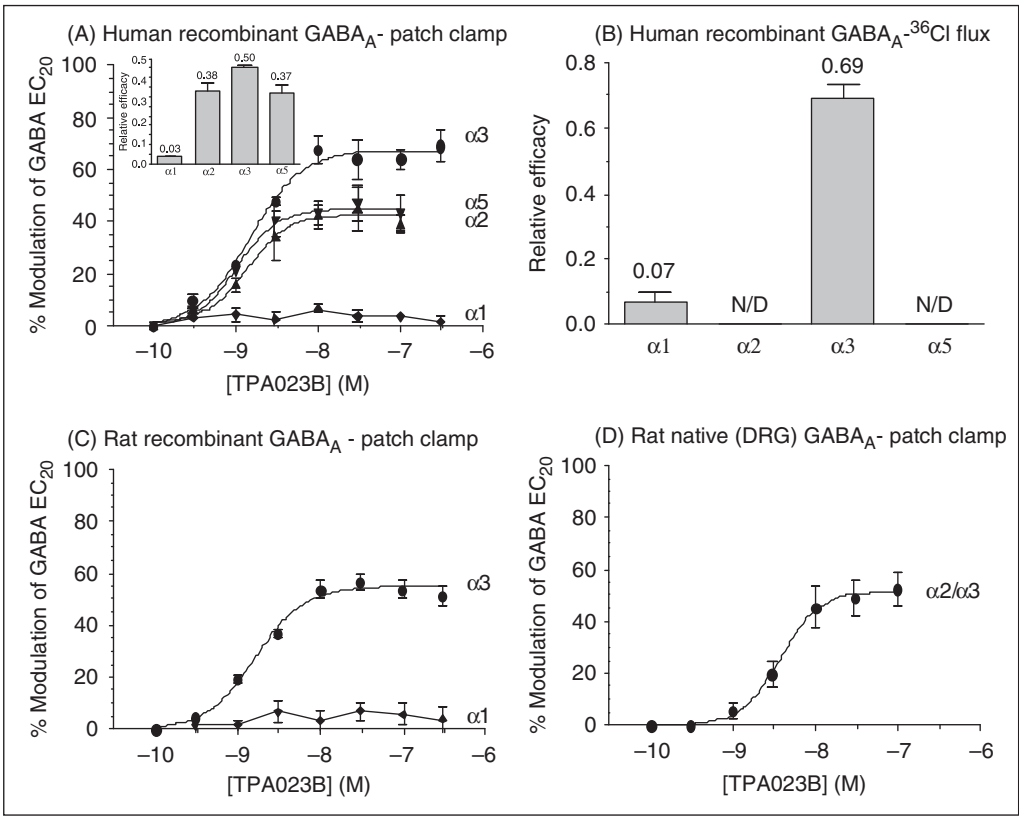

Figure 2. TPA023B behaves as an α2/α3 partial agonist and α1 antagonist at human and rat $GABA_A$ receptors. Modulation by TPA023B of a GABA EC_{20} at human recombinant $GABA_A$ receptors containing β3, γ2 and either an α1, α2, α3 or α5 subunit stably expressed in mouse fibroblast L(tk^-) cells as measured using either an (A) whole-cell patch clamp assay with data expressed as % increase in the GABA EC_{20}-induced current or relative to chlordiazepoxide (inset) or (B) whole-cell [$^{36}Cl^-$] flux uptake assay with data being expressed relative to the potentiation produced by the non-selective full agonist chlordiazepoxide. N/D = not determined. TPA023B has efficacy comparable to human $GABA_A$ receptors when measured using whole-cell patch clamping at either (C) rat recombinant $GABA_A$ receptors containing β3, γ2 and either an α1 or α3 subunit transiently transfected into HEK cells or (D) rat native α2/α3-containing $GABA_A$ receptors in rat dorsal root ganglia (DRG) cells. All values shown are mean ± SEM (n = 4–7/data point).

Table 1. Affinity of TPA023B for the benzodiazepine site of recombinant human and native rat brain $GABA_A$ receptors

K_i, nM								
Human recombinant $GABA_A$ receptors containing β3, γ2 plus						Native rat brain receptors		
α1	α2	α3	α4	α5	α6	Cerebellum	Spinal cord	Frontal cortex
0.73 ± 0.21	2.0 ± 0.4	1.8 ± 0.4	3300 ± 700	1.1 ± 0.2	4700 ± 1200	0.32 ± 0.03	0.99 ± 0.02	0.67 ± 0.08

Data shown are the mean ± SEM (n = 8–10 separate determinations).

half of that observed with chlordiazepoxide). When efficacy was measured in these same cell lines using a [$^{36}Cl^-$] flux assay (Figure 2B), similar data were obtained, with TPA023B having much greater efficacy at the α3 compared to α1 subtype (efficacy values relative to chlordiazepoxide = 0.07 and 0.69 at the α1 and α3 subtypes; Table 2). Given the resolution of the flux assay, the efficacy at the α1β3γ2 subtype should be considered as essentially antagonist-like.

The responses obtained with rat cDNAs transiently transfected into HEK cells were similar to those obtained with human stable cell lines, with the exception that the relative efficacy of TPA023B was slightly higher in the rat (0.61) than in the human α3 subtype (0.50). Finally, the maximum efficacy of TPA023B on native rat (dorsal root ganglion neuron) α2/α3-containing $GABA_A$ receptors was 54 ± 6% (n = 4), which, when normalized to the full agonist chlordiazepoxide, gave a value of 0.40 ± 0.01 (Figure 2D).

In an additional study (data not shown), TPA023B antagonized the ability of chlordiazepoxide to potentiate the GABA EC_{20}-induced current in cells expressing the α1 subtype. More specifically, 3 μM chlordiazepoxide potentiated the GABA EC_{20} current by 105 ± 6% (mean ± SEM, n = 6) and this effect could be reduced to 8 ± 3% in the presence of 100 nM TPA023B.

In vivo binding

The ability of TPA023B to occupy rodent brain $GABA_A$ receptors was assessed using a [^{3}H]flumazenil in vivo binding assay. In both mouse and rat, TPA023B was potent, with the doses required to produce 50% occupancy (Occ_{50}) 0.75 h after dosing being 0.11 and 0.22 mg/kg respectively (Figure 3A). The occupancy of rat brain binding sites was time-dependent, since TPA023B was more potent 8 h than 0.75 h after dosing (respective Occ_{50} values = 0.09 and 0.22 mg/kg). Based upon the occupancy data obtained 0.75–1 h after dosing, the estimated occupancy values at doses ranging from 0.03 to 10 mg/kg were estimated (Table 3) and were used to interpret data from behavioural experiments in which occupancy was not directly measured.

The time-dependency of rat brain occupancy was explored in more detail in a time course experiment in which occupancy was assessed at various times after a dose of TPA023B of 0.3 mg/kg (Figure 3B). These data show that TPA023B gives sustained occupancy, with reasonably similar occupancy over the 2–8 h time period, and that this corresponds to relatively constant plasma drug concentrations. More specifically, the occupancies 2, 4 and 8 h after dosing were 82 ± 2, 83 ± 1 and 87 ± 2%, corresponding to plasma drug concentrations of 62 ± 6, 73 ± 7 and 64 ± 4 ng/mL, respectively (Figure 3B, inset). Furthermore, 54 ± 2% occupancy remained 24 h after dosing, with a corresponding plasma concentration of 22 ± 2 ng/mL. The prolonged occupancy of TPA023B is consistent with the 11 h plasma half-life reported previously in rats following i.v. dosing (Russell et al., 2006).

Table 3. Occupancy of rat brain benzodiazepine binding sites at various p.o. doses of TPA023B 0.75–1 h post dose

Dose, mg/kg	Estimated % Occupancy
0.03	8
0.1	27
0.3	61
1	87
3	96
10	99

Table 2. Efficacy of TPA023B at human and rat recombinant and rat native $GABA_A$ receptors

	Human $GABA_A$ receptors[a]						Rat $GABA_A$ receptors		
	Whole cell patch clamp				[$^{36}Cl^-$] uptake		Recombinant[a]		Native
	α1	α2	α3	α5	α1	α3	α1	α3	DRG
Max % modulation[b]	4 ± 1	43 ± 5	67 ± 5	45 ± 3	N/A	N/A	5 ± 1	55 ± 4	54 ± 6
Efficacy vs. CDP	0.03 ± 0.01	0.38 ± 0.04	0.50 ± 0.02	0.37 ± 0.04	0.07 ± 0.03[c]	0.69 ± 0.04[c]	0.04 ± 0.01	0.61 ± 0.07	0.40 ± 0.10
EC_{50}, nM[d]	N/D	1.6	1.4	1.0	N/D	N/D	N/D	1.6	4.4

[a]Recombinant $GABA_A$ receptors containing β3, γ2 and various α subunits.
[b]Values are mean ± SEM of modulation observed in each individual cell (n = 4–6).
[c]Values for $^{36}Cl^-$ flux data are mean ± SEM (n = 7).
[d]Calculated from the curve fitted through the mean data.
N/A: not assayed; CDP = chlordiazepoxide; N/D: not determined.

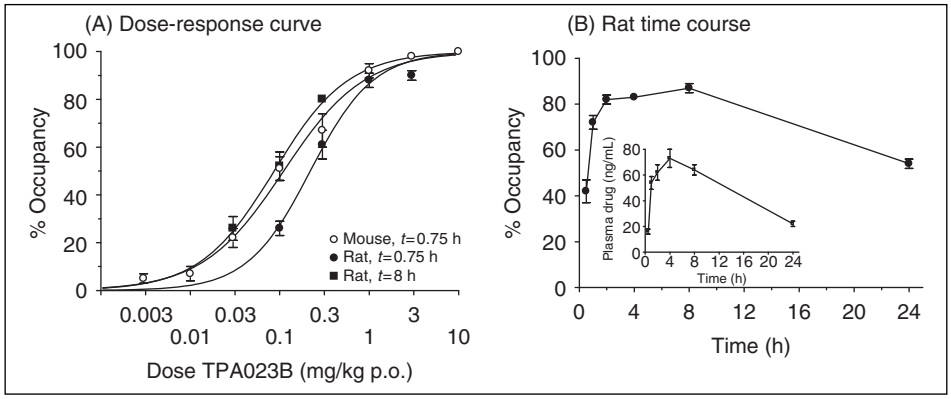

Figure 3. Occupancy of rodent brain benzodiazepine binding sites by TPA023B was dose- and time-dependent. (A) Mice or rats received various p.o. doses of TPA023B and occupancy was measured either 0.75 (mice and rats) or 8 h (rats) later using a [^{3}H]flumazenil in vivo binding assay. Values shown are mean ± SEM of data obtained from three separate experiments (mouse and rat t = 0.75 h) or represent the variability within a single experiment (rat t = 8 h), in which case n = 5–7/dose group. (B) The time course of rat brain occupancy after a single dose (0.3 mg/kg p.o.) of TPA023B. Values shown are mean ± SEM (n = 4–6/time point). The inset shows the corresponding plasma concentrations measured in the same animals.

From the rat and mouse occupancy and corresponding plasma drug concentrations, plasma-occupancy curves were constructed for both rat and mouse (Figure 4). In rat, the plasma-occupancy relationship was comparable at 0.75 and 8 h post-dose (respective EC_{50} values of 22 and 16 ng/mL). The EC_{50} for the combined rat data was 19 ng/mL, which was similar to that observed in mouse (25 ng/mL), indicating that the blood–brain barrier penetrability of TPA023B is similar in both species.

TPA023B has anxiolytic-like activity in rats

TPA023B had anxiolytic-like activity in an unconditioned anxiety assay (the elevated plus maze) as well as two conditioned assays (conditioned suppression of drinking and fear-potentiated startle). Thus, Figure 5A shows that TPA023B produced a dose-proportional increase in the time spent on the open arms of the elevated plus maze during the 5 min trial. More specifically, 0.1, 0.3 and 1 mg/kg TPA023B increased the open-arm time to 17 ± 2, 22 ± 3 and $32 \pm 2\%$ relative to vehicle-treated control animals ($15 \pm 3\%$), although only the 1 mg/kg dose was significantly different from control. However, the extent of the effect at 1 mg/kg (time on open arms $= 32 \pm 2\%$) was similar to that observed in animals dosed with the positive control, chlordiazepoxide ($30 \pm 2\%$). Following the elevated plus maze trial, rat brain occupancy was measured, and the comparable anxiolytic-like effects of 1 mg/kg TPA023B and 5 mg/kg chlordiazepoxide occurred at occupancies of 88 and 32%, respectively.

In the conditioned suppression of drinking test, all groups of rats receiving TPA023B (1, 3 and 10 mg/kg p.o.) had mean suppression ratios significantly greater than the vehicle control group, indicating that the suppression of drinking produced by the presentation of the conditioning stimulus was alleviated by TPA023B (Figure 5B). The levels of occupancy at doses of 1, 3 and 10 mg/kg were 87, 96 and 99%, respectively (Table 3). Since TPA023B had anxiolytic-like activity even at the lowest dose tested (1 mg/kg), a minimal effective dose was not established in this assay.

Figure 5C shows the mean startle amplitudes observed in either dark or light conditions as well as the mean difference scores. Compared with vehicle-treated rats, baseline startle responses recorded in the dark were not significantly different in rats treated with TPA023B (0.1, 0.3 and 1 mg/kg p.o.). Whilst there was an enhanced startle response during presentation of the conditioning stimulus (light) in rats treated with 0.1, 0.3 and 1 mg/kg TPA023B (open bar significantly greater in magnitude than filled bar at each dose: Figure 5C), the extent of this startle response was significantly reduced relative to vehicle-treated animals at 0.3 and 1 mg/kg TPA023B as measured in terms of either the absolute startle response (left-hand panel) or the difference scores (right-hand panel).

TPA023B is non-sedating in rodents

Figure 6 shows the effects of TPA023B and diazepam on performance in the rat chain-pulling assay (a test for sedation, myorelaxation and/or ataxia). Although diazepam (10 mg/kg p.o.) produced a marked and significant decrease in responding throughout the trial, there was no significant effect of TPA023B even at a dose (10 mg/kg) corresponding to 99% occupancy.

Figure 7 shows the effects of TPA023B and diazepam on mouse rotarod performance in the absence and presence of ethanol. In the absence of ethanol, rotarod performance was not significantly impaired by TPA023B even at a dose (10 mg/kg) that gave essentially complete occupancy. Moreover, even at this high dose, the effect of ethanol was only modest (Figure 7B), with mice lasting for 65 ± 19 sec before falling off, with no significant ethanol interaction being observed at the lower dose (3 mg/kg), which gave only slightly lower levels of occupancy ($\geq$97%). On the other hand, there was a marked enhancement of diazepam's impairing effects in the presence of ethanol, such that, in the presence of ethanol, the impairment produced by 3 mg/kg diazepam (time on the

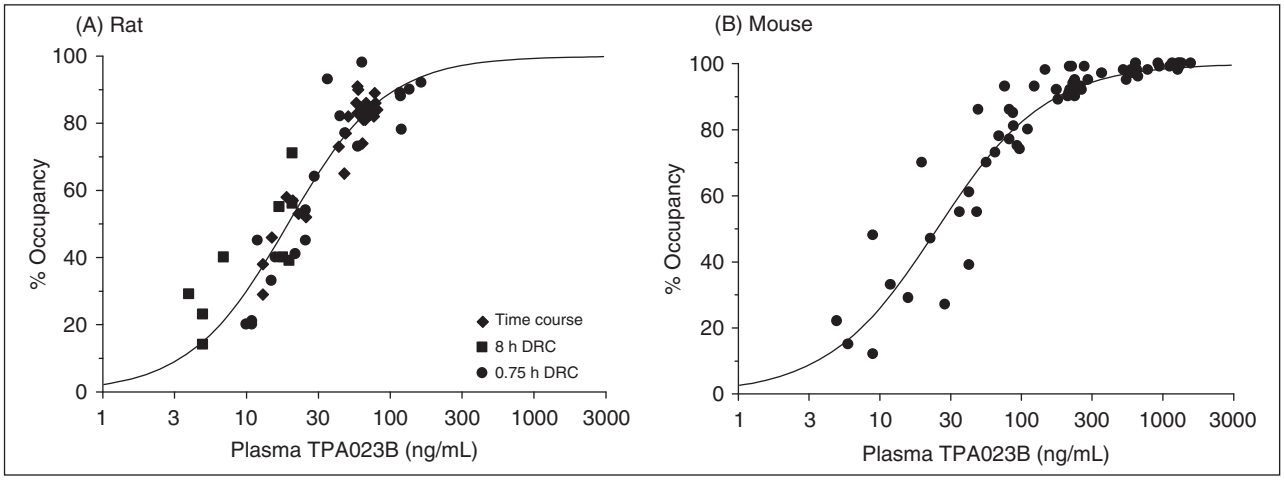

Figure 4. Relationship between plasma drug concentrations and occupancy in (A) rat and (B) mouse brain. Each data point represents an individual animal. EC_{50} values for rat and mouse were 19 and 25 ng/mL, respectively. DRC = dose-response curve.

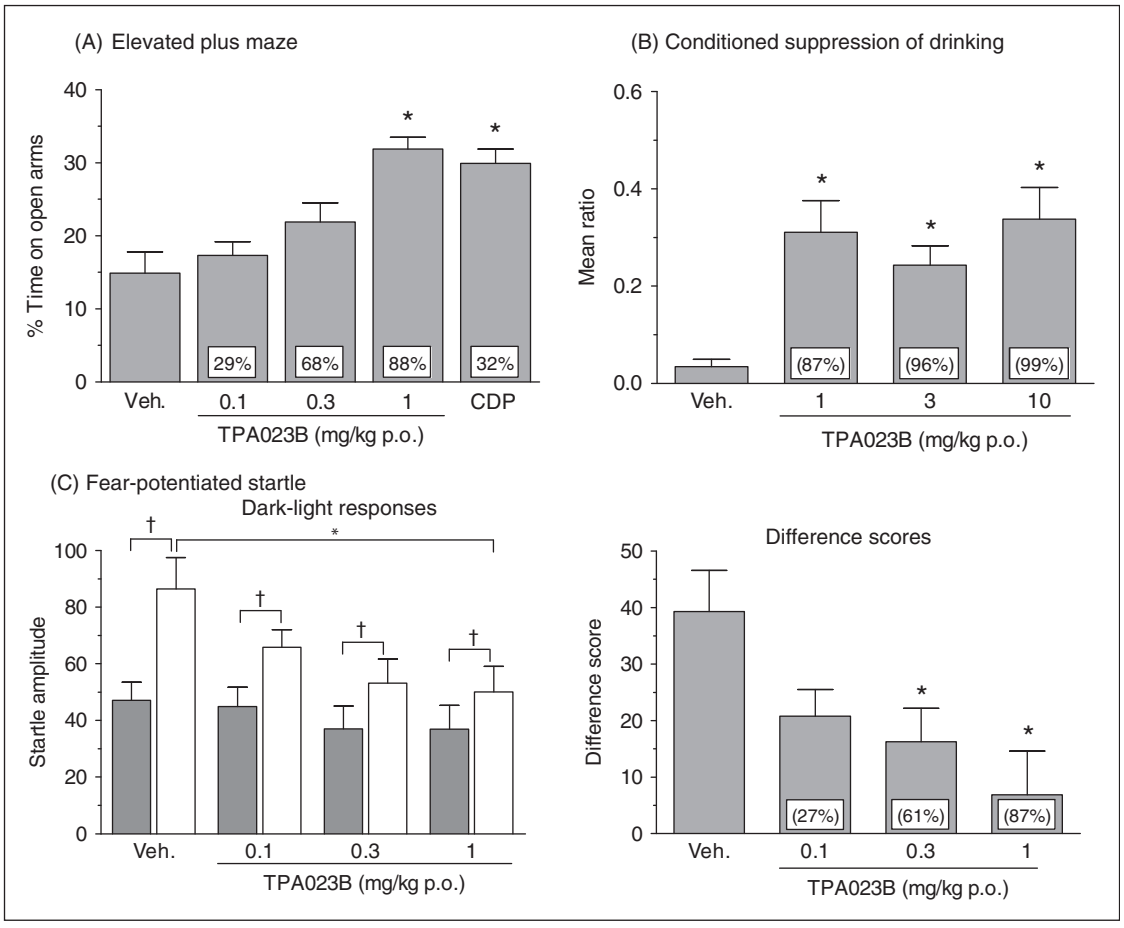

Figure 5. TPA023B has anxiolytic-like activity in the rat elevated plus maze, conditioned suppression of drinking and fear-potentiated startle assays. (A) TPA023B significantly increased the percentage of time spent on the open arms of the elevated plus maze at a dose of 1 mg/kg p.o. compared with vehicle (0.5% methyl cellulose, 10 mL/kg)-treated animals. The extent of the increase produced by TPA023B was similar to the positive control chlordiazepoxide (CDP; 5 mg/kg i.p.). Values shown are mean ± SEM ($n = 18$/group), with percentage values representing the occupancy measured immediately following completion of the plus maze trial. (B) In the conditioned suppression of drinking assay, data are presented as the mean ratio (±SEM; $n = 9$/group) of the lick rate in the presence and absence of a conditioning stimulus in rats given either vehicle (0.5% methyl cellulose, 10 mL/kg p.o.) or TPA023B (1, 3 and 10 mg/kg p.o.) 45 min prior to testing. Percentage figures in parentheses represent the occupancy values corresponding to the doses used (N.B. these values differ from those in the elevated plus maze, since the former are estimates derived from a mean of three dose response curves (Table 3) whereas the latter were measured in the same animals used for the plus maze). (C) Rats were trained to associate the presentation of light with a mild electric foot-shock and then tested for acoustic startle responses to 100 dB tones delivered either in the dark or in the presence of light 45 min following treatment with TPA023B (0.1, 0.3 or 1 mg/kg) or vehicle (0.5% methyl cellulose). Data shown are the mean ± SEM ($n = 10$) of the startle responses at 100 dB and the difference scores. Figures in parentheses represent estimated occupancy values for the doses used (Table 3). Comparisons between drug-treated and vehicle control groups were made using an analysis of variance followed by Dunnett's post hoc test; * = $p < 0.05$. In the fear-potentiated startle assay, data were also analysed within subjects; † = $p < 0.05$ compared with startle responses measured in the dark.

rotarod = 32 ± 11 sec) was greater than that seen in the absence of ethanol at a dose of 10 mg/kg (58 ± 19 sec).

TPA023B is a non-sedating anxiolytic in primates

The anxiolytic-like effects of TPA023B in the squirrel monkey conditioned emotional response assay are presented in Figure 8A. Hence, following vehicle treatment, lever pressing rates during illumination of the light that had previously been associated with tail-shock were 11 ± 4% of those observed in the minute preceding its illumination (Figure 8A). This suppression in responding was alleviated in a dose-dependent manner by TPA023B, such that responding rates were significantly increased relative to vehicle at doses of 0.3, 1 and 3 mg/kg p.o. (responding rates = 48 ± 13, 59 ± 13 and 66 ± 11% of baseline, respectively).

In order to assess the sedative, myorelaxant and/or ataxic effects of the compound in squirrel monkeys, the effects of TPA023B on lever pressing rates during a 30 min test session were measured (Figure 8B) and expressed as a percentage of

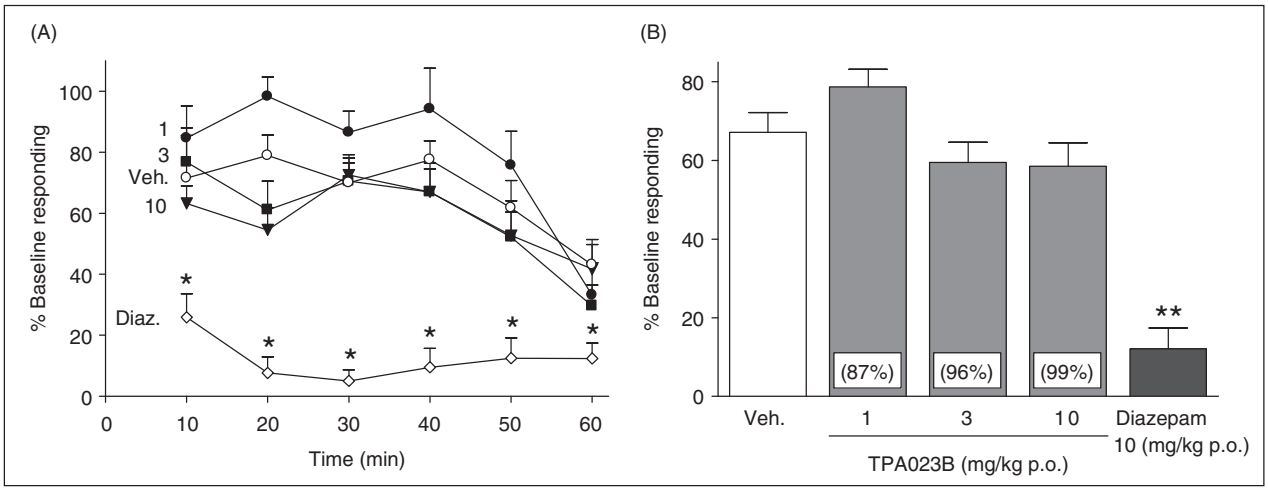

Figure 6. TPA023B did not significantly affect performance in the rat chain-pulling assay of sedation. Rats were dosed p.o. with either vehicle (0.5% methyl cellulose), TPA023B (1, 3 or 10 mg/kg) or diazepam (10 mg/kg), and the rate of chain-pulling was recorded over the subsequent 1 h period with data being expressed as a percentage of baseline responding. (A) The number of chain pulls/min at 10 min intervals during the 60 min session. (B) The mean chain pulls/min averaged across the session. Figures within bars represent estimated occupancy values (Table 3). Values shown are mean ± SEM ($n = 12$/group). *, **= $p < 0.05$ and $p < 0.01$ versus vehicle-treated animals (ANOVA followed by Student Newman-Keuls post hoc t-test).

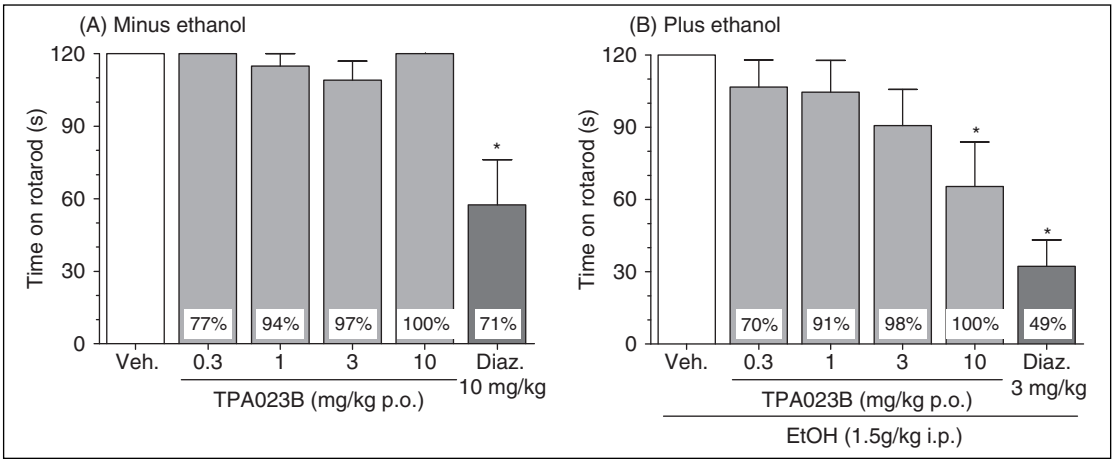

Figure 7. Effects of TPA023B on rotarod performance in the absence and presence of ethanol. The latency to fall off the rotarod during a 2 min trial was measured in mice 30 min after p.o. dosing with either vehicle (0.5% methyl cellulose), TPA023B (0.3, 1, 3 or 10 mg/kg) or diazepam (3 or 10 mg/kg) in (A) the absence or (B) the presence of ethanol (1.5 g/kg i.p., 30 min pretreatment). Figures within bars are the mean occupancy values measured immediately after completion of the trial. Values shown are mean ± SEM ($n = 7$–8/group). * = $p < 0.05$ versus vehicle group.

the previous day's performance (baseline). However, 1, 3 and 10 mg/kg TPA023B did not significantly affect lever pressing rates (response rates = 80 ± 10, 76 ± 7 and 82 ± 9%) relative to vehicle-treated animals (95 ± 4%).

Separate pharmacokinetic analyses showed that 0.5 h after oral dosing of 0.3 and 3 mg/kg, respective plasma drug concentrations were 61 ± 39 and 319 ± 102 ng/mL (data not shown). Assuming proportionality between doses of 3 and 10 mg/kg, the estimated plasma concentration at 10 mg/kg would be around 1000 ng/mL. Thus, and further assuming that the plasma-occupancy in squirrel monkeys is comparable to that seen in rats (Figure 4), the minimum effective dose in the conditioned emotional response assay (0.3 mg/kg) corresponds to 80% occupancy, whereas there are no overt signs of sedation in the lever pressing assay at a dose (10 mg/kg) corresponding to >98% occupancy.

Baboon PET

In baboon [^{11}C]flumazenil PET studies, a dose of 0.0032 mg/kg i.v. did not significantly alter the kinetics of radiotracer in the brain (Figure 9A). Since the limits of detection of the PET assay are in the region of 10%, these data suggest that a dose of 0.0032 mg/kg i.v. produced occupancy of ≤10%. On the other hand, a dose of 0.32 mg/kg i.v. displaced brain [^{11}C]flumazenil to essentially baseline (pons) levels, consistent with TPA023B giving essentially complete occupancy (≥95%) of baboon brain benzodiazepine binding sites. A dose of

0.032 mg/kg produced occupancy in between these two extremes, with occupancy averaged across the cerebellum and frontal, parietal, temporal and occipital cortices being 67 ± 9% ($n = 3$). When occupancy was plotted as a function of plasma drug concentration (Figure 9B), the plasma EC_{50} was 10 ng/mL, which is comparable to corresponding values for rat and mouse (19 and 25 ng/mL; Figure 4).

Pharmacokinetics and tolerability of TPA023B in man

The safety and tolerability studies of doses of TPA023B ranging from 0.05 to 3 mg total dose (administered as dry-filled capsules) were performed in healthy young men ($n = 7$–8/dose with 2 placebo and 5–6 active per dose) and the pharmacokinetic data from these studies are presented in Figure 10, with parameters derived from these data, along with clinical observations, being summarized in Table 4. Absorption occurred fairly rapidly, with T_{max} values ranging from 2 to 4 h, and the total exposures (estimated area under the curve from $t = 0$ to infinity; $AUC_{0-\infty}$) were reasonably dose-proportional (Figure 10, inset), as were the C_{max} values (Table 5). The apparent half-life of TPA023B was in the region of 40 h, which is appreciably longer than the half-lives reported in rat, dog or rhesus monkey following i.v. dosing (11, 6.5 and 4.9 h, respectively; Russell et al., 2006).

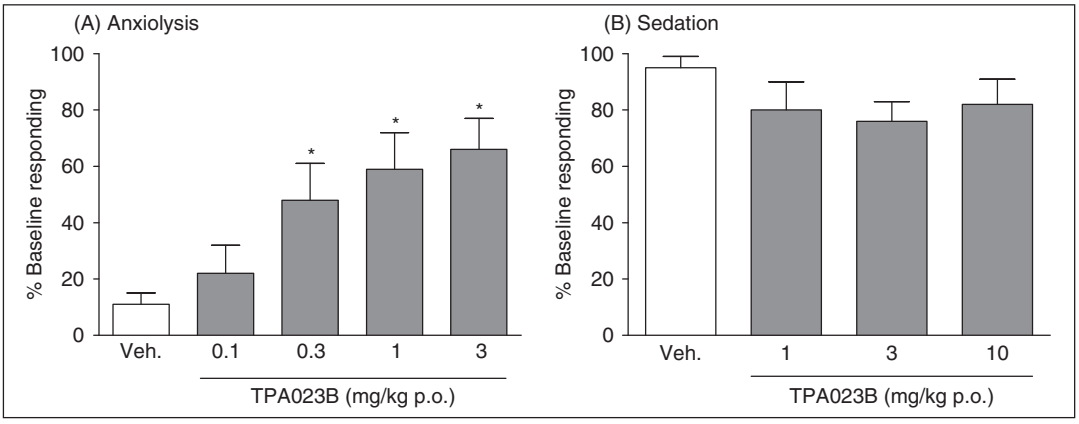

Figure 8. TPA023B had anxiolytic-like activity in a squirrel monkey conditioned emotional response assay but was devoid of overt sedation in a lever pressing response assay. (A) Effect of TPA023B, administered 30 min prior to testing, on lever pressing rates suppressed by a red cue light that had previously been associated with mild electric tail-shock. Values shown are mean ± SEM ($n = 11$/group). * = $p < 0.05$ vs. vehicle control (paired t-test). (B) The number of lever presses per minute during the 60 min session (expressed as a percentage of baseline responding) are shown following administration of TPA023B (1–10 mg/kg p.o.) given 30 min before the test session. Values shown are mean ± SEM ($n = 8$–9/group). There were no statistically significant differences between groups as assessed using paired t-tests ($p > 0.05$ versus vehicle treatment).

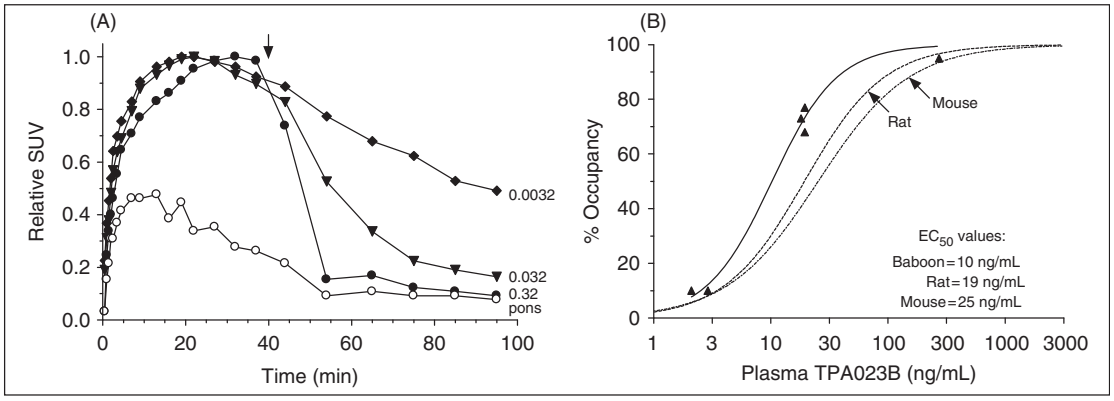

Figure 9. Displacement of baboon brain [^{11}C]flumazenil binding by TPA023B. (A) Time-activity curves for radioactivity in frontal cortex of the same baboon after separate doses of 0.0032, 0.032 and 0.32 mg/kg TPA023B (i.v. in 50% PEG400:50% saline vehicle). In order to compare the standardized uptake values (SUVs) across different scans, values were normalized relative to the maximum SUV in each scan (actual maximal SUV values = 5.4, 5.8 and 3.8 for the 0.0032, 0.032 and 0.32 mg/kg scans). For comparative purposes the data from a brain region with minimal expression of benzodiazepine binding sites, the pons, is also shown as an index of the level of non-specific uptake. Arrow denotes the time of drug administration ($t = 40$ min). (B) The occupancy measured 45–55 min after drug administration (scan time = 85–95 min) plotted as a function of plasma drug concentration. Occupancy values at the 0.0032 and 0.32 mg/kg doses were defined as 10% and >95%, respectively, which are the limits of reliable detection using the PET scanner. Plasma samples were not available from each baboon at each time point, and hence only six rather than nine values were used to construct the curve. The EC_{50} value was 10 ng/mL.

In fasted, healthy young men, TPA023B was generally well tolerated at doses ranging from 0.05 to 1 mg (Table 4), with mild adverse experiences at the 1 mg dose including headache, fatigue/tiredness and somnolence/drowsiness. In the initial cohort dosed with 2 mg, a single subject experienced a severe, reversible ataxia that lasted for about 10 h and resulted in this subject discontinuing. However, in two additional cohorts, the 2 mg dose was generally well tolerated. At a dose of 3 mg, adverse experiences, including fatigue/tiredness and somnolence/drowsiness, were observed in four subjects and precluded further dose advancement. Accordingly, 2 mg was defined as the maximum tolerated single dose in healthy young men.

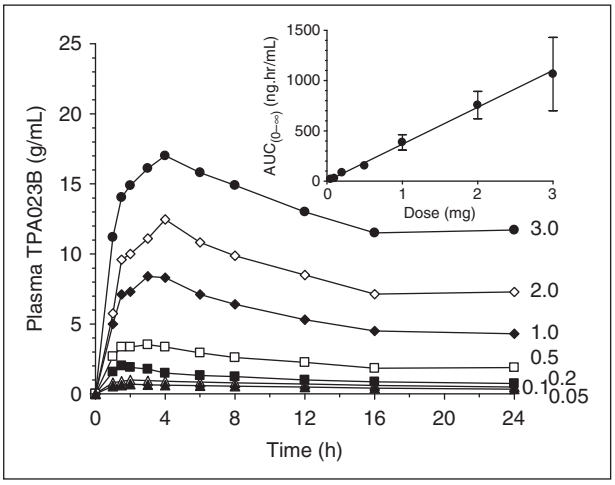

Figure 10. Plasma drug concentrations in fasted, healthy young men at various times after oral doses of TPA023B ranging from 0.05 to 3 mg total dose. Values shown are mean ($n = 5$–6/group) with error bars being omitted for clarity. Inset shows the mean (±SD) of the total exposure (estimated area under the curve from $t = 0$ to infinity; $AUC_{0-\infty}$) as a function of dose. Although plasma concentrations were measured for 7 days after dosing, for clarity, only data from the first day are shown here.

The effect of a high-fat meal was to increase the T_{max} of a 0.5 mg dose by 2–3 h. Hence, the T_{max} values in the fasted and fed states ranged from 2–4 to 3–6 h, respectively. However, neither the mean C_{max} (3.5 versus 3.4 ng/mL) nor the total area under the curve (153 and 150 ng.h/mL) differed between the fasted and fed groups.

Across-species comparison of plasma-occupancy relationship for TPA023B

The distribution of brain [^{11}C]flumazenil uptake in four healthy normal volunteers, one given placebo and three given a single 1.5 mg oral dose of TPA023B, is shown in Figure 11 (modified from Van Laere et al., 2008). Cortical $GABA_A$ receptor occupancy by TPA023B 5 and 24 h after dosing were 53 ± 1 and 46 ± 6%, respectively, with corresponding plasma drug concentrations, 7.6 ± 0.7 and 5.6 ± 0.7 ng/mL, that were consistent with those observed in the safety and tolerability studies (Figure 10). When occupancy was plotted as a function of plasma drug concentration, the resulting plasma-occupancy relationship (Figure 11B) gave an EC_{50} of 5.8 ng/mL, compared with values of 25, 19 and 10 ng/mL in mouse, rat and baboon, respectively.

Table 4. Pharmacokinetic parameters and adverse events noted after administration of single oral doses of TPA023B and placebo to young healthy male volunteers

		Pharmacokinetic parameters				Most frequent adverse events				
Dose, mg	n[a]	T_{max}, h	C_{max}, ng/mL	$AUC^{b}_{0-\infty}$ ng.h/mL	$T_{½}$, h	Headache	Fatigue/ tiredness	Somnolence/ drowsiness	Dizziness	Ataxia
0.05	8	1.5	0.6 ± 0.1	20 ± 8	~26[c]	1				
0.1	8	2	0.9 ± 0.1	29 ± 4	~31[c]	1	1			
0.2	8	1.5	2.0 ± 0.2	85 ± 10	~40	2				
0.5	8	3	3.5 ± 0.5	153 ± 35	~38			1		
0.5 (Fed)	8	6	3.4 ± 0.3	150 ± 30	~33	1	1			
1.0	8	3	8.4 ± 2.0	386 ± 78	~41	3	2	2		
1.5 (PET)[d]	3	N/D[e]	7.6 ± 0.7	N/D[e]	N/D[e]					
2.0 (I)	7	2	10.8 ± 3.8	545 ± 122	~35		1			1[f]
2.0 (II)	8	4	12.5 ± 2.1	756 ± 136	~41		1	4		
2.0 (III)	8	4	13.4 ± 3.5	723 ± 263	~40		1	4	2	
3.0	8	4	17.0 ± 3.9	1065 ± 365	~41	2	4	4	1	

[a]n refers to group size (two subjects were given placebo and five to six received TPA023B; PK data shows mean values of the five to six subjects, whereas the adverse events are unblinded and therefore represent observations from the total cohort of seven to eight subjects).
[b]AUC: area under the (plasma concentration versus time) curve.
[c]Concentrations below limits of quantification after 36–48 h post dose.
[d]Six adverse events were noted (five pain/discomfort due to arterial catheter insertion, one diarrhoea 3 days after dosing) and were considered definitely not drug-related (van Laere et al., 2008).
[e]Although plasma samples were obtained during the PET scan procedure, there were insufficient data to determine pharmacokinetic parameters.
[f]Severe ataxia resulting in discontinuation.
N/D: not determined.

Table 5. Summary of the preclinical non-sedating anxiolytic properties of TPA023B

Behaviour	Assay	Observation	Dose, mg/kg p.o.	Occupancy[b]
Anxiety assays				
Rat anxiety	Elevated plus maze	Anxiolysis	1[a]	88%
	Conditioned suppression of drinking	Anxiolysis	(1)[c]	(87%)[c]
	Fear-potentiated startle	Anxiolysis	0.3	61%
Primate anxiety	Conditioned emotional response	Anxiolysis	0.3	80%
Sedation assays				
Mouse sedation	Rotarod	No effect	10	99%
Rat sedation	Chain-pulling	No effect	10	100%
Primate sedation	Response sensitivity	No effect	10	>98%
Other assay				
Mouse ethanol interaction	Rotarod	Mild ethanol potentiation	10	100%

[a]Anxiolytic-like doses are minimal effective doses unless otherwise stated.
[b]Occupancy for elevated plus maze and rotarod experiments are measured values; all others are predicted, either from the rat dose–response curve (Table 3) or, in the case of the squirrel monkey, from the squirrel monkey plasma drug concentrations and the rat plasma-occupancy relationship (Figure 4).
[c]Dose and occupancy for the conditioned suppression of drinking are in parentheses, since these are not minimal effective doses because all doses tested had anxiolytic-like activity.

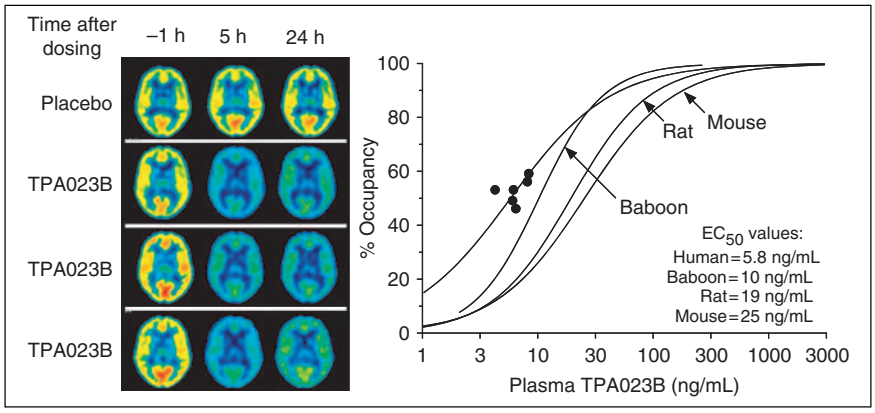

Figure 11. TPA023B has prolonged occupancy at the benzodiazepine binding site of human brain $GABA_A$ receptors. A: Pseudocolour images showing the inhibition of [^{11}C]flumazenil binding produced by a single 1.5 mg oral dose of TPA023B in PET scans performed 1 h before and 5 and 24 h after dosing. B. Occupancy plotted as a function of the corresponding plasma TPA023B concentrations. Images modified from van Laere et al. (2008).

Discussion

In vitro properties of TPA023B

In the search for α2/α3 $GABA_A$ receptor subtype-selective modulators, we have previously described compounds from the triazolopyridazine series, including L-838417 (McKernan et al., 2000), TPA023 (Atack et al., 2006) and MRK-409 (Atack et al., 2010), as well as the α3-selective fluoroimidazopyridine agonist TP003 (Dias et al., 2005) and the α3-selective pyridone inverse agonist α3IA (Atack et al., 2005). In the present article, we describe the characterization of the imidazotriazine TPA023B (Compound 11; Russell et al., 2006). It has an efficacy profile that is a hybrid of the efficacy profiles of other clinical candidates from this project (Figure 12), namely TPA023 and MRK-409. Hence, TPA023B has, like TPA023, essentially antagonist efficacy at the α1 subtype, but its α2/α3 efficacy (α2/α3 relative efficacy = 0.38 and 0.50) is higher than that of TPA023 (0.11 and 0.21) and is more comparable to that of MRK-409 (0.23 and 0.45).

In vivo properties of TPA023B in preclinical species

TPA023B showed anxiolytic-like efficacy in unconditioned and conditioned rat assays (elevated plus maze, fear-potentiated startle and conditioned suppression of drinking) as well as a primate conditioned anxiety model, conditioned suppression of drinking. More specifically, TPA023B produced a robust anxiolytic-like effect, with efficacy being observed at ~60–90% $GABA_A$ receptor occupancy, yet very little sign of overt sedation at doses 10–30-fold higher, corresponding to occupancy of >98% (Table 5). In addition, in mice TPA023B potentiated the rotarod-impairing effects of ethanol to a mild extent, but only at essentially complete occupancy, as well as also affording complete protection against PTZ (120 mg/kg s.c.)-induced seizures at a dose of

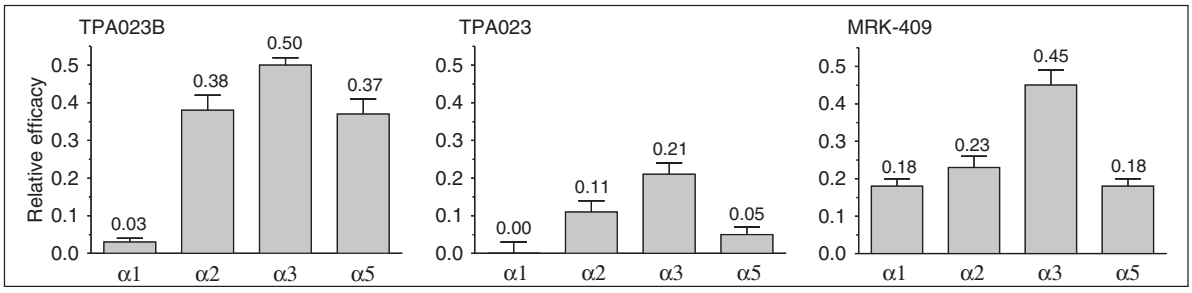

Figure 12. A comparison of the relative efficacy profiles of TPA023B, TPA023 and MRK-409.

Table 6. Comparison of the in vitro affinity and in vivo properties of TPA023B, TPA023 and MRK-409

								Human studies				
	In vitro affinity	Rat occupancy		Occupancy at anxiolytic MEDs				PET			Tolerability	
	K_i, nM	Occ_{50}, mg/kg	EC_{50}, ng/mL	EPM	FPS	CSD	CER	Dose, mg	% Occ.	EC_{50}, ng/mL	MTD, mg	Dose-limiting AEs
TPA023B	0.73–2.0	0.09/0.22[a]	19	88%	61%	87%	80%	1.5	50–55	5.8	2	Fatigue, drowsiness
TPA023	0.19–0.41	0.42	25	70%	70%	88%	65%	2.0	35–65	9.0	2	Dizziness, drowsiness, motor incoordination
MRK-409	0.21–0.40	2.2	115	43%	63%	63%	35%	1.0	$<$10%	$\gg$30	1.0	Sedation

[a]Occ, ED_{50} values for TPA023B are quoted for 0.75–1 h and 8 h post dose, respectively.
AEs: adverse events, CER: squirrel monkey conditioned emotional response, CSD: rat conditioned suppression of drinking, Occ, ED_{50} and EC_{50}: dose and plasma drug concentrations required to produce 50% occupancy, EPM: rat elevated plus maze, FPS: rat fear-potentiated startle, MED: minimal effective dose, MTD: maximal tolerated dose.

0.3 mg/kg p.o. (72% occupancy), with the ID_{50} for seizure protection (0.04 mg/kg) corresponding to 27% occupancy (based upon an occupancy ED_{50} of 0.11 mg/kg, $t = 0.5$ h; data not shown).

Like TPA023 and MRK-409 (as well as L-838417; McKernan et al., 2000), the higher α2/α3 versus α1 efficacy of TPA023B translated into a non-sedating anxiolytic-like behavioural phenotype in preclinical species. For these three compounds, a comparison of the levels of occupancy at a minimum effective dose in the various assays shows that no consistent pattern emerges (Table 6). For example, it might be predicted that less occupancy would consistently be required for efficacy in the milder anxiety provoked by the elevated plus maze relative to the presumably more severe anxiety provoked by the fear-potentiated startle and conditioned suppression of drinking models, but this is clearly not the case (although assumptions about relative anxiety levels in ethological compared with conditioned assays are largely anthropomorphic). Moreover, the higher α2/α3 relative efficacy of TPA023B (0.38 and 0.50) relative to TPA023 (0.11 and 0.21) does not result in the former requiring lower occupancy for anxiolytic-like efficacy. Indeed, both compounds required similar levels of occupancy (60–90%) for anxiolytic-like efficacy.

A possible confound in the various preclinical as well as clinical studies with TPA023B, TPA023 and MRK-409 is the extent to which metabolites of the parent drugs may be responsible for the observed pharmacological actions. As regards TPA023B, after intravenous or oral dosing of [^{14}C]-labelled compound to rats, only relatively low levels of metabolites of the isopropyl group (hydroxylated and demethylated versions of the parent) were detected in plasma, whereas only TPA023B could be detected in the brain (unpublished observations). Furthermore, the in vitro metabolite profile produced by incubating TPA023B with human hepatocytes was qualitatively similar to that produced by rat hepatocytes. These data indicate, therefore, that the effects observed with TPA023B are indeed due to parent compound rather than metabolite(s). Similarly, the pharmacological actions of TPA023 and MRK-409 are also attributable to parent compound (Atack, 2009; Atack et al., 2010).

Properties of TPA023B in man

In the human PET studies, 1.5 mg TPA023B did not produce any sedation (Table 4). Moreover, of the six adverse events noted in the three subjects studied, all were considered definitely not drug-related (Van Laere et al., 2008). There were some signs of somnolence in two out of the three cohorts dosed with 2 mg, yet this was not a consistent observation, since in a third cohort there were no signs of somnolence/drowsiness (Table 4). The adverse events were self-reported and/or observed, and are therefore subjective rather than objective measurements, which may, in part, explain the variability between different cohorts.

α5-containing $GABA_A$ receptors have been implicated in cognitive function, with inverse agonism at this subtype enhancing cognitive function (Dawson et al., 2006) and

agonism presumed to be associated with cognitive impairment. However, despite TPA023B possessing significant partial agonist efficacy at the α5 subtype (relative efficacy = 0.37), cognitive impairment did not feature as a significant adverse event, even at the poorly tolerated dose of 3 mg.

TPA023B produced long-lasting occupancy significant with a single dose of 1.5 mg giving 53 ± 1% occupancy 5 h after dosing and 46 ± 6% after 24 h. This prolonged occupancy is consistent with the long plasma half-life of this compound in man (~36 h) and the observation that plasma drug concentrations 5 and 24 h post dose were 7.6 ± 0.7 and 5.6 ± 0.7 ng/mL indicates that occupancy tracks plasma drug concentration. The plasma EC_{50} in man, 5.8 ng/mL, was slightly less than in baboon (10 ng/mL), which in turn was around half that observed in rodents (EC_{50} values = 19 and 25 ng/mL in rat and mouse, respectively), a trend also observed with TPA023 (Table 6).

The fact that TPA023B and TPA023 both gave 50% occupancy in man using respective single doses of 1.5 and 2.0 mg without sedation being observed clearly differentiates these compounds as being much less sedating than existing non-selective benzodiazepines that cause sedation at occupancies of less than 25%. For example, clonazepam, which produced sleep and ataxia at a dose (0.03 mg/kg p.o.) that occupied only 15–24% of benzodiazepine sites (Shinotoh et al., 1989) whereas, doses of diazepam (30 mg p.o.) and alprazolam (0.5 mg every 6 h) that caused sedation were associated with respective occupancies of 24% and 16% (Fujita et al., 1999; Pauli et al., 1991). In addition, a 20 mg dose of the α1-subtype-preferring hypnotic compound zolpidem produced 26–29% occupancy (at a plasma drug concentration of ~100 ng/mL), suggesting that a typical clinically efficacious dose of zolpidem (10 mg) would correspond to around 15% occupancy (Abadie et al., 1996). The decreased sedative liability of TPA023 relative to non-selective benzodiazepine full agonists is also reflected in its reduced liability in quantitative measures of sedative liability such as saccadic eye movement latency, body sway and a visual analogue scale measure of alertness relative to lorazepam (de Haas et al., 2007).

The main outcome from the present studies was that in man TPA023B was more like TPA023, in that it did not cause sedation in healthy young male volunteers even at relatively high levels of occupancy (~50%) compared with MRK-409, which was associated with sedation at relatively low levels of occupancy (Atack et al., 2010; de Haas et al., 2008). This similarity of TPA023B and TPA023 is also reflected preclinically by the fact that in rats TPA023B generalizes to TPA023 but not lorazepam (or zolpidem), suggesting that the interoceptive cues produced by both compounds are comparable (Kohut and Ator, 2008). In addition, in baboons TPA023 did not generalize to lorazepam, nor was it self-administered, suggesting it has no abuse potential (Ator, 2005). Given that in rats TPA023B generalizes to TPA023 yet the latter compound has no abuse potential in baboons, it is tempting to assume that TPA023B might also have no abuse potential, although this obviously needs to be demonstrated experimentally.

A comparison of the efficacy profiles of TPA023 and MRK-409 does not provide many clues as to which subtype may be responsible for the sedating effects of MRK-409. Hence, relative to TPA023, MRK-409 has increased efficacy at all four subtypes (Figure 12). However, a comparison of the efficacy profiles of TPA023B and MRK-409 suggests that the α2, α3 and α5 subtypes are not associated with the sedating effects of MRK-409; rather, the sedating effects of MRK-409 are likely mediated by the α1 subtype (Table 7). More specifically, the major difference in the efficacy profiles of these two compounds is that TPA023B is an antagonist at the α1 subtype whereas MRK-409 is a weak partial agonist. Moreover, although the α1 efficacy of MRK-409 is relatively weak (relative efficacy = 0.18), this does nevertheless appear to be sufficient to cause sedation in man. As a corollary, these data also indicate that humans are particularly sensitive to even very modest levels of α1 agonist efficacy, highlighting the need for antagonist efficacy at this $GABA_A$ subtype.

In summary, the clinical experience with TPA023B confirms the preclinical observations that this compound has a much reduced sedative liability in man relative to existing nonselective benzodiazepines, such as diazepam, lorazepam or alprazolam. Moreover, the lack of sedation observed with TPA023B suggests that the sedation observed in man with MRK-409 (Atack et al., 2010) is probably associated with the weak partial agonism of this latter compound at the α1 subtype. However, it is worth adding the caveat that α1 efficacy per se may not be the sole determinant of sedative liability in man, since the pyrazolopyrimidine Ocinaplon and its major metabolite both have appreciable efficacy at the α1 subtype yet do not produce sedation in man (Berezhnoy et al., 2008; Lippa et al., 2005) and suggest that there are aspects of $GABA_A$ receptor subtype pharmacology which remain

Table 7. Potential role of different $GABA_A$ subtypes in mediating the sedating effects of MRK-409

$GABA_A$ subtype	Association with sedating effects of MRK-409	Reasoning
α1	Probable	MRK-409, which causes sedation, has α1 efficacy that is much greater than TPA023 and TPA023B, both of which do not produce sedation
α2	Unlikely	TPA023B has greater α2 efficacy than MRK-409 but is non-sedating
α3	Unlikely	TPA023B and MRK-409 have similar α3 efficacies, yet TPA023B is non-sedating, whereas MRK-409 produces marked sedation
α5	Unlikely	TPA023B has greater α5 efficacy than MRK-409 but is non-sedating

poorly understood. Nevertheless, the fact that at the very least subtype-selective $GABA_A$ receptor modulators can produce effects in man that are distinct from the non-selective benzodiazepines, namely a reduced sedation liability (albeit without as yet clinically proven anxiolytic efficacy; de Haas et al., 2007) should encourage the pursuit of such novel compounds not only as potential non-sedating anxiolytics (Mirza et al., 2008) but also for other indications such as schizophrenia (Lewis et al., 2008) and pain (Knabl et al., 2008; Munro et al., 2008, 2009).

MRK-409, 7-cyclobutyl-6-(2-methyl-2*H*-1,2,4-triazol-3-ylmethoxy)-3-(2,6-difluorophenyl)-1,2,4-triazolo[4,3-*b*]pyridazine; PET, positron emission tomography; TPA023, 7-(1,1-Dimethylethyl)-6-(2-ethyl-2*H*-1,2,4-triazol-3-ylmethoxy)-3-(2-fluorophenyl)-1,2,4-triazolo[4,3-*b*]pyridazine; TPA023B, 6,2'-difluoro-5'-[3-(1-hydroxy-1-methylethyl)imidazo[1,2-*b*][1,2,4]triazin-7-yl]biphenyl-2-carbonitrile.

Acknowledgements

The authors would like to acknowledge Lingling Xue and Rohini Ramakrishnan for their excellent support in the analysis of clinical samples.

References

Abadie P, Rioux P, Scatton B, etal. (1996) Central benzodiazepine receptor occupancy by zolpidem in the human brain as assessed by positron emission tomography. Eur J Pharmacol 295: 35–44.

Atack JR (2005) The benzodiazepine binding site of $GABA_A$ receptors as a target for the development of novel anxiolytics. *Expert Opin Investig Drugs* 14: 601–618.

Atack JR (2009) Subtype-selective $GABA_A$ receptor modulation yields a novel pharmacological profile: The design and development of TPA023. *Adv Pharmacol* (in press).

Atack JR, Hutson PH, Collinson N, et al. (2005) Anxiogenic properties of an inverse agonist selective for α3 subunit-containing $GABA_A$ receptors. *Br J Pharmacol* 144: 357–366.

Atack JR, Wafford KA, Tye SJ, et al. (2006) TPA023 [7-(1,1-dimethylethyl)-6-(2-ethyl-2H-1,2,4-triazol-3-ylmethoxy)-3-(2-fluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine], an agonist selective for α2- and α3-containing $GABA_A$ receptors, is a nonsedating anxiolytic in rodents and primates. *J Pharmacol Exp Ther* 316: 410–422.

Atack JR, Wafford KA, Street LJ, et al. (2010) MRK-409 (MK-0343), a $GABA_A$ receptor subtype-selective agonist, is a non-sedating anxiolytic in preclinical species but causes sedation in man, *Journal of Psychopharmacology*, in press.

Ator NA (2005) Contributions of $GABA_A$ receptor subtype selectivity to abuse liability and dependence potential of pharmacological treatments for anxiety and sleep disorders. *CNS Spectr* 10: 31–39.

Ballard TM, Knoflach F, Prinssen E, et al. (2009) RO4938581, a novel cognitive enhancer acting at $GABA_A$ α5 subunit-containing receptors. *Psychopharmacol* 202: 207–223.

Barnard EA, Skolnick P, Olsen RW, et al. (1998) International Union of Pharmacology. XV. Subtypes of γ-aminobutyric $acid_A$ receptors: Classification on the basis of subunit structure and receptor function. *Pharmacol Rev* 50: 291–313.

Berezhnoy D, Gravielle MC, Downing S, et al. (2008) Pharmacological properties of DOV 315,090, an ocinaplon metabolite. *BMC Pharmacol* 8: 11.

Cheng VY, Martin LJ, Elliott EM, et al. (2006) α5$GABA_A$ receptors mediate the amnestic but not sedative-hypnotic effects of the general anesthetic etomidate. *J Neurosci* 26: 3713–3720.

Crestani F, Keist R, Fritschy J-M, et al. (2002) Trace fear conditioning involves hippocampal α5 $GABA_A$ receptors. *Proc Natl Acad Sci USA* 99: 8980–8985.

Dawson GR, Maubach KA, Collinson N, et al. (2006) An inverse agonist selective for α5 subunit-containing $GABA_A$ receptors enhances cognition. *J Pharmacol Exp Ther* 316: 1335–1345.

de Haas SL, de Visser SJ, van der Post JP, et al. (2007) Pharmacodynamic and pharmacokinetic effects of TPA023, a $GABA_A$ $\alpha_{2,3}$ subtype-selective agonist, compared to lorazepam and placebo in healthy volunteers. *J Psychopharmacol* 21: 374–383.

de Haas SL, de Visser SJ, van der Post JP, et al. (2008) Pharmacodynamic and pharmacokinetic effects of MK-0343, a $GABA_A$ $\alpha_{2,3}$ subtype selective agonist, compared to lorazepam and placebo in healthy male volunteers. *J Psychopharmacol* 22: 24–32.

Dias R, Sheppard WFA, Fradley RL, et al. (2005) Evidence for a significant role of α3-containing $GABA_A$ receptors in mediating the anxiolytic effects of benzodiazepines. *J Neurosci* 25: 10682–10688.

Fujita M, Woods SW, Verhoeff NPLG, et al. (1999) Changes of benzodiazepine receptors during chronic benzodiazepine administration in humans. *Eur J Pharmacol* 368: 161–172.

Gauthier DR, Jr, Limanto J, Devine PN, et al. (2005) Palladium-catalyzed regioselective arylation of imidazo[1,2-b][1,2,4]triazine: synthesis of an $\alpha_{2/3}$-selective GABA agonist. *J Org Chem* 70: 5938–5945.

Knabl J, Witschi R, Hösl K, et al. (2008) Reversal of pathological pain through specific spinal $GABA_A$ receptor subtypes. *Nature* 451: 330–334.

Kohut SJ, Ator NA (2008) Novel discriminative stimulus effects of TPA023B, subtype-selective γ-aminobutyric-$acid_A$/benzodiazepine modulator: Comparisons with zolpidem, lorazepam, and TPA023. *Biochem Pharmacol Behav* 90: 65–73.

Lewis DA, Cho RY, Carter CS, et al. (2008) Subunit-selective modulation of GABA type A receptor neurotransmission and cognition in schizophrenia. *Am J Psychiatry* 165: 1585–1593.

Lippa A, Czobor P, Stark J, et al. (2005) Selective anxiolysis produced by ocinaplon, a $GABA_A$ receptor modulator. *Proc Natl Acad Sci USA* 102: 7380–7385.

Löw K, Crestani F, Keist R, et al. (2000) Molecular and neuronal substrate for the selective attenuation of anxiety. *Science* 290: 131–134.

McKernan RM, Rosahl TW, Reynolds DS, et al. (2000) Sedative but not anxiolytic properties of benzodiazepines are mediated by the $GABA_A$ receptor α1 subtype. *Nature Neurosci* 3: 587–592.

Mirza NR, Larsen JS, Mathiasen C, et al. (2008) NS11394 [3′-[5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitrile], a unique subtype-selective $GABA_A$ receptor positive allosteric modulator: in vitro actions, pharmacokinetic properties and in vivo anxiolytic efficacy. *J Pharmacol Exp Ther* 327: 954–968.

Möhler H (2007) Molecular regulation of cognitive functions and developmental plasticity: impact of $GABA_A$ receptors. *J Neurochem* 102: 1–12.

Möhler H, Fritschy JM, Rudolph U (2002) A new benzodiazepine pharmacology. *J Pharmacol Exp Ther* 300: 2–8.

Morris HV, Dawson GR, Reynolds DS, Atack JR, Stephens DN (2006) Both α2 and α3 $GABA_A$ receptor subtypes mediate the anxiolytic properties of benzodiazepine site ligands in the conditioned emotional response paradigm. *Eur J Neurosci* 23: 2495–2504.

Munro G, Lopez-Garcia JA, Rivera-Arconada I, et al. (2008) Comparison of the novel subtype-selective $GABA_A$ receptor-positive allosteric modulator NS11394 [3′-[5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitrile] with diazepam, zolpidem, bretazenil, and gaboxadol in rat models of inflammatory and neuropathic pain. *J Pharmacol Exp Ther* 327: 969–981.

Munro G, Ahring PK, Mirza NR (2009) Developing analgesics by enhancing spinal inhibition after injury: $GABA_A$ receptor subtypes as novel targets. *Trends Pharmacol Sci* 30: 453–459.

Pauli S, Farde L, Halldin L, Sedvall G (1991) Occupancy of the central benzodiazepine receptors during benzodiazepine treatment determined by PET. *Eur Neuropsychopharmacol* 1: 229.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G (2000) $GABA_A$ receptors: Immunocytochemical distribution of 13 subunits in the adult brain. *Neurosci* 101: 815–850.

Rudolph U, Crestani F, Benke D, et al. (1999) Benzodiazepine actions mediated by specific γ-aminobutyric $acid_A$ receptor subtypes. *Nature* 401: 796–800.

Russell MGN, Carling RW, Street LJ, et al. (2006) Discovery of imidazo[1,2-b][1,2,4]triazines as $GABA_A$ α2/3 subtype selective agonists for the treatment of anxiety. *J Med Chem* 49: 1235–1238.

Sanger DJ (2004) The pharmacology and mechanisms of action of new generation, non-benzodiazepine hypnotic agents. *CNS Drugs* 18(Suppl 1): 9–15.

Shinotoh H, Iyo M, Yamada T, et al. (1989) Detection of benzodiazepine receptor occupancy in the human brain by positron emission tomography. *Psychopharmacology* 99: 202–207.

Sieghart W (1995) Structure and pharmacology of γ-aminobutyric $acid_A$ receptor subtypes. *Pharmacol Rev* 47: 181–234.

Sieghart W (2006) Structure, pharmacology, and function of $GABA_A$ receptor subtypes. *Adv Pharmacol* 54: 231–263.

Sieghart W, Sperk G (2002) Subunit composition, distribution and function of $GABA_A$ receptor subtypes. *Curr Top Med Chem* 2: 795–816.

Sigel E (2002) Mapping of the benzodiazepine recognition site on $GABA_A$ receptors. *Curr Top Med Chem* 2: 833–839.

Simon J, Wakimoto H, Fujita N, Lalande M, Barnard EA (2004) Analysis of the set of $GABA_A$ receptor genes in the human genome. *J Biol Chem* 279: 41422–41435.

Smith AJ, Alder L, Silk J, et al. (2001) Effect of α subunit on allosteric modulation of ion channel function in stably expressed human recombinant γ-aminobutyric $acid_A$ receptors determined using ^{36}Cl ion flux. *Mol Pharmacol* 59: 1108–1118.

Van Laere K, Bormans G, Sanabria-Bohórquez SM, et al. (2008) In vivo characterization and dynamic receptor occupancy imaging of TPA023B, an α2/α3/α5 subtype selective γ-aminobutyric acid-A partial agonist. *Biol Psychiatry* 64: 153–161.

Wieland HA, Lüddens H, Seeburg PH (1992) A single histidine in $GABA_A$ receptors is essential for benzodiazepine agonist binding. *J Biol Chem* 267: 1426–1429.