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MRK-409 (MK-0343), a $GABA_A$ receptor subtype-selective partial agonist, is a non-sedating anxiolytic in preclinical species but causes sedation in humans

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Abstract

MRK-409 binds to α1-, α2-, α3- and α5-containing human recombinant $GABA_A$ receptors with comparable high affinity (0.21–0.40 nM). However, MRK-409 has greater agonist efficacy at the α3 compared with α1 subtypes (respective efficacies relative to the full agonist chlordiazepoxide of 0.45 and 0.18). This compound readily penetrates the brain in rats and occupies the benzodiazepine site of $GABA_A$ receptors, measured using an in vivo [^{3}H]flumazenil binding assay, with an Occ_{50} of 2.2 mg/kg p.o. and a corresponding plasma EC_{50} of 115 ng/mL. Behaviourally, the α3-preferring agonist efficacy profile of MRK-409 produced anxiolytic-like activity in rodent and primate unconditioned and conditioned models of anxiety with minimum effective doses corresponding to occupancies, depending on the particular model, ranging from ~35% to 65% yet there were minimal overt signs of sedation at occupancies greater than 90%. In humans, however, safety and tolerability studies showed that there was pronounced sedation at a dose of 2 mg, resulting in a maximal tolerated dose of 1 mg. This 2 mg dose corresponded to a C_{max} plasma concentration of 28 ng/mL, which, based on the rodent plasma EC_{50} for occupancy of 115 ng/mL, suggested that sedation in humans occurs at low levels of occupancy. This was confirmed in human positron emission tomography studies, in which [^{11}C]flumazenil uptake following a single dose of 1 mg MRK-409 was comparable to that of placebo, indicating that occupancy of $GABA_A$ receptor benzodiazepine binding sites by MRK-409 was below the limits of detection (i.e. <10%). Taken together, these data show that MRK-409 causes sedation in humans at a dose (2 mg) corresponding to levels of occupancy considerably less than those predicted from rodent models to be required for anxiolytic efficacy (~35–65%). Thus, the preclinical non-sedating anxiolytic profile of MRK-409 did not translate into humans and further development of this compound was halted.

Keywords

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

Introduction

The many pharmacological actions of benzodiazepines, including sedation, anxiolysis, myorelaxation and the anticonvulsant and cognition impairing activities, are mediated via interactions with a specific recognition site on $GABA_A$ receptors. These receptors are composed of various combinations of the $GABA_A$ gene family (α1-6, β1-3, γ1-3, δ, ε, θ, π; Simon et al., 2004). More specifically, those that contain a benzodiazepine binding site comprise a β, γ2 and either α1, α2, α3 and α5 subunits (Sieghart, 2006) arranged in a pentamer with a α:β:γ subunit stoichiometry of 2:2:1 (Sieghart and Sperk, 2002). Around three-quarters of the total number of brain $GABA_A$ receptors contain a benzodiazepine binding site (McKernan and Whiting, 1996), the location of which is at the interface of the γ2 and α subunits (Sieghart, 2006). The pharmacology of this site is dictated to a large extent by the α subunit (Sieghart, 2006); an observation best illustrated by the fact that receptors containing either an α4 or α6 subunit do not bind diazepam, a property that can be solely attributed to a single amino acid (Wieland et al., 1992).

The fact that the pharmacological effects of benzodiazepines are mediated by four different $GABA_A$ subtypes, namely those receptors containing either an α1, α2, α3 or α5 subunit, has led to the search for compounds which

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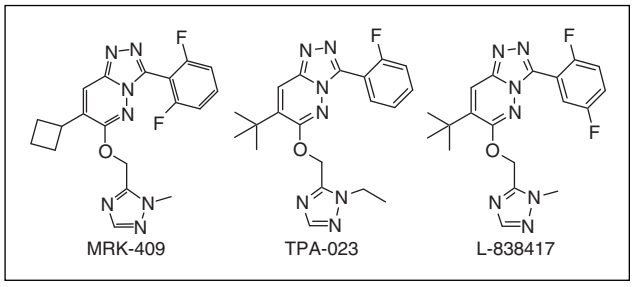

Figure 1. Comparison of the structures of 7-cyclobutyl-6-(2-methyl-2*H*-1,2,4-triazol-3-ylmethoxy)-3-(2,6-difluorophenyl)-1,2,4-triazolo[4,3-*b*] pyridazine (MRK-409, also known as MK-0343 (de Haas et al., 2008)), TPA023 (Atack et al., 2006) and L-838417 (McKernan et al., 2000).

selectively interact with only certain of these subtypes and might therefore possess novel pharmacological profiles (Atack, 2005; Maubach, 2003). These studies have been facilitated by the use of transgenic mouse models (Rudolph and Mohler, 2004) that have helped ascribe particular pharmacological properties of benzodiazepines to certain $GABA_A$ subtypes. As a result, compounds which selectively interact with the α2/α3 subtypes have been reported to be non-sedating anxiolytics in preclinical species (Atack et al., 2006; McKernan et al., 2000; Mirza et al., 2008) whilst α5 selective compounds have been shown to enhance cognition in rodent models (Ballard et al., 2009; Chambers et al., 2004; Dawson et al., 2006). It should be emphasized that to date there is no data to suggest that these novel preclinical profiles translate into clinical efficacy and that the predictive value of the preclinical assays of such novel compounds remains to be established.

In the present study, we describe the properties of a compound, 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(MRK-409, also known as MK-0343; de Haas et al., 2008), which is structurally related to the previously-described compounds TPA023 and L-838417 (Figure 1 and Atack et al., 2006; McKernan et al., 2000). More specifically, the anxiolytic-like and sedative properties of this compound were evaluated in preclinical species (rodents and non-human primates) following which the safety, tolerability, pharmacokinetics and receptor occupancy of MRK-409 were assessed in healthy young male volunteers.

Materials and methods

All animal procedures were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines.

Drugs

MRK-409 was synthesized on the milligram scale using methods analogous to those described elsewhere (Carling et al., 2005). The gram-scale synthesis was performed as summarized in Figure 2. Briefly, 2,6-difluorobenzoyl chloride was reacted with 3-chloro-4-cyclobutyl-6-hydrazinopyridazine (3) to produce 6-chloro-7-cyclobutyl-3-(2,6-difluorophenyl)-pyridazine (4). This latter product was coupled with (2-methyl-(1,2,4)triazol-3-yl)methanol to produce the final compound

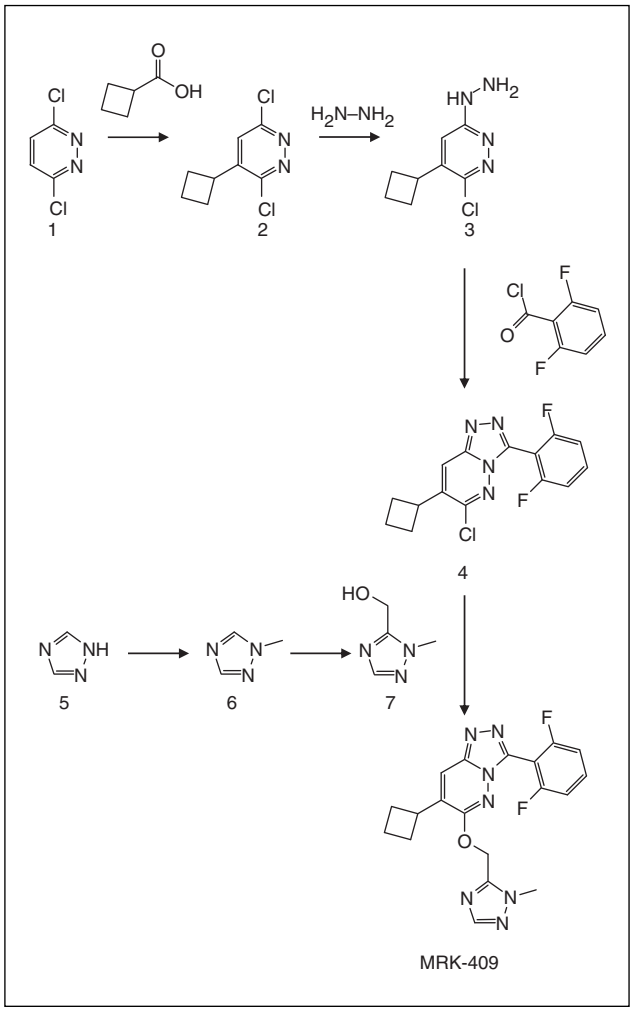

Figure 2. Scheme for the synthesis of gram quantities of MRK-409. Initial, smaller scale synthesis was carried out using methods described previously for other triazolopyridazines in this series (Carling et al., 2005). Briefly, the reaction of cyclobutane carboxylic acid with 3,6-dichloropyridazine (1) produced 2 which was then reacted with hydrazine to produce the hydrazinopyridazine 3, the latter of which was reacted with 2,6-difluorobenzoyl chloride to produce the triazolopyridazine 4. The 1,2,4-triazole 5 was first *N*-methylated (6) and then hydroxymethylated (7) before being coupled to the triazolopyridazine 4 to produce 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; MRK-409). 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 of MRK-409 for the benzodiazepine binding site of $GABA_A$ receptors was measured in mouse fibroblast

L(tk$^-$) cells expressing human recombinant $GABA_A$ receptors containing β3, γ2 plus either α1, α2, α3, α4, α5 or α6 subunits using [^{3}H]flumazenil for α1-, α2-, α3- and α5-containing receptors or [^{3}H]Ro 15-4513 for the α4- and α6-containing subtypes: (Hadingham et al., 1993, 1996; Wafford et al., 1996). The affinity of MRK-409 for native rat cerebellum and spinal cord P2 membranes was measured using [^{3}H]flumazenil as described elsewhere (Atack et al., 2006).

The same cell lines were used to measure the intrinsic efficacy of MRK-409 using whole cell patch clamp electrophysiology (Atack et al., 2006; Brown et al., 2002). Having separately established the concentration of GABA that produced a maximal response, the intrinsic efficacy of MRK-409 was defined as the ability of the compound (applied for 30 sec prior to a 5 sec application of a GABA) to modulate the currents produced by a GABA concentration equivalent to an EC_{20}. A concentration of the non-selective benzodiazepine agonist chlordiazepoxide which produced a maximal modulation (3 μM) was used as an internal standard and gave an increase in the GABA EC_{20}-equivalent current amplitude in the region of 75–150%. Concentration-effect curves were constructed for each cell with curve-fitting being performed using a non-linear least squares method (GraphPad Prism, GraphPad Software Inc., San Diego, CA, USA). From these analyses, the maximum efficacy and an EC_{50} were determined for each cell (Atack et al., 2006; Brown et al., 2002). In addition, the maximum efficacy was also expressed relative to that observed with chlordiazepoxide (3 μM).

MRK-409 $GABA_A$ receptor occupancy

The inhibition of the in vivo binding of [^{3}H]flumazenil by MRK-409 was used to measure the occupancy of rat (male 260–300g Sprague-Dawley; B&K Universal, Hull, UK) or mouse (male 23–31g Swiss-Webster; B&K Universal) brain $GABA_A$ receptor benzodiazepine binding sites (Atack et al., 2006). Rat occupancy studies were performed following p.o. dosing of MRK-409 (1 mL/kg in 0.5% methyl cellulose vehicle) in either separate in vivo binding studies (3, 10 and 30 mg/kg dose–response curve or 3 mg/kg 1 h vs. 6 h comparison) or in conjunction with elevated plus maze experiments (0.3, 1 and 3 mg/kg and 1, 2 and 3 mg/kg studies). Mouse brain occupancy was determined immediately after rotarod experiments using oral doses of 1, 3 or 10 mg/kg MRK-409 (10 mL/kg in 0.5% methyl cellulose). 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.).

Animals pretreated with either vehicle or drug were given an intravenous (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, 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 order to establish the extent of non-specific in vivo binding of [^{3}H]flumazenil, separate groups of rats or mice were given a dose of bretazenil (5 mg/kg i.p. in 100% PEG 300) that occupies essentially all rat or mouse brain benzodiazepine binding sites. In rat 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 high performance liquid chromatography (HPLC) (Kromasil KR100 C18 150 mm × 3.2 mm column with a 2:3 ratio 25 mM ammonium formate pH 3.0:acetonitrile mobile phase) and tandem mass spectrometry detection (mass transition 398.2 to 370.1 a.m.u.).

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

In order to establish the anxiolytic properties of MRK-409, three different assays were employed, one using an ethological measure of anxiety (the elevated plus maze) and two using conditioned models of anxiety (fear-potentiated startle and conditioned suppression of drinking). An abbreviated description of these methods is outlined below, although more detailed methodology is presented elsewhere (Atack et al., 2006).

Elevated plus maze: Male Sprague-Dawley rats (approximately 250–300g, $n = 17$–18/group; B&K Universal, Hull, UK) were given p.o. doses of either vehicle (0.5% methyl cellulose; dose volume = 1 mL/kg), and either in an initial experiment, 0.3, 1 or 3 mg/kg, or a subsequent experiment, 1, 2 or 3 mg/kg MRK-409. Additional animals were given the benzodiazepine full agonist chlordiazepoxide as a positive control (5 mg/kg i.p. in isotonic saline). Half an hour later rats were given a 5 min trial on the elevated plus maze (Dawson and Tricklebank, 1995) with their movements being monitored by a video tracking system. The primary measure of anxiolytic-like activity was the percent time spent on the open arms of the maze, with increases in this parameter being used as an index of anxiolytic activity. At completion of the trial, alternate animals were taken and occupancy measured as described above.

Fear-potentiated startle: Male Sprague–Dawley rats (260–310 g, $n = 15$/group; B&K Universal) were trained to associate the presentation of a light with a mild foot-shock. Following this training period, rats were dosed (p.o.) with either vehicle (0.5% methyl cellulose, 1 mL/kg) or MRK-409 (0.3, 1 or 3 mg/kg) and 0.5 h later placed in the startle apparatus (San Diego Instruments, San Diego, CA, USA). Rats were allowed to acclimatize for 5 min following

which they received 10 × 100 dB tones (50 msec duration, 30 sec inter-trial interval) to partially habituate them to the startle stimulus. During the test session itself, rats were presented with random noise stimuli (10 × 100 dB, half given 3200 msec after the presentation of the conditioning, light stimulus and half given in darkness). All responses were measured over a 100 msec window beginning immediately upon presentation of the tone and the mean startle amplitude for each stimulus type was calculated. For each animal, the startle amplitude in the light minus the startle amplitude in the dark (the 'difference score') was also calculated.

Conditioned suppression of drinking: Water-deprived male hooded-Lister rats (200–250 g; B&K Universal) were trained to lick a metal spout for a reward of 0.1 mL water (Dawson et al., 1994). Following this training period, a house light was illuminated for 60 sec and in the last second a 0.4-mA foot-shock was delivered; after only three light-shock pairings, the licking rates (as measured by the ratio of the lick rates during compared with prior to presentation of the light) were markedly suppressed. Rats that had learned the light–shock association (as judged by a suppression ratio of $\leq$0.25) were dosed with either vehicle (0.5% methyl cellulose, 1 mL/kg p.o.) or one of three doses of MRK-409 (1, 3, 10 mg/kg p.o.) 0.5 h prior to the test session ($n = 11$–12/group). A significant increase in the suppression ratio was used as an indicator of anxiolytic-like activity.

Rodent sedation assays

Rat chain-pulling assay: Male PVG rats (270–350g; B&K Universal) were trained to pull a chain in order to receive a food pellet reward. On the testing day, rats ($n = 12$/group) were dosed p.o. with either vehicle (0.5% of methyl cellulose, 1 mL/kg), MRK-409 (10 or 30 mg/kg) or diazepam (10 mg/kg) and then immediately placed in the testing chamber with chain-pulling activity being measured 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.), MRK-409 (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 latency 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 MRK-409 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. In other words, ethanol was given immediately prior to p.o. doses of either vehicle, MRK-409 or diazepam.

Primate anxiolysis and sedation assays

Conditioned emotional response: Twelve adult male squirrel monkeys (*Saimiri sciureus*, 0.7–1.2 kg) were trained to press a lever to obtain a fruit juice reward. In subsequent sessions, a red cue light was illuminated for 60 sec and during the last 0.5 sec there was a 10% chance of a mild electric tail-shock (1–7 mA) being delivered. The association between the red light and a possible tail-shock produced a marked reduction in lever pressing (Atack et al., 2006).

On drug-testing days, animals were given either vehicle (0.5% methyl cellulose, 2 mL/kg p.o.) or MRK-409 (0.03 and 0.1 mg/kg p.o. in Experiment 1 or 0.3, 1 or 3 mg/kg p.o. in Experiment 2) 0.5 h prior to testing in a pseudo-Latin square design in which every animal received vehicle and each dose of MRK-409. On test days, the schedule was as above except that tail-shocks were not delivered when the cue light was illuminated. The suppression of lever pressing produced by the cue light (ratio of responding during and prior to illumination of the red light) was measured with a anxiolytic-like activity being defined as a significant increase in the suppression ratio relative to vehicle-treated animals.

Squirrel monkey lever pressing: This assay is analogous to the rat chain pulling assay in so far as behavioural disruption, for example sedation or myorelaxation, is assessed by the ability of a compound to reduce the rate of responding to obtain a fruit juice reward. Nine adult male squirrel monkeys (*S. sciureus*, 0.7–1.2 kg) were trained to press a lever to obtain a fruit juice reward. On the test day, animals were dosed with vehicle (0.5% methyl cellulose, 2 mL/kg p.o.) or MRK-409 (1, 3 or 10 mg/kg p.o.) in a pseudo-Latin square design with every animal receiving, on separate days, vehicle and each dose of MRK-409. The rate of lever pressing measured on the test day was expressed as a percentage of that observed on the preceding day.

Human studies

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

Positron emission tomography: PET studies were performed at the Katholieke Universiteit Leuven, Belgium using an HR + PET camera (Siemens, Germany) as described elsewhere (Van Laere et al., 2008). In brief, prior to the first scan, a radial artery cannula was inserted under local anaesthesia for blood sampling and a venous cannula was inserted for tracer injection. For each scan a bolus of approximately 7.5 mCi of [^{11}C]flumazenil was injected and scans consisted of 20 frames with a progressive increase in frame duration (4 × 0.25, 4 × 1, 2 × 2.5, 10 × 5 min) to give a total duration of 1 h. A metabolite-corrected arterial input function for the tracer was generated from blood sampling during the scan by measuring the plasma concentration of [^{11}C]flumazenil and its labelled metabolites. Two 1-h scans were performed, one

commencing 1.5 to 0.5 h before and the second 1.5 to 2.5 h after dosing with either placebo or 1 mg MRK-409.

Separately, standard T1 weighted volumetric MRI scans were obtained for each subject and these were used for the manual anatomical delineation of the occipital cortex, frontal cortex, cerebellum and pons as regions of interest (ROIs). The [^{11}C]flumazenil PET images obtained in the second scans were aligned with the first study in order to define the same ROIs and [^{11}C]flumazenil time–activity curves (TACs) were generated for each ROI. These [^{11}C]flumazenil TACs were used together with the metabolite-corrected plasma curve and a single-tissue compartment model to estimate the rate constant for forward capillary exchange (K_1, expressed in mL/min/cm^3 tissue), the total volume of distribution of flumazenil (V_T) and the cerebral blood volume. V_T is proportional to the ratio of benzodiazepine binding sites (Salmi et al., 2008) 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. Comparison of the V_T values obtained from the [^{11}C]flumazenil scans provides information on receptor occupancy at the studied times.

Safety, tolerability and pharmacokinetics: An escalating single oral dose study was performed in healthy young (18–45 years old) male volunteers using doses of 0.05, 0.1, 0.25, 0.5, 1.0, 1.5 and 2.0 mg MRK-409. Tolerability was assessed by careful questioning for adverse events, ECGs, monitoring of vital signs and laboratory safety. Blood samples were removed 0.5, 1, 2, 4, 6, 8, 12, 16 and 24 h after dosing and plasma drug concentrations were measured.

Results

In vitro affinity and efficacy of MRK-409

MRK-409 has equivalent high affinity for the benzodiazepine site of human recombinant $GABA_A$ receptors containing either an α1, α2, α3 or α5 subunit (*Ki* values ranging from 0.21 to 0.40 nM) but its affinity for the α4 and α6 subtypes (78 and 980 nM, respectively) was much lower (Table 1). The affinities measured in native rat cerebellum and spinal cord receptors (0.28 and 0.27 nM, respectively; Table 1) were similar to those seen in human recombinant receptors (0.21–0.40 nM).

Whole cell patch clamp electrophysiology showed that MRK-409 potentiated the GABA EC_{20}-induced currents in human recombinant α1-, α2-, α3- and α5-containing $GABA_A$ receptors expressed in mouse fibroblast L(tk$^-$) cells by 20, 36, 74 and 26%, respectively (Figure 3, Table 2). When expressed in comparison with the non-selective agonist chlordiazepoxide, the respective relative efficacy values of MRK-409 were 0.18, 0.23, 0.45 and 0.18 at the α1, α2, α3 and α5 subtypes (inset, Figure 3). Hence, the maximal efficacy of MRK-409 was higher at the α3-subtype than at other $GABA_A$ subtypes. A similar profile (i.e. α1 efficacy < α2 and/or α3) was also observed in human $GABA_A$ receptors transiently-expressed in *Xenopus laevis* oocytes, rat α1 and α3 subtypes transiently-expressed in human embryonic kidney cells as well as in L(tk$^-$) stably-expressing the human subtypes but using a [$^{36}Cl^-$] flux rather than electrophysiological assay (data not shown).

In comparison, the prototypic non-selective benzodiazepine diazepam has an affinity at the different $GABA_A$ receptors ranging from 11 to 20 nM (Atack et al., 1999). Moreover, at each subtype diazepam has an efficacy comparable to chlordiazepoxide (a potentiation of a GABA EC_{20} in the region of 100–150%; Dawson et al., 2006) such that at each

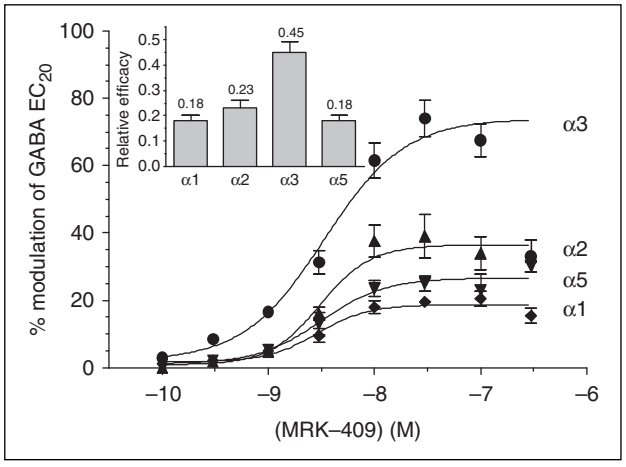

Figure 3. Efficacy of MRK-409 at human recombinant $GABA_A$ receptors. Potentiation of GABA EC_{20}-equivalent currents in human recombinant $GABA_A$ receptors containing β3, γ2 plus either α1, α2, α3 or α5 subunits stably expressed in mouse L(tk$^-$) cells was measured using whole-cell patch clamp electrophysiology. Inset shows maximal potentiation at each cell type expressed relative to the full agonist chlordiazepoxide. Data shown is the mean ± SEM (n = 5–6).

Table 1. Affinity of MRK-409 for the benzodiazepine site of recombinant human and native rat brain $GABA_A$ receptors

Ki, nM							
Human recombinant $GABA_A$ receptors containing β3, γ2 plus						Native rat brain receptors	
α1	α2	α3	α4	α5	α6	Cerebellum	Spinal cord
0.22 ± 0.02	0.40 ± 0.07	0.21 ± 0.03	78 ± 12	0.23 ± 0.04	980 ± 160	0.28 ± 0.06	0.27 ± 0.04

Data shown is the mean ± SEM (n = 6–8 separate determinations).

of the α1, α2, α3 and α5 subtypes its efficacy relative to chlordiazepoxide is ~1.0.

In vivo binding

Following oral dosing, MRK-409 was rapidly absorbed, with occupancy 1 h after a 3 mg/kg dose being 77 ± 6% (Figure 4A). However, by 6 h post-dose, occupancy fell to 36 ± 5%. Plasma drug concentrations were measured in terminal samples collected from these same animals and showed that the drop in occupancy from 77% at 1 h post-dose to 36% at 6 h was associated with a decrease in plasma drug concentrations from 241 ± 35 ng/mL to 47 ± 9 ng/mL. This data is consistent with more detailed pharmacokinetic studies in which the time at which maximum plasma drug concentrations were achieved following oral dosing was, depending upon dose, between 0.5 and 1 h (data not shown).

In order to calculate the Occ_{50} for MRK-409 at 0.75–1 h after dosing, data from a number of separate studies (the 1 and 6 h time course, the two elevated plus maze experiments and a 3, 10, 30 mg/kg dose–response curve) were combined (Figure 4B). There was a degree of experiment-to-experiment variability at some doses but not at others. For example, a dose of 1 mg/kg gave occupancy of 11% and 31% in separate studies whereas at a dose of 3 mg/kg, data was much less variable (occupancy of 52, 62, 74 and 77%). Nevertheless, these data show that between doses of 0.3 and 30 mg/kg, and at 0.75–1 h post-dose, occupancy was dose-dependent with an Occ_{50} of 2.2 mg/kg and a Hill slope of 1.65 (Figure 4B). Based upon these values, the percent occupancy at doses ranging between 0.3 and 30 mg/kg was calculated (Table 3).

Plasma samples were collected from the various rat occupancy studies and plasma drug concentrations measured. This allowed occupancy to be plotted as a function of plasma MRK-409 drug concentrations (Figure 5). This data was fitted to a single-site model with a Hill slope of 1.11 and the mean plasma level required to occupy 50% of brain receptors was 115 ng/mL (equivalent to a plasma concentration of 290 nM).

MRK-409 has anxiolytic-like activity in rats

Elevated plus maze: Two separate elevated plus maze experiments were performed, the first using doses of 0.3, 1 and 3 mg/kg p.o. (Experiment 1) and the second 1, 2 and 3 mg/kg (Experiment 2), with Figure 6 showing the time spent on the open arms of the maze expressed as a percent of the total time (5 min). In both Experiment 1 and

Table 2. Efficacy of MRK-409 at human recombinant $GABA_A$ receptors containing different α subunits stably expressed in mouse fibroblast L(tk⁻) cells

Human recombinant $GABA_A$ receptors containing β3, γ2 plus				
	α1	α2	α3	α5
Maximum modulation	20 ± 2%	36 ± 5%	74 ± 5%	26 ± 2%
Efficacy relative to CDP	0.18 ± 0.02	0.23 ± 0.03	0.45 ± 0.04	0.18 ± 0.02
EC_{50} (nM)	3.2 ± 0.6	3.1 ± 0.3	3.4 ± 0.5	3.0 ± 0.6

CDP = chlordiazepoxide.
Values shown are mean ± SEM (n = 5–6).

Table 3. Occupancy of rat brain benzodiazepine binding sites estimated for various p.o. doses of MRK-409

Dose (mg/kg, p.o.)	Estimated % occupancy
0.3	4
1	22
3	65
10	93
30	99

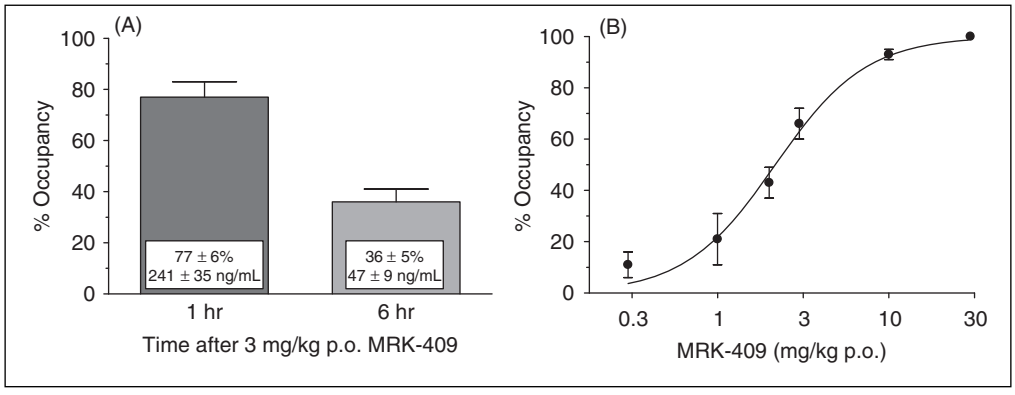

Figure 4. Occupancy of rat brain benzodiazepine binding sites by MRK-409. (A) Following a dose of 3 mg/kg (p.o.) occupancy was greater at 1 h (77 ± 6%) compared with 6 h (36 ± 5%) after dosing. Values shown are mean ± SEM (n = 6/group). (B) Occupancy 0.75–1 h after dosing was dose-dependent with an Occ_{50} of 2.2 mg/kg. Values shown are mean ± SEM (n = 5–18 animals measured in 1–4 separate experiments).

Experiment 2, vehicle-treated animals spent a similar proportion of time on the open arms (16 ± 2% and 17 ± 1%, respectively). Similarly, chlordiazepoxide produced a significant increase in time spent on the open arms in both Experiment 1 (time on open arms, 35 ± 4%) and Experiment 2 (31 ± 3%) at comparable levels of receptor occupancy (respective occupancy values = 30 ± 2% and 24 ± 2%). MRK-409 induced a dose-proportional increase in the time spent on the open arms which achieved significance at doses of 3 mg/kg in Experiment 1 and 2 mg/kg in Experiment 2, corresponding to receptor occupancies of 74 ± 3% and 43 ± 6%, respectively.

Fear-potentiated startle: Figure 7 shows that MRK-409 attenuated fear-potentiated startle in the rat. Baseline startle responses recorded in the dark were not significantly different in rats treated with any of the doses of MRK-409 (0.3–3 mg/kg p.o.), consistent with MRK-409 not having overt sedative properties (Figure 7A). However, there was a dose-dependent decrease in the startle responses recorded during presentation of the conditioning stimulus (light) but this was only significantly from vehicle-treated rats at a dose of 3 mg/kg (Figure 7A). With regard to the differences scores (i.e. the fear-potentiated startle) MRK-409 again produced a dose-dependent decrease which was significantly different from vehicle at a dose of 3 mg/kg. Unlike the elevated plus maze experiments, occupancy was not measured directly after testing (Figure 7B). Nevertheless, in comparison with data from other experiments, the minimal effective dose of 3 mg/kg p.o. corresponds to 65% occupancy (Table 3).

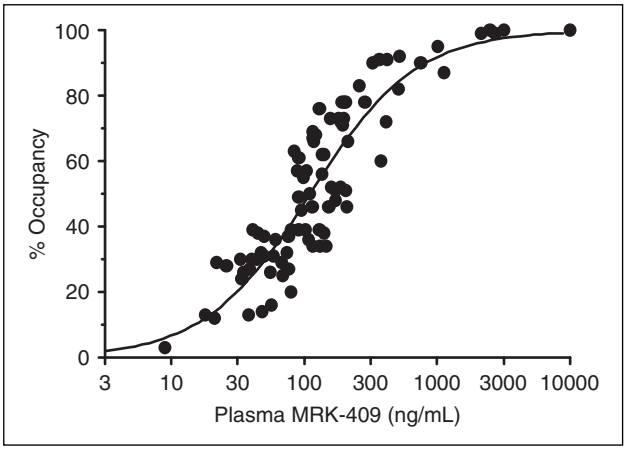

Figure 5. Relationship between plasma drug concentrations and occupancy in rat brain. Each data point represents an individual animal ($n = 90$). The EC_{50} was 115 ng/mL with the Hill slope being 1.11.

Conditioned suppression of drinking: The rate at which rats trained to associate the presentation of a light with an electric shock licked a waterspout prior to and during presentation of the conditioning stimulus (i.e. light) is shown in Figure 8A. This data demonstrates that prior to the presentation of the light stimulus the lick rate was not significantly altered, even at a dose of 10 mg/kg MRK-409. In vehicle-treated rats, presentation of the light produced a marked (77%) reduction in the lick rate. However, this suppression of responding was alleviated in a dose-dependent manner by MRK-409 such that the rate of responding at a dose of 10 mg/kg was significantly greater than in vehicle-treated rats (Figure 8A).

From this primary data, the suppression ratio, which is an index of the light (i.e. fear)-induced reduction in responding, was calculated (Figure 8B). Analysis of this parameter showed a dose-dependent reversal of the response suppression such that responding at doses of 3 and 10 mg/kg p.o. was significantly greater than in vehicle-treated animals. The minimal effective dose (3 mg/kg) corresponds to

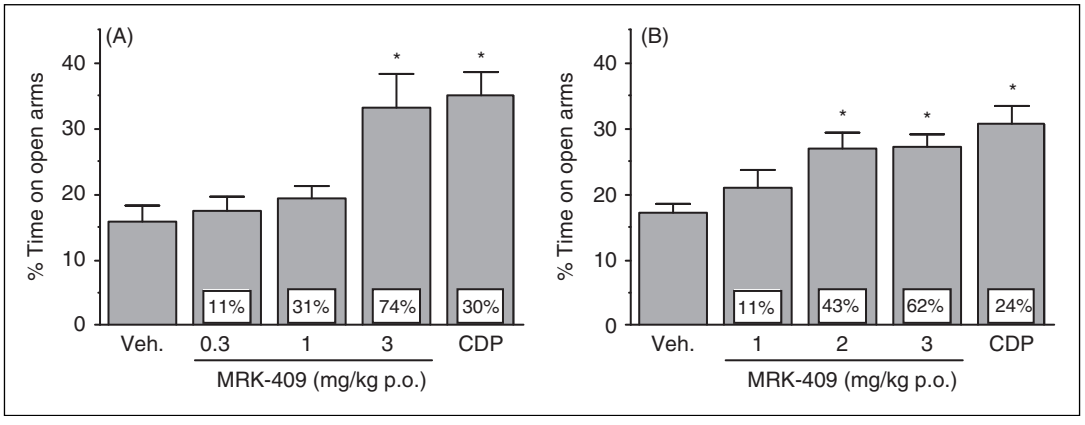

Figure 6. Effects of MRK-409 on the rat elevated plus maze. Date shown is the mean time (± SEM, $n = 17$–18/group) spent on the open arms (expressed as a percentage of the total 5 min trial time) after 0.5 h pre-treatment with: (A) 0.3, 1 or 3 mg/kg p.o. (Experiment 1) or 1, 2 or; (B) 3 mg/kg p.o. (Experiment 2) MRK-409. CDP = chlordiazepoxide. *$p < 0.05$ versus vehicle-treated animals using ANOVA followed by Dunnett's post-hoc *t*-test. Figures within bars are the mean occupancy values obtained from a subset of animals tested ($n = 9$).

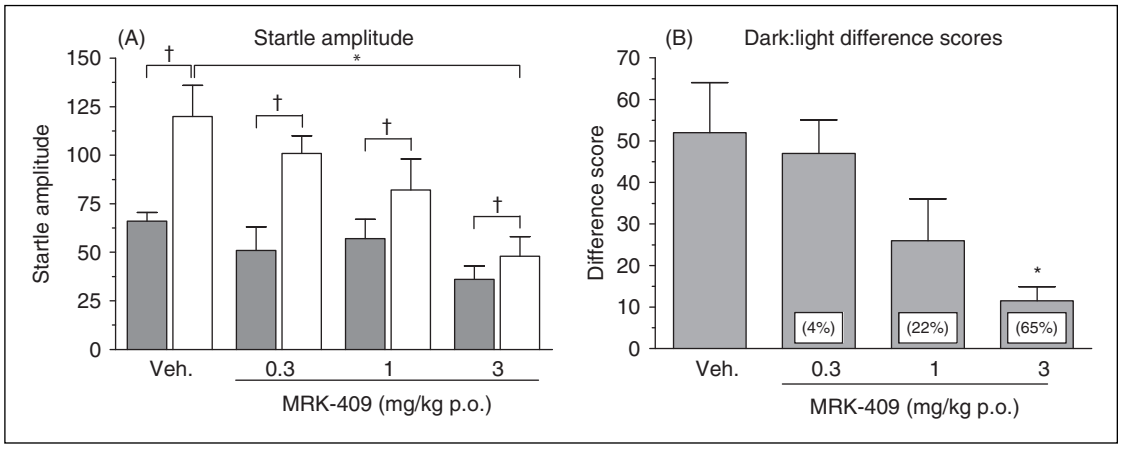

Figure 7. Effects of MRK-409 in rat fear-potentiated startle. Rats were conditioned to associate the presentation of light with a mild electric foot-shock and then the extent to which they startled in response to a 100 dB tone was measured in the dark or the presence of light. Results are expressed as the mean ± SEM ($n = 15$ group) of (A) the startle amplitude under non-threat (dark) or threat (light) conditions or (B) the mean of the difference of each animal's startle amplitude in the light compared with dark (safe) conditions (i.e. the extent of the fear-potentiated startle). Figures within bars represent estimated occupancy values (Table 3). Within-group comparisons were made between the startle amplitude in the light and dark ($^{\dagger}p < 0.05$ compared with startle responses measured in the dark) and the difference scores were analyses across groups using an analysis of variance followed by Dunnett's post hoc *t*-test). $^{*}p < 0.05$ compared with startle responses in vehicle-treated control animals).

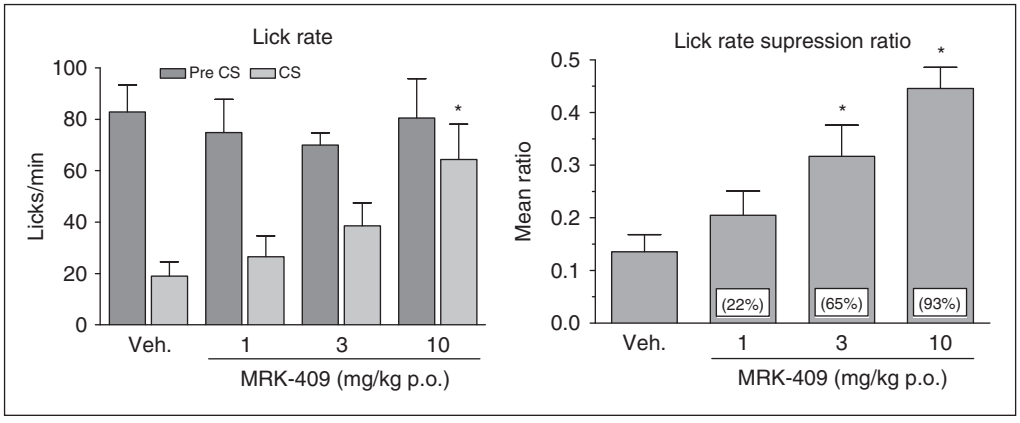

Figure 8. MRK-409 reverses fear-induced suppression of drinking in rats. Rats were dosed with either vehicle (0.5% methyl cellulose) or MRK-409 (1, 3 or 10 mg/kg p.o.) and 0.5 h later their rates of responding (water spout licking) were measured prior to and during a 60-sec presentation of a light-stimulus that had previously been associated with an electric shock. Figures within bars represent estimated occupancy values (Table 3). Values are mean ± SEM ($n = 11$–12/group). $^{*}p < 0.05$ versus corresponding vehicle-treated animals (ANOVA followed by Dunnett's post-hoc *t*-test).

occupancy of 65% whereas the maximum dose tested (10 mg/kg p.o.), and at which no overt sedation was observed, is equivalent to occupancy of 93% (Table 3).

MRK-409 is non-sedating in rodents

Rat chain-pulling: Figure 9A shows the effects of vehicle (0.5% methyl cellulose), MRK-409 (10 and 30 mg/kg p.o.) and diazepam (10 mg/kg p.o.) on the number of chain pulls/minutes at successive 10 min intervals during the 1-h session (expressed as a percentage of the baseline response rate obtained from the previous drug-free session; left-hand panels). Statistical analysis of the chain-pulling rates showed that at each 10-min interval during the 1-h testing session, diazepam (10 mg/kg p.o.) significantly reduced chain-pulling rates. At a dose of 30 mg/kg p.o. MRK-409 the response rate at each 10-min time point was consistently lower than in vehicle-treated rats. However, when the mean chain pulls/min were averaged over the whole 1-h session (Figure 9B), there was not only a profound effect of diazepam but also a statistically significant, albeit modest (25%), decrement in responding at the 30 mg/kg (but not 10 mg/kg) dose. Nevertheless, it should be noted that this modest effect on response rates was achieved only at a dose (30 mg/kg) that corresponds to 99% occupancy (Table 3).

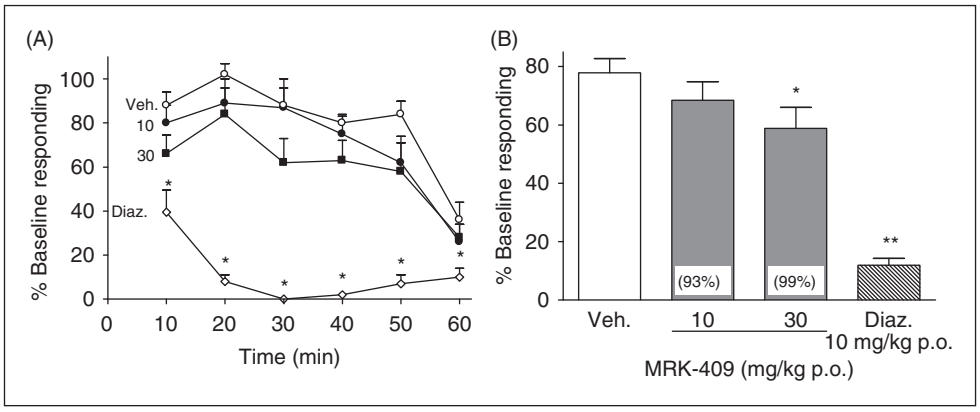

Figure 9. Effects of MRK-409 in the rat chain-pulling assay of sedation. Rats were dosed p.o. with either vehicle (0.5% methyl cellulose), MRK-409 (10 or 30 mg/kg) or diazepam (10 mg/kg) and the rate of chain pulling was recorded over the subsequent 1 h period with data being expressed as a percentage of baseline responding. (A) The number of chain pulls/min at 10 min intervals during the 1 h 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).

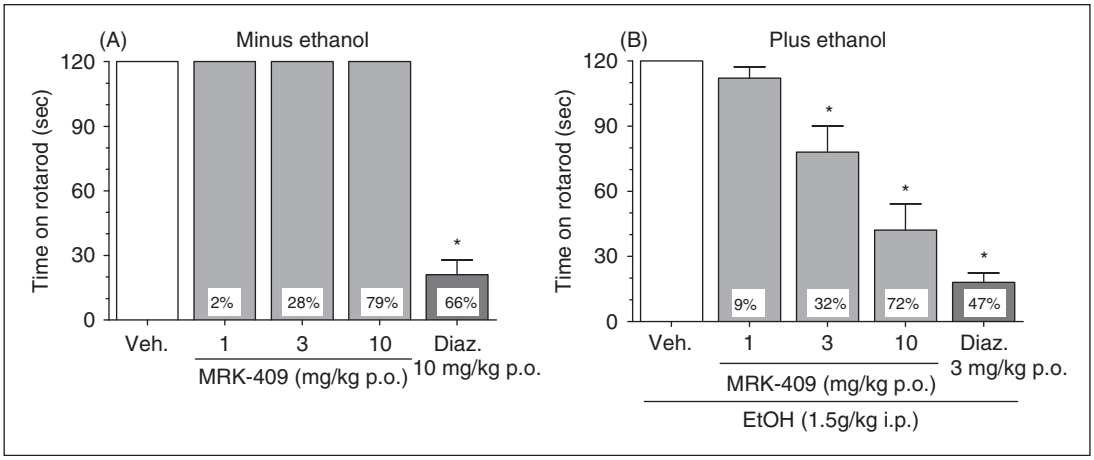

Figure 10. Effects of MRK-409 on rotarod performance in the absence and presence of ethanol. The latency to fall-off the rotarod during a 2-min trial was measured in mice 0.5 h after p.o. dosing with either vehicle (0.5% methyl cellulose), MRK-409 (1, 3 or 10 mg/kg) or diazepam (3 or 10 mg/kg) in (A) the absence or (B) the presence of ethanol (1.5 g/kg i.p., 0.5 h pretreatment). Figures within bars are the mean occupancy values measured immediately after completion of the trial. In these studies, the occupancy ED_{50} values were 5.5 and 5.4 mg/kg in the absence and presence of ethanol, respectively. Values shown are mean ± SEM ($n = 7$–8/group). *$p < 0.05$ versus vehicle group.

Mouse rotarod: Mice were dosed p.o. with either vehicle (0.5% methyl cellulose), MRK-409 (1, 3 or 10 mg/kg p.o.) and diazepam (10 mg/kg p.o.) and then subjected to a trial on the rotarod lasting up to 2 min. Immediately following completion of the trial, mice were taken and occupancy measured. Figure 10A shows that diazepam had a pronounced effect on rotarod performance, with mice lasting on average 21 ± 7 sec before falling off. Occupancy in these animals was 66 ± 2%. In contrast, MRK-409 had no effect on performance with all animals completing the 2-min trial, even at a dose of 10 mg/kg that corresponds to occupancy of 79 ± 4%.

The same experiment described above was repeated but with pre-treatment using a dose of ethanol (1.5 g/kg i.p.) which did not in its own right produce any impairment of rotarod performance (Figure 10B). However, ethanol pre-treatment did potentiate the effects of not only diazepam but also MRK-409. More specifically, doses of MRK-409 which did not impair performance in the absence of ethanol (i.e. trial latency = 120 sec) did so when mice had been pre-treated with ethanol such that at 3 and 10 mg/kg average trial durations were 78 ± 12 and 42 ± 15 sec, respectively. Furthermore, these effects were not a consequence of ethanol affecting MRK-409 occupancy since in the absence and presence of ethanol occupancy values at 3 mg/kg were 28 ± 3% and 32 ± 5% and at 10 mg/kg were 79 ± 4% and 72 ± 3%. Hence, MRK-409 demonstrates a significant ethanol interaction at a dose (3 mg/kg) corresponding to ~30% occupancy.

MRK-409 is a non-sedating anxiolytic in primates

MRK-409 is anxiolytic-like in the conditioned emotional response assay: Two separate conditioned emotional response experiments were performed (Figure 11A) and in both, the presentation of the conditioning stimulus produced a marked reduction in the response rate (response rates = 6 ± 2% and 4 ± 1% of baseline responding in Experiments 1 and 2, respectively). In the initial experiment, 0.1 mg/kg MRK-409 p.o. gave a significant increase in the rate of responding, to 35 ± 10% of the baseline responding rate, whereas in the second experiment, all of the doses of MRK-409 induced a statistically significant release in lever pressing with respective response rates being 55 ± 9%, 60 ± 9% and 60 ± 10% at 0.3, 1 and 3 mg/kg p.o.

MRK-409 is non-sedating in the lever-pressing assay: Lever pressing rates on drug days, averaged throughout the 30 min test session, were expressed as a percentage of the previous day's performance (baseline). As illustrated in Figure 11B, lever-pressing rates following vehicle treatment were approximately 96 ± 9% of those recorded on drug-free baseline days. MRK-409 did not have any significant effect on lever-pressing rates with doses of 1, 3 and 10 mg/kg p.o., producing response rates of 88 ± 9%, 90 ± 11% and 80 ± 9%, respectively.

In separate pharmacokinetic studies in squirrel monkeys (data not shown) the minimum effective dose of 0.1 mg/kg gave plasma drug concentrations 1 h after dosing of 69 ng/mL whereas 3 mg/kg gave a plasma drug concentration of 699 ng/mL, which (and assuming linearity of exposure between 3 and 10 mg/kg) would correspond to a plasma drug concentration at a dose of 10 mg/kg in the region of 2000 ng/mL. Assuming further that the plasma occupancy relationship in squirrel monkey is similar to that observed in rat, the respective occupancies at the minimal effective dose for anxiolytic-like activity (0.1 mg/kg p.o., 69 ng/mL) and at the highest non-sedating dose tested (10 mg/kg p.o., 2000 ng/mL) are 35% and 95%.

Single-dose pharmacokinetics and tolerability of MRK-409 in humans

Healthy male volunteers were given doses of MRK-409 ranging from 0.05 to 2.0 mg and plasma drug concentrations were measured. As can be seen from Figure 12, the drug was rapidly absorbed with the maximum plasma drug concentrations being achieved within 0.8–1.3 h after dosing (Table 4). Thereafter, drug was rapidly cleared with a half-life that ranged from 2.3 to 2.8 h (Table 4). There was a reasonably linear relationship between dose and total drug exposure (measured as area under the curve (AUC); Figure 12, inset) as well as C_{max} (Table 4).

The effect of food as measured by comparing the pharmacokinetic profiles of a 0.5 mg dose of MRK-409 in fasted and fed men showed that feeding reduced the C_{max} and delayed the T_{max} (3.9 ng/mL and 3.3 h) relative to fasted men (7.6 ng/mL and 0.9 h). However, the total exposures were similar in the fasted and fed subjects (respective AUCs of 29.6 and 27.7 ng.h/mL).

The most frequent adverse events associated with MRK-409 are summarized in Table 4. Drowsiness/sleepiness and tiredness were dose-limiting at doses of 1.5 and 2 mg. At the highest single oral dose evaluated (2 mg) there was drowsiness in five out of six subjects and dose-limiting moderate and severe drowsiness in two subjects, one of whom had difficulty

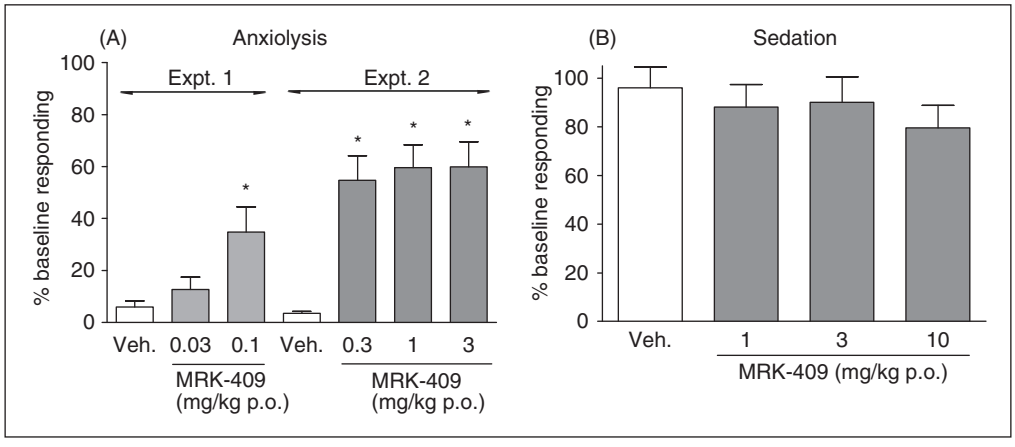

Figure 11. MRK-409 had anxiolytic-like activity in a squirrel monkey conditioned emotional response assay but was devoid of overt sedation in a lever-pressing response assay. (A) Two separate conditioned emotional response experiments were performed, using MRK-409 doses of either 0.03 and 0.1 (Experiment 1) or 0.3, 1 and 3 mg/kg. MRK-409 or vehicle (0.5% methyl cellulose) was administered p.o. 0.5 h prior to testing during which the suppression of lever pressing rates in response to a red cue light that had previously been associated with mild electric tail-shock was measured. Values shown are mean ± SEM ($n = 12$/group). *$p < 0.05$ vs. vehicle control (paired t-test). (B) The number of lever presses per minute during the 1 h session (expressed as a percentage of baseline responding) are shown following administration of MRK-409 (1–10 mg/kg, p.o.) given 0.5 h before the test session. Values shown are mean ± SEM ($n = 9$/group). There were no statistically significant differences between groups as assessed using paired t-tests ($p > 0.05$ vs. vehicle treatment).

staying awake. The severe drowsiness commenced approximately one hour after dosing and lasted for three to six hours. Other less frequent adverse experiences included headache, tiredness and dizziness. There were no laboratory adverse events and none of the clinical adverse events was considered serious. Based upon these findings, the maximum tolerated single oral dose was determined to be 1 mg.

The 2 mg dose produced marked somnolence in five out of six subjects and this dose corresponds to a plasma C_{max} of 28 ng/mL (Table 4). Using the rat plasma-occupancy relationship (Figure 5), a plasma MRK-409 concentration of 28 ng/mL corresponds to an occupancy of around 20%, which is surprising given that no overt signs of sedation were observed in preclinical species even at levels of receptor occupancy greater than 90%. However, it is possible that the blood–brain barrier permeability and/or central nervous system (CNS) distribution of MRK-409 is markedly different in humans compared with rat and that as a consequence the human plasma-occupancy relationship is different from that in rats. As a consequence, the plasma-occupancy relationship was evaluated in humans using [^{11}C]flumazenil PET.

[^{11}C]Flumazenil PET studies

Figure 13 shows the distribution of brain radioactivity in three healthy volunteers given either placebo ($n = 1$)

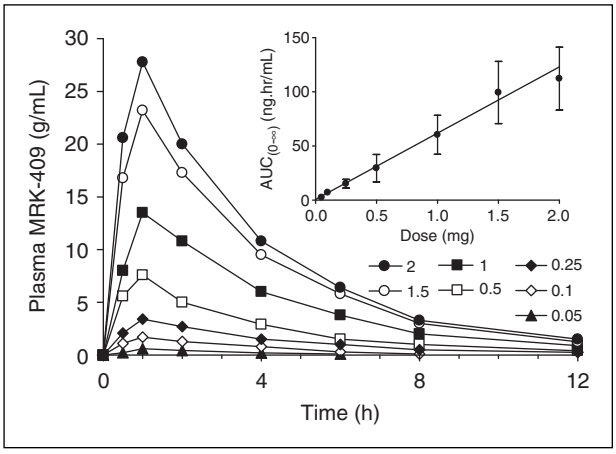

Figure 12. Plasma drug concentrations following different single oral doses of MRK-409 in healthy, fasted male volunteers. Values shown are mean plasma concentrations at doses ranging from 0.05 to 2 mg ($n = 5$–6/dose group) with error bars being omitted for clarity. However, within each dose group, the total exposure (area under the curve, AUC) and the maximum plasma concentration (C_{max}) varied by less than 40%. The inset shows that the AUC was linearly related to dose within the range of 0.05–2.0 mg.

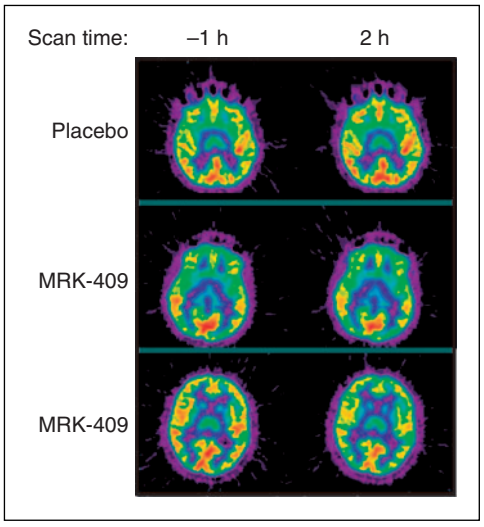

Figure 13. A pseudocolour representation of the [^{11}C]flumazenil total distribution volume V_T in the brains of three healthy human volunteers, one of whom received placebo, the other two being given MRK-409 (1 mg). In subjects receiving drug, there was no marked decrease in [^{11}C]flumazenil V_T demonstrating that there was limited occupancy of the benzodiazepine binding site of human brain $GABA_A$ receptors by MRK-409.

Table 4. Pharmacokinetic parameters and adverse events noted after administration of single oral doses of MRK-409 to young healthy male volunteers

		Pharmacokinetic parameters				Most frequent adverse events				
Dose (mg)	*N*	T_{max} (h)	C_{max} (ng/mL)	$AUC_{(0-\infty)}$ [a] (ng.h/mL)	$T_{½}$ (h)	Headache	Nausea	Tiredness	Drowsiness/ sleepiness	Dizziness
Placebo	16	N/A	N/A	N/A	N/A	3		2	1	
0.05	6	1.2 ± 0.4	0.6 ± 0.1	2.5 ± 0.6	2.4					
0.1	5	0.8 ± 0.3	1.7 ± 0.3	7.1 ± 2.7	2.5	1				
0.25	6	1.3 ± 0.5	3.4 ± 0.6	15.3 ± 4.0	2.6	2	1		1	
0.5	6	0.9 ± 0.2	7.6 ± 1.8	29.6 ± 12.6	2.3	3		1		1
0.5 (fed)	6	3.3 ± 1.0	3.9 ± 0.8	27.7 ± 10.4	2.5	2		4	2	1
1.0	6	1.2 ± 0.7	13.5 ± 4.6	60.4 ± 18.0	2.7			2	1	1
1.5[b]	6	0.8 ± 0.3	23.2 ± 5.4	99.4 ± 28.7	2.8			2	2	
2.0[b]	6	0.9 ± 0.2	27.8 ± 4.1	112.2 ± 29.0	2.5			2	5	

NA = not applicable.
[a]AUC = area under the (plasma concentration versus time) curve.
[b]Doses highlighted in bold were poorly tolerated and defined the maximum tolerated dose as 1.0 mg.

or 1.0 mg MRK-409 ($n = 2$). As can be clearly seen, a dose of 1.0 mg MRK-409 produced very little inhibition of the binding of [^{11}C]flumazenil to benzodiazepine binding sites in human brain and regional brain radioactivity in MRK-409-treated subjects was not distinguishable from that observed for placebo.

Discussion

In vitro properties in MRK-409

MRK-409 is a triazolopyridazine that is structurally related to TPA023 and the prototypic efficacy-selective compound L-838417 (Figure 1 and Atack et al., 2006; McKernan et al., 2000). These compounds are all α2/α3 efficacy selective compounds. Thus, although they all bind with equivalent affinity to α1-, α2-, α3- and α5-containing $GABA_A$ receptors, they have higher partial agonist efficacy at the α2 and/or α3 compared with α1 subtypes. The α2 and/or α3 subtypes are thought to be associated with the anxiolytic properties of benzodiazepines whereas agonism at the α1 subtype is associated with sedation (Atack et al., 2005; Löw et al., 2000; McKernan et al., 2000; Morris et al., 2006; Rudolph et al., 1999; Savic et al., 2008). It is noteworthy, however, that whereas L-838417 and TPA023 have essentially antagonist efficacy at the α1 subtype, MRK-409 has a degree of weak partial α1 agonist efficacy, albeit much lower than at the α3 subtype (relative efficacies at the α1 and α3 subtypes of 0.18 and 0.45, respectively; Table 2).

In in vitro rat, dog, rhesus monkey and human liver microsome turnover assays, the major metabolite of MRK-409 was the 3-hydroxycyclobutyl analogue and this compound had around 10-fold lower $GABA_A$ receptor affinity than the parent (unpublished data). The *O*-dealkylated metabolite of MRK-409 was detected in vivo in rat and dog but the $GABA_A$ receptor affinity of this compound was more than 500-fold lower than the parent. In addition, neither metabolite was detected in rat brain after a dose of 3 mg/kg, highlighting their poor CNS penetration. Accordingly, it is assumed that the $GABA_A$-mediated pharmacological effects of MRK-409 are indeed associated with this compound rather than a metabolite.

In vivo properties of MRK-409 in preclinical species

In rats, MRK-409 gave good occupancy after oral dosing with an Occ_{50} of 2.2 mg/kg. However, this is around five-fold less potent than TPA023, which had an Occ_{50} of 0.42 mg/kg (Atack et al., 2006). This difference in potency is not related to affinity since the *Ki* values of both compounds were comparable against not only recombinant human but also native rat receptors (*Ki* values ranging from 0.21 to 0.40 nM for MRK-409 and 0.19 to 0.41 nM for TPA023). Rather, the difference in potency seems to be related to blood–brain barrier penetration and/or distribution within the CNS since the plasma concentrations of MRK-409 required to give 50% occupancy (in rat 115 ng/mL) were around 5 times more than the corresponding value for TPA023 (25 ng/mL; Atack et al., 2006).

In preclinical species, MRK-409 produced anxiolytic-like effects not only in an unconditioned model (rat elevated plus maze) but also in conditioned anxiety models (rat fear-potentiated startle and conditioned suppression of drinking and squirrel monkey conditioned emotional response; see Table 5). The minimal effective doses for these anxiolytic effects corresponded to occupancies in the region of 35–65%, which is consistent with the fact that the lower efficacy compound TPA023 required higher levels of occupancy for anxiolytic-like efficacy (65–88%; Atack et al., 2006). MRK-409 had no overt sedative properties at receptor occupancies of up to 95% and it was only at a dose (30 mg/kg) corresponding to receptor occupancy of 99% that a modest sedative effect was observed in the rat chain-pulling assay (Table 5). Similarly, TPA023 had no sedative effect at occupancies up to 99% (Atack et al., 2006).

Table 5. Summary of the preclinical non-sedating anxiolytic properties of MRK-409

Behaviour	Assay	Observation	Dose (mg/kg, p.o.)	Occupancy[a]
Anxiety assays				
Rat anxiety	Elevated plus maze	Anxiolysis	2[b]	43%
	Conditioned suppression of drinking	Anxiolysis	3	65%
	Fear-potentiated startle	Anxiolysis	3	65%
Primate anxiety	Conditioned emotional response	Anxiolysis	0.1	35%
Sedation assays				
Mouse sedation	Rotarod	No effect	10	79%
Rat sedation	Chain-pulling	No effect	10	93%
Rat sedation	Chain-pulling	Slight effect	30	99%
Primate sedation	Response sensitivity	No effect	10	95%
Other assay				
Mouse EtOH interaction	Rotarod	Potentiates EtOH effects	3	32%

[a]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 5).
[b]Anxiolytic-like doses are minimal effective dose.

Consequently, MRK-409 behaved as a non-sedating anxiolytic in not only rodents but also primates.

The non-sedating anxiolytic properties of MRK-409 in preclinical species can be attributed to its partial agonism at the α2 and α3 subtypes with lower agonism being measured at the α1 ('sedative') subtype. In this regard, MRK-409 is consistent with previous published data describing the non-sedating anxiolytic effects of a number of compounds with higher agonist efficacy at α2- and/or α3- compared with α1-containing receptors, such as L-838417, TPA023, TPA003 and NS11394 (Atack et al., 2006; Dias et al., 2005; McKernan et al., 2000; Mirza et al., 2008; Munro et al., 2008; Rowlett et al., 2005).

Properties of MRK-409 in humans

In young healthy male volunteers, MRK-409 was rapidly absorbed having a T_{max} in the region of 1 h, with total exposure (Figure 12, inset) and C_{max} (Table 4) both being dose-dependent (C_{max} values at doses of 0.05 and 2.0 mg = 0.6 and 27.8 ng/mL, respectively). This data agrees with that from a separate study performed to assess the effects of MRK-409 (also known as MK-0343) on saccadic eye movements using single doses of 0.25 and 0.75 mg in which the C_{max} occurred within the first hour, with values in the region of 3.5 and 9 ng/mL, respectively (de Haas et al., 2008).

Most notable, however, was the observation that MRK-409 produced marked sedation in five out of six and two out of six subjects receiving the 2.0 and 1.5 mg dose, respectively. A dose of 0.75 mg has been reported to impair saccadic eye movement peak velocity, visual analogue scale (VAS) alertness scores and postural stability (increased body sway) and to increase saccadic latency in a manner comparable to 2 mg lorazepam, although MRK-409 did not affect memory performance whereas lorazepam did (de Haas et al., 2008). Non-selective benzodiazepines usually show comparable effects on memory, alertness and postural stability (see discussion in de Haas et al., 2007) and therefore MRK-409 differentiates itself from non-selective benzodiazepines in that it showed no memory deficits. However, the significant effect of 0.75 mg MRK-409 on the VAS alertness score suggests a sedative effect of this compound at this dose.

Although MRK-409 has an effect on saccadic peak velocity that is comparable to lorazepam, it is uncertain whether this is a biomarker for sedative or anxiolytic efficacy. Thus, although this effect might be related to sedation (de Visser et al., 2003), an effect on saccadic peak velocity does not necessarily track the effects on VAS alertness scores. For example, 0.5 and 1.5 mg of TPA023 (doses chosen based upon the tolerability of TPA023 in healthy volunteers) affected saccadic peak velocity to a similar extent as lorazepam (2 mg) yet both doses were without effect not only on the VAS alertness score but also on memory performance and postural stability (de Haas et al., 2007).

It could be argued that aside from having efficacy at the α3 subtype higher than the other subtypes, the efficacy profile of MRK-409 at α1-, α2-, α3- and α5-containing $GABA_A$ receptors (0.18, 0.23, 0.45 and 0.18) is similar to the non-selective partial agonist bretazenil (Atack, 2003). In this regard, and like MRK-409, a dose of 0.5 mg bretazenil (which gave a plasma C_{max} of 5 ng/mL) showed little evidence of dissociation between sedative effects measured using the VAS alertness scale and saccadic eye movements with subjects tending to fall asleep during testing (van Steveninck et al., 1996). Moreover, and again like MRK-409, bretazenil had previously been shown to behave as a non-sedating anxiolytic in preclinical rodent and primate models (Atack, 2003).

Although the sedation observed with MRK-409 was unexpected it nevertheless remained possible that these effects might occur at high levels of occupancy and that anxiolysis could occur at lower doses, thereby maintaining the separation between anxiolytic and sedative doses observed in preclinical species, albeit to a reduced extent. Consequently, it was important to establish the level of receptor occupancy produced by the maximum tolerated dose of MRK-409 (1.0 mg) in healthy normal volunteers. This data (Figure 13) shows that MRK-409 gave occupancy below the regional test–retest values for [^{11}C]flumazemil PET (i.e. <10%; Salmi et al., 2008). This is much less than the occupancy produced by a 2 mg dose of TPA023 (~50–60%). As a consequence of these results, the development of MRK-409 as a non-sedating anxiolytic was halted.

Comparison of MRK-409 with TPA023

There was a clear difference between the properties of MRK-409, which caused sedation at relatively low levels of occupancy (~10%), and TPA023, which was non-sedating at a dose (2 mg) that gave 50–60% occupancy (de Haas et al., 2007, 2008), raising the question of which aspect of the efficacy profiles of MRK-409 and TPA023 is responsible for the sedation seen with MRK-409 but not with TPA023. The three obvious differences in the intrinsic efficacy between these compounds are: (1) MRK-409 has partial agonist efficacy at the α1 subtype whereas TPA023 does not; (2) MRK-409 has relative efficacy at the α2 and α3 subtypes (0.23 and 0.45) greater than the corresponding values for TPA023 (0.11 and 0.21; Atack et al., 2006); (3) MRK-409 has partial agonist efficacy at the α5 subtype (relative efficacy = 0.18) but TPA023 is essentially an antagonist at this subtype. In order to try to resolve this issue, we characterized a compound which had an efficacy profile at the α2, α3 and α5 subtypes very similar to that of MRK-409 but was devoid of efficacy at the α1 subtype, and in this regard was more like TPA023. The properties of this compound, TPA023B are presented in the accompanying manuscript and the data show that the sedating properties of MRK-409 are probably related to the weak partial agonism at the α1 subtype (Atack et al., 2010).

$GABA_A$ subtype-selective modulators – does the hypothesis need refining?

It is also important to note that not all compounds conform to the hypothesis that the α1 subtype mediates sedation and that high efficacy at this subtype is accompanied by sedation. For example, the structurally related compounds Ocinaplon and DOV 51892 have appreciable efficacy at the α1 subtype (which, in the case of DOV 51892 might be classified as 'super-agonism') yet both are non-sedating anxiolytics in

preclinical models (Lippa et al., 2005; Popik et al., 2006). Moreover, Ocinaplon showed no signs of sedation at anxiolytic doses in humans (Lippa et al., 2005) and this cannot be attributed to an active metabolite since both Ocinaplon and the active metabolite DOV 315,090 possess similar in vitro efficacy profiles (Berezhnoy et al., 2008). Whilst the reason for the lack of sedation in these apparently anomalous compounds is unclear, at the very least this data suggests that efficacy as measured in recombinant α1-containing $GABA_A$ receptors may not necessarily be predictive of sedative liability (Popik et al., 2006).

The pyridoindole SL65.1498 possesses appreciable efficacy at the α1 subtype (relative efficacy of 0.45 versus zolpidem; Griebel et al., 2001) yet even at a dose of 25 mg there were no overt signs of sedation. However, although the 25 (but not 2.5 or 7.5)-mg dose of SL65.1498 did decrease saccadic peak velocity, these effects were much less than those produced by not only lorazepam (de Haas et al., 2009) but also TPA023 and MRK-409 (de Haas et al., 2007, 2008). The maximal plasma concentration achieved with 25 mg SL65.1498 was 375 ng/mL, which was much higher than achieved for the highest tested doses of TPA023 (1.5 mg, $C_{max} = 13$ ng/mL) and MRK-409 (0.75 mg, $C_{max} = 9$ ng/mL; de Haas et al., 2007, 2008, 2009). Nevertheless, the affinity of SL65.1498 at the α1, α2 and α3 subtypes (17–80 nM; Griebel et al., 2001) is ≥50-fold less than that of either TPA023 and MRK-409 and therefore in the absence of receptor occupancy data, the pharmacodynamic effects of SL65.1498 are difficult to compare with those of TPA023 or MRK-409.

In addition, the triazolopyrimidine Adipiplon (NG2-73) is claimed to be an α3-preferring partial agonist that was being evaluated in Phase II/III clinical studies for the treatment of insomnia until development for this indication was halted in July 2008 due to a higher than anticipated rate of unwanted next-day effects (www.neurogen.com). The fact that a drug that is claimed to be selective for the 'anxiolytic' α3 subtype of $GABA_A$ receptors has hypnotic properties would appear contrary to the prevailing hypothesis that the α1 $GABA_A$ subtype mediates sedation whereas the α2 and/or α3 subtypes are anxiolytic and α5-containing receptors mediate aspects of cognition. However, when efficacy is expressed relative to standard non-selective benzodiazepines (such as lorazepam, triazolam, diazepam and alprazolam) rather than as a maximal potentiation, Adipiplon did not appear to have a marked α3-selectivity (Krause et al., 2007). Indeed, this compound had partial agonist efficacy at the α1 subtype that might account for its clinical properties (Krause et al., 2007).

The apparently paradoxical observations with Ocinaplon and, possibly SL65.1498 and Adipiplon, raises the issue of to what extent the in vitro measurement of efficacy reflects the in vivo situation. More specifically, the intrinsic efficacy of a compound is measured as the extent to which the peak current produced by a submaximal concentration of GABA (normally an EC_{20}) can be either potentiated or attenuated (benzodiazepine site agonism or inverse agonism, or, alternatively, positive or negative allosteric modulation, respectively). Such a measure does not take into account other components of the electrophysiological response, such as the rates of activation, desensitization and deactivation, that might be more relevant to synaptic transmission.

A better understanding of the clinical relevance of $GABA_A$ subtype selective modulators will emerge from the clinical evaluation of additional compounds. In this regard, the properties TPA023B are presented in the accompanying manuscript. These data indicate that the sedating properties of MRK-409 are probably related to its weak partial agonism at the α1 subtype (Atack et al., 2010).

Abbreviations

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-*2H*-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.

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