

In Vitro and in Vivo Properties of 3-*tert*-Butyl-7-(5-methylisoxazol-3-yl)-2-(1-methyl-1*H*-1,2,4-triazol-5-ylmethoxy)-pyrazolo[1,5-*d*]-[1,2,4]triazine (MRK-016), a GABA_A Receptor α 5 Subtype-Selective Inverse Agonist

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ABSTRACT

3-*tert*-Butyl-7-(5-methylisoxazol-3-yl)-2-(1-methyl-1*H*-1,2,4-triazol-5-ylmethoxy)-pyrazolo[1,5-*d*][1,2,4]triazine (MRK-016) is a pyrazolotriazine with an affinity of between 0.8 and 1.5 nM for the benzodiazepine binding site of native rat brain and recombinant human α 1-, α 2-, α 3-, and α 5-containing GABA_A receptors. It has inverse agonist efficacy selective for the α 5 subtype, and this α 5 inverse agonism is greater than that of the prototypic α 5-selective compound 3-(5-methylisoxazol-3-yl)-6-[(1-methyl-1,2,3-triazol-4-yl)methoxy]-1,2,4-triazolo[3,4-*a*]phthalazine (α 5IA). Consistent with its greater α 5 inverse agonism, MRK-016 increased long-term potentiation in mouse hippocampal slices to a greater extent than α 5IA. MRK-016 gave good receptor occupancy after oral dosing in rats, with the dose required to produce 50% occupancy being 0.39 mg/kg and a corresponding rat plasma EC₅₀ value of 15 ng/ml that was similar to the rhesus monkey plasma EC₅₀ value of 21 ng/ml

obtained using [¹¹C]flumazenil positron emission tomography. In normal rats, MRK-016 enhanced cognitive performance in the delayed matching-to-position version of the Morris water maze but was not anxiogenic, and in mice it was not proconvulsant and did not produce kindling. MRK-016 had a short half-life in rat, dog, and rhesus monkey (0.3–0.5 h) but had a much lower rate of turnover in human compared with rat, dog, or rhesus monkey hepatocytes. Accordingly, in human, MRK-016 had a longer half-life than in preclinical species (~3.5 h). Although it was well tolerated in young males, with a maximal tolerated single dose of 5 mg corresponding to an estimated occupancy in the region of 75%, MRK-016 was poorly tolerated in elderly subjects, even at a dose of 0.5 mg, which, along with its variable human pharmacokinetics, precluded its further development.

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ABBREVIATIONS: DMCM, methyl-6,7-dimethoxy-4-ethyl- β -carboline-3-carboxylate; L-655,708, ethyl[S]-11,12,13,13a-tetrahydro-7-methoxy-9-oxo-9*H*-imidazo[1,5-*a*]pyrrolo[2,1-*c*][1,4]benzodiazepine-1-carboxylate; α 5IA, 3-(5-methylisoxazol-3-yl)-6-[(1-methyl-1,2,3-triazol-4-yl)methoxy]-1,2,4-triazolo[3,4-*a*]phthalazine; RO4938581, 3-bromo-10-difluoromethyl-9*H*-imidazo(1,5-*a*)(1,2,4)triazolo(1,5-*d*)(1,4)benzodiazepine; MRK-016, 3-*tert*-butyl-7-(5-methylisoxazol-3-yl)-2-(1-methyl-1*H*-1,2,4-triazol-5-ylmethoxy)-pyrazolo[1,5-*d*][1,2,4]triazine; FG-7142, *N*-methyl- β -carboline-3-carboxamide; Ro 15-1788, [flumazenil, 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylic acid, ethyl ester]; Ro 15-4513, ethyl 8-azido-6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*]-[1,4]benzodiazepine-3-carboxylate; PPF, paired pulse facilitation; LTP, long-term potentiation; fEPSP, field excitatory postsynaptic potential; ANOVA, analysis of variance; Occ₅₀, dose required to produce 50% occupancy; PET, positron emission tomography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; ROI, region of interest; PTZ, pentylenetetrazole; AUC, area under the curve; AE, adverse event.

Classical benzodiazepines, such as diazepam (Valium), exert their effects via GABA_A receptors that are GABA-gated chloride ion channels. These receptors are pentameric assemblies of members of the GABA_A gene family (α 1–6, β 1–3, γ 1–3, δ , ϵ , θ , and π), the most abundant of which contain α , β , and γ subunits in a 2:2:1 stoichiometry (Barnard et al., 1998). Benzodiazepines interact with GABA_A receptors containing either an α 1, α 2, α 3, or α 5 subunit along with β and γ 2 subunits, and their specific recognition site occurs at the interface of the α subunit and γ 2 subunit. Clinically used benzodiazepines have agonist activity at the benzodiazepine binding site in that they enhance the inhibitory effects of GABA (positive allosteric modulation), thereby reducing neuronal excitability (Sieghart, 1995, 2006). In contrast, benzodiazepine site inverse agonists (negative allosteric modulators), the prototypic example of which is the β -carboline DMCM, attenuate the inhibitory effects of GABA, with a consequent increase in neuronal excitability. The opposing effects of benzodiazepine site agonists and inverse agonists at the molecular level are reflected in vivo. Hence, benzodiazepine site agonists possess a variety of pharmacological actions in human, including anxiolytic, sedative, myorelaxant, anticonvulsant, and cognition-impairing activities (Argyropoulos and Nutt, 1999; Buffett-Jerrott and Stewart, 2002), whereas inverse agonists are anxiogenic and convulsant or proconvulsant and also enhance vigilance and improve cognitive performance in preclinical species (Sieghart, 2006). Unfortunately, the anxiogenic and proconvulsant properties of nonselective inverse agonists have precluded their clinical use as cognition and/or vigilance enhancers (Dorow et al., 1983; Horowski and Dorow, 2002). Clearly, however, a compound that retained the cognition-enhancing properties yet was devoid of the anxiogenic and proconvulsant liabilities would offer the potential for clinical utility.

The use of molecular genetic (knockout and point-mutated mice) and pharmacological (subtype-selective compound) approaches has begun to define which of the pharmacological features of benzodiazepine site ligands are associated with particular GABA_A receptor subtypes (Rudolph et al., 1999; McKernan et al., 2000; Rudolph and Möhler, 2004). As a consequence, molecular targets for the development of compounds that selectively interact with specific GABA_A receptor subtypes and that therefore have unique pharmacological profiles relative to the nonselective benzodiazepines have been identified (McKernan et al., 2000; Dias et al., 2005; Atack et al., 2006b). With respect to cognition, the α 5 subtype is preferentially localized within the hippocampus (Fritschy and Möhler, 1995; Sur et al., 1999) and has been shown to play a role in aspects of cognitive performance (Collinson et al., 2002; Crestani et al., 2002; Maubach, 2003). More specifically, compounds that possess either α 5-selective affinity, α 5-selective efficacy, or a mixture of both these features (e.g., L-655,708, α 5IA, and RO4938581, respectively) have been shown to enhance cognition in rodents (Atack et al., 2006a; Collinson et al., 2006; Dawson et al., 2006; Ballard et al., 2009) as well as primates (Ballard et al., 2009). Regarding human data, α 5IA did not improve performance in the paired-associate learning task in normal elderly (mean age, 72 years) volunteers (indeed, if anything, performance got worse; Atack, 2009), but it did attenuate the ethanol-induced impairment of word recall in healthy young (25-year-old) volunteers (Nutt et al., 2007). However, due to preclinical

hepatotoxicity issues, development of this compound was stopped (Atack, 2009). Here, we provide a more detailed description of the in vitro and in vivo properties of the back-up compound MRK-016, an initial characterization of which has been presented previously (compound 13 in Chambers et al., 2004).

Materials and Methods

Drugs

FG-7142, diazepam, and Ro 15-1788 (flumazenil) were purchased from Sigma-Aldrich (Gillingham, UK), and bretazenil was a gift from F. Hoffman-La Roche (Basel, Switzerland). [³H]Flumazenil (70–87 Ci/mmol) and [³H]Ro 15-4513 (20–40 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). MRK-016 (Fig. 1) was synthesized as described previously (Chambers et al., 2004).

In Vitro Binding

The affinity of MRK-016 was measured at human recombinant GABA_A receptors stably expressed in mouse fibroblast L-cells essentially as described in detail previously (Atack et al., 2006b; Dawson et al., 2006). In brief, the ability of MRK-016 to inhibit the binding of either [³H]flumazenil to GABA_A receptors containing β 3, γ 2, and either α 1, α 2, α 3, or α 5 subunits or [³H]Ro 15-4513 to α 4 β 3 γ 2 and α 6 β 3 γ 2 receptors was measured. From the resulting IC₅₀ value, the K_i was calculated from the Cheng-Prusoff equation, using K_D values for the binding of [³H]flumazenil to the α 1, α 2, α 3, and α 5 subtypes of 0.92, 1.05, 0.58, and 0.45 nM, respectively, and for the binding of [³H]Ro 15-4513 to the α 4 and α 6 subtypes of 4.0 and 6.5 nM, respectively.

Whole-Cell Patch-Clamp Electrophysiology

The intrinsic efficacy of MRK-016 was measured using whole-cell patch-clamp electrophysiology of mouse fibroblast L-cells stably expressing human recombinant GABA_A receptors containing β 3, γ 2, and either an α 1, α 2, α 3, or α 5 subunit as described in detail previously (Atack et al., 2006b; Dawson et al., 2006). In brief, a monolayer of cells was grown on glass coverslips, and cells were patch-clamped on the stage of a Diaphot inverted microscope (Nikon, Tokyo, Japan) using a patch pipette with a diameter of approximately 2 μ m and 4-M Ω resistance. A double-barrelled pipette was used for rapid drug application, and the efficacy was defined as the extent to which the current produced by an EC₂₀-equivalent concentration of GABA was altered by MRK-016 applied for 30 s before a GABA EC₂₀. Under these conditions, a potentiation or attenuation of the GABA EC₂₀ reflects benzodiaz-

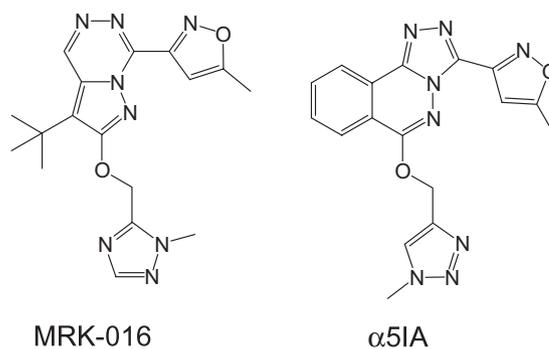


Fig. 1. Comparison of the structures of MRK-016 (compound 13, Chambers et al., 2004), and α 5IA (Dawson et al., 2006). Note that the 5-methyl-3-isoxazolyl and triazolymethoxy groups are found in both compounds, albeit that the triazole group is in a 1,2,4 configuration in MRK-016 and a 1,2,3 arrangement in α 5IA.

epine site agonist (positive allosteric modulation) or inverse agonist efficacy (negative allosteric modulation), respectively.

Mouse Brain Slice Electrophysiology

Paired pulse facilitation (PPF) and long-term potentiation (LTP) were measured electrophysiologically in brain slices prepared from 6- to 9-month-old C57 male mice as described previously (Collinson et al., 2002; Dawson et al., 2006). To summarize, parasagittal 350- μm -thick brain slices were cut on a Vibratome sectioning system, and glass microelectrodes placed in the stratum radiatum of the hippocampal CA1 region recorded the field excitatory postsynaptic potentials (fEPSPs) in response to stimulation of the fibers of the Schaffer collateral-commissural pathway. The effects of MRK-016 (100 nM) and $\alpha 5\text{IA}$ (100 nM) on presynaptic calcium-dependent release mechanisms were studied by measuring the fEPSPs with interstimulus intervals ranging from 25 to 300 ms, and the PPF ratio was calculated as the slope of the second fEPSP response divided by the slope of the first fEPSP response. To produce LTP, a brief tetanus (10 stimuli at 100 Hz) or a θ -burst protocol (four pulses delivered with a frequency of 100 Hz, repeated 10 times with an interval of 200 ms) was used in slices pretreated with either vehicle (0.1% dimethyl sulfoxide), MRK-016 (100 nM), or $\alpha 5\text{IA}$ (100 nM). Comparisons between groups were performed using an ANOVA followed by post hoc Student-Newman-Keuls tests.

In Vivo Experiments

All procedures involving animals were performed under the auspices of the UK Animals (Scientific Procedures) Act 1986 and related guidelines. Rhesus monkey PET studies were performed after review and approval by the Merck Research Laboratories' West Point Institutional Animal Care and Use Committee in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Institutes of Health, Bethesda, MD).

Receptor Occupancy

Rodent in Vivo [^3H]Flumazenil Binding. The occupancy of the benzodiazepine binding site of mouse and rat brain GABA_A receptors by MRK-016 was measured using an in vivo [^3H]flumazenil binding assay (Atack et al., 2006b; Dawson et al., 2006). In brief, mice or rats were dosed with compound or vehicle (0.5% methylcellulose), and 3 min before killing, [^3H]flumazenil (70–87 Ci/mmol diluted 1:150 with saline) was administered via a tail vein (5 $\mu\text{g}/\text{g}$ for mice; 1 $\mu\text{g}/\text{g}$ for rats). Animals were killed by decapitation, and the brains were rapidly removed, homogenized in 10 volumes of ice-cold buffer (10 mM phosphate buffer and 100 mM KCl, pH 7.4) and 300- μl aliquots of homogenate were filtered and washed over Whatman GF/B glass fiber filters (Whatman, Maidstone, UK). To determine the extent of nonspecific in vivo [^3H]flumazenil binding, a separate group of animals was pretreated for 30 min with bretazenil (5 mg/kg i.p. in 100% polyethylene glycol vehicle). The extent by which MRK-016 reduced the specific in vivo binding of [^3H]flumazenil relative to the binding in vehicle-treated animals was defined as the occupancy. For the dose-response study, rats were given doses of MRK-016 ranging from 0.3 to 30 mg/kg p.o., and occupancy was measured 0.5 h later. For the time course study, rats were treated with 1, 3, or 10 mg/kg p.o. MRK-016, and occupancy was measured 0.5, 1, 2, 4, 8, or 24 h after dose. Values shown are presented as mean \pm S.E.M.

Rhesus Monkey [^{11}C]Flumazenil PET. PET studies of MRK-016 were conducted in two male rhesus monkeys weighing ~ 10 kg. Fasted monkeys were initially anesthetized with ketamine intramuscularly (10 mg/kg), and subsequent anesthesia was maintained using propofol infused through an intravenous catheter inserted into the right cephalic and left saphenous veins with an induction dose of 5 mg/kg, followed by a constant infusion at 0.4 mg/kg/min for the duration of the study. The anesthetized monkey was intubated and placed on ventilated medical grade compressed air at 50 ml/breath

and approximately 25 respirations per minute. Physiological parameters were monitored with a temperature probe, pulse oximeter, and end tidal CO₂ monitor, and body temperature was maintained using heating pads. It should be noted that propofol as well as isoflurane have been reported to affect the uptake and/or binding of [^{11}C]flumazenil to GABA_A receptors in humans (Gyulai et al., 2001; Salmi et al., 2004). However, the plasma EC₅₀ value for lorazepam obtained in rat isoflurane-anesthetized [^{11}C]flumazenil micro-PET studies was comparable with that obtained in awake rats using [^3H]flumazenil in vivo binding methods (respective values of 96 and 134 ng/ml) (Atack et al., 2007), suggesting that anesthesia does not appreciably alter the plasma EC₅₀ value for benzodiazepine site ligands. Nevertheless, the possibility that propofol may affect the rhesus monkey MRK-016 plasma EC₅₀ value cannot be excluded.

PET scans were performed on an ECAT EXACT HR+ scanner (Siemens/CTI, Knoxville, TN) with the monkey in the supine position using [^{11}C]flumazenil as the tracer. MRK-016 was administered intravenously to anesthetized monkeys in an ~ 0.2 -mg/ml solution in 20% hydroxypropyl- β -cyclodextrin using either a single bolus (1 mg/kg) or a bolus followed by a constant infusion to reach plasma steady-state levels of MRK-016. Regarding the latter, the bolus (range, 0.003–0.22 mg/kg) was administered over 5 min followed by a constant infusion ranging from 0.004 to 0.44 mg/kg/h. A stepwise protocol was used in some of the studies, in which two separate steady-state plasma levels were reached for two independent PET scans on the same day.

For the intravenous bolus study, two [^{11}C]flumazenil injections were made at 0.5 and 3 h, respectively, after the drug administration, whereas in the bolus plus infusion studies, [^{11}C]flumazenil was injected at ~ 1 h after the start of the intravenous infusion. For each tracer injection, ~ 5 mCi of [^{11}C]flumazenil was administered intravenously as a bolus and image acquisition started simultaneously and lasted for approximately 60 min. During scanning, ~ 1 -ml venous whole blood samples were collected into heparinized tubes at various times and centrifuged. The plasma was then removed and stored at -80°C until subsequent analysis of plasma drug concentrations using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Computerized image reconstruction was used to obtain dynamic data sets in the form of multiple image frames. A PET image was thus obtained for each scan, by summing the dynamic frames acquired during the acquisition. Regions of interest (ROIs) were drawn on the occipital cortex and pons, with the latter being used to define the level of nonspecific binding. The ROIs were then projected into the dynamic data sets to obtain the corresponding kinetic imaging data in the form of time-activity curves. The time-activity curves were expressed in standard uptake value units, which represent normalized tissue tracer uptake using the individual monkey body weight and the injected tracer dose in each study, and therefore more suitable for cross-comparisons of different studies. MRK-016 occupancy was defined as the extent by which the specific binding of [^{11}C]flumazenil was reduced in the occipital cortex relative to baseline scans performed using vehicle injections.

Behavioral Pharmacology

All assays were performed as described in detail in previous publications (Atack et al., 2006b; Dawson et al., 2006). All animals were purchased from B&K Universal (Hull, UK), except the CD1 mice used for the kindling study, which were obtained from Charles River (Margate, Kent, UK).

Rat Elevated Plus Maze. Male Sprague-Dawley rats (250–300 g) received either vehicle (0.5% methylcellulose p.o.; dose volume, 1 ml/kg), MRK-016 (3, 10, or 30 mg/kg p.o.), or the nonselective partial inverse agonist FG-7142 (30 mg/kg i.p. in a 70% polyethylene glycol vehicle). Thirty minutes later, rats were given a 5-min trial on the elevated plus maze, and the times spent on the open and closed arms and the central area of the plus maze were calculated, with the time spent in the closed arms being used as an index of anxiety. Data for

each measurement are expressed as mean \pm S.E.M. ($n = 18$ /group). Statistically significant differences between MRK-016- and vehicle-pretreated rats were determined using one-way ANOVA and Dunnett's post hoc test. A subset of rats ($n = 9$ /dose group) were taken immediately after completion of the plus maze trial, and occupancy was measured as described above.

Mouse Rotarod. Male BTKO mice (26–30 g; $n = 8$ /group) were trained to walk on the Rotarod (rotating at 16 rpm) until they were able to complete three consecutive 2-min sessions without falling off. Mice were then dosed per os with either vehicle (0.5% methylcellulose), MRK-016 (1, 3, 10, or 30 mg/kg), or diazepam (10 mg/kg), 30 min before being placed on the Rotarod for a 2-min trial. The time before either falling from the Rotarod or completing the trial was then recorded. Data for each measurement are expressed as mean \pm S.E.M. Statistically significant differences between MRK-016 and vehicle-pretreated mice were determined using two-way ANOVA, followed by Dunnett's post hoc test. Immediately after completion of the trial, mice were taken and receptor occupancy was measured as described above.

Mouse Proconvulsant Activity. Male Swiss-Webster mice (25–30 g; $n = 12$ /group) were injected intraperitoneally with either vehicle [polyethylene glycol 300/water; 70:30 (v/v)] or one of three doses of MRK-016 (1, 3, and 10 mg/kg). Thirty minutes later, mice were infused via a marginal tail vein with pentylenetetrazole (PTZ; 15 mg/ml at an infusion rate of 0.2 ml/min), and the PTZ doses that induced clonic and tonic seizures were determined. Comparisons between the dose of PTZ required to induce clonic and tonic convulsions in vehicle- and MRK-016-treated mice were assessed using a one-way ANOVA. Separate groups of mice ($n = 4$ –8/group) were dosed and used to measure receptor occupancy as described above.

Mouse Kindling. Male CD1 mice (27–33 g; $n = 8$ /group) were dosed daily for 20 days with either vehicle (separate groups of either 0.2% Tween 80 i.p. or 0.5% methylcellulose p.o.), FG-7142 (40 mg/kg i.p.; 0.2% Tween 80), or MRK-016 (30 mg/kg p.o. in 0.5% methylcellulose). Mice were injected in batches of six after which they were placed into Perspex boxes and observed for 45 min for signs of abnormal behavior (e.g., hypolocomotion, Straub tail, slit eyes, and flattened ears) as well as the incidence of myoclonic jerks and generalized seizures characterized by clonic or tonic contraction of the limbs including loss of righting reflex. Animals with tonic extension of the limbs were culled immediately. After the 45-min observation period, surviving mice were returned to their home cage.

Rat Morris Water Maze. The delayed matching-to-position version of the Morris water maze was performed as described previously (Dawson et al., 2006). Male hooded Lister rats (280–350 g; B&K Universal) were given four trials a day (with an intertrial interval of 30 s) for 9 days to learn that the position of the hidden platform in relation to external visual cues was the same during each of the four trials on the same day but that the platform position changed from day to day. As rats learn the position of the platform, the latency to find the platform during trials 2, 3, and 4 is significantly less than on trial 1, with the difference between the time to find the platform in trials 1 and 2 being used as an index of the ability of the rat to "remember" the platform location.

Two separate experiments each of 5-day duration were performed. In the first, rats were dosed per os with either vehicle (0.5% methylcellulose; 1 ml/kg) or MRK-016 (0.3, 1, or 3 mg/kg). In a second experiment, the specificity of the effects of MRK-016 (3 mg/kg p.o.) on water maze performance was evaluated by determining the ability of the benzodiazepine antagonist flumazenil (10 mg/kg i.p.) to block its effects. In both experiments, MRK-016 or vehicle was dosed 30 min before trial 1 ($n = 9$ –10/group), and there was a 4-h delay between trial 1 and trial 2. In the flumazenil-blockade experiment, vehicle or flumazenil was dosed 15 min before trial 2 ($n = 10$ /group). The savings score was calculated for each animal on each day and then averaged over 5 days. These data were used as the primary indicator of changes in cognitive performance.

After completion of the water maze experiments, rats were used to

measure the receptor occupancy 0.5 and 4.5 h after dosing with MRK-016 at 0.3, 1, or 3 mg/kg p.o. as well as 15 min after dosing with 10 mg/kg i.p. flumazenil, using methods described above. Because there was a 30-min pretreatment period before trial 1 and the interval between trials 1 and 2 is 4 h, the receptor occupancies at $t = 0.5$ and 4.5 h correspond to the times at which trials 1 and 2 were performed, respectively.

Data for each measurement are expressed as mean \pm S.E.M. Statistically significant differences between treatment group were determined using one-way ANOVA followed by post hoc Student-Newman-Keuls tests.

Preclinical Pharmacokinetics and Metabolism

Pharmacokinetics and Bioanalysis. To measure intravenous pharmacokinetics, MRK-016 (1 mg/kg of a 1 mg/ml solution in 50% polyethylene glycol 300) was administered after overnight food deprivation to either male Sprague-Dawley rats (~ 300 g; $n = 6$), female beagle dogs (~ 12 kg; $n = 3$), or male rhesus monkeys (~ 4 kg; $n = 3$). Serial blood samples were then collected into heparinized tubes at various time points up to 8 h after dosing and then they were centrifuged. Plasma samples removed and frozen at -80°C for subsequent bioanalysis.

For oral dosing studies, the animal species, weights, and sex were the same as for the intravenous dosing described above. Animals were dosed with 5 ml/kg MRK-016 made up as a suspension in 0.5% methylcellulose and dosed at a concentration of 0.2, 0.6, or 6 mg/ml (equivalent to 1, 3, and 30 mg/kg, respectively). Doses of either 1 or 30 mg/kg p.o. were given to rats ($n = 6$ /dose); 1, 3, or 30 mg/kg to dogs ($n = 3$ /dose); and 1 and 30 mg/kg to rhesus monkeys ($n = 3$ /dose). Blood samples were taken, and the plasma was prepared and stored before analysis as described above.

Concentrations of MRK-016 in plasma were determined using a quality-controlled LC-MS/MS assay. Aliquots of plasma with added internal standard were subjected to protein precipitation using acetonitrile, and the supernatant was diluted with 25 mM ammonium formate, pH 3.0, before analysis by LC-MS/MS (Micromass Quattro; Micromass Ltd., Manchester, UK). The lower limit of quantification was between 0.3 and 1 ng/ml.

In Vitro Metabolism. [^{14}C]MRK-016 (50 μM) was incubated at 37°C for 2 h in microsomes, and S9 fraction and cytosol were prepared from rat, dog, rhesus monkey, and human liver. Tissue for the human studies was from a pool of 12 individuals. In all cases, reactions were terminated by the addition of acetonitrile. Samples were analyzed by liquid-mass spectrometry, with additional UV or radiochemical detection. Initial structural characterization was by mass spectrometry fragmentation or NMR, with final confirmation by coelution with independently synthesized material.

Hepatocyte Turnover. MRK-016 (0.5 μM) was incubated in triplicate in aerobic Hanks' balanced salt solution containing 20 mM HEPES at 37°C , pH 7.4, with hepatocytes (10^6 cells/ml) either freshly prepared from male Sprague-Dawley rats, female beagle dogs, and male rhesus monkeys or from cryopreserved human hepatocytes. At various times up to 6 h, aliquots were removed, acetonitrile was added, and levels of MRK-016 were determined by LC-MS/MS.

For the hepatocyte turnover data, intrinsic clearance values were calculated using the AUCs of the concentration versus time curves for each species and converted to whole animal values using published scaling factors (Davies and Morris, 1993; Cross and Bayliss, 2000). In vivo hepatic clearances were predicted using the restrictive well stirred model (Pang and Rowland, 1977) using respective rat, dog, rhesus monkey, and human plasma protein binding values of 69, 49, 57, and 69% (data not shown).

Clinical Pharmacology

The safety, tolerability, and pharmacokinetics of MRK-016 were evaluated in healthy human volunteers in three separate studies: 1) a single-dose escalation study in eight healthy young males of

which two received placebo and six were given MRK-016 with a dose range of 0.2 to 10 mg; 2) a multiple-dose study in eight healthy young males of which two received placebo and six were given either 1 or 2 mg of MRK-016 every 6 h for 3.25 days for a planned total of 13 doses; and 3) an intended single-dose escalation study in eight healthy elderly males and eight healthy elderly females that, due to poor tolerability, was terminated after the initial 0.5-mg dose.

Blood samples were taken for up to 24 h after dosing in the single-dose studies and up to 6 h after dosing on day 1 and up to 24 h after dosing on day 4 in the multiple-dose study. Blood samples were kept on ice before being centrifuged within 0.5 h of collection, after which plasma was removed and then frozen at -20°C for subsequent analysis of MRK-016 concentrations by LC-MS/MS using methods similar to those described above for preclinical samples.

The safety and tolerability of MRK-016 were assessed by physical examination and the measurement of vital signs and routine blood biochemistry and hematology. The occurrence of adverse events was noted.

Results

Affinity of MRK-016 at Human Recombinant GABA_A Receptors

MRK-016 had high and essentially equivalent affinity for the benzodiazepine binding site of human recombinant GABA_A receptors containing either an $\alpha 1$, $\alpha 2$, $\alpha 3$, or $\alpha 5$ subunit (K_i value range, 0.83–1.4 nM; Table 1). However, and as with prototypic benzodiazepines such as diazepam, the affinity was much lower at GABA_A receptors containing either an $\alpha 4$ or $\alpha 6$ subunit (the so-called diazepam-insensitive GABA_A receptors). The affinity of MRK-016 at native rat brain receptors (0.95 and 1.5 nM in cerebellum and spinal cord, respectively) was similar to that measured in recombinant human GABA_A receptors.

Intrinsic Efficacy of MRK-016

Figure 2 shows a comparison of the intrinsic efficacies of MRK-016 and $\alpha 5\text{IA}$ (Dawson et al., 2006). Like $\alpha 5\text{IA}$, MRK-016 is clearly an $\alpha 5$ -selective inverse agonist in that it has much greater efficacy at the $\alpha 5$ subtype (maximal efficacy, -55%) compared with the $\alpha 1$, $\alpha 2$, or $\alpha 3$ subtypes (respective

maximal efficacy values, -16 , $+6$, and -9% , respectively). Most notably, however, the efficacy of MRK-016 at the $\alpha 5$ subtype (-55%) is greater than that observed for $\alpha 5\text{IA}$ (-40%) and is comparable with the $\alpha 5$ inverse agonism of the nonselective full inverse agonist DMCM (-57%) (Dawson et al., 2006).

Effects of MRK-016 Mouse Hippocampal Brain Slice Electrophysiology

PPF is the enhancement of the fEPSP of a second stimulus when it follows shortly after an initial, equivalent stimulation. Figure 3A shows the ratio of the slopes of second (fEPSP2) to the first (fEPSP1) synaptic potential at each different stimulus interval. Under baseline (i.e., vehicle) conditions, there was clear PPF, with the fEPSP of the second stimulus being approximately 1.85 times the size of the initial stimulus when the interval was from 25 to 50 ms. The PPF response decreased as the interval between the two pulses increase such that the second stimulus was approximately 1.25 times the size of the initial stimulus when the interval was 300 ms. There was a trend for PPF to be increased by $\alpha 5\text{IA}$, whereas MRK-016 significantly enhanced PPF at intervals of 50 and 100 ms ($P < 0.05$).

LTP, which is a measure of increased synaptic efficacy, was induced using a standard paradigm that involves a tetanic stimulus followed 30 min later by a θ -burst stimulus (Fig. 3B). In the period from 85 to 90 min after the θ -burst stimulus, the fEPSP slope in vehicle-treated slices was $150 \pm 7\%$ of baseline conditions, with this long-lasting increase in synaptic efficacy representing LTP. During this corresponding period, the fEPSP slopes observed in the presence of $\alpha 5\text{IA}$ and MRK-016 were 175 ± 9 and $210 \pm 11\%$, both of which were significantly increased relative to vehicle-treated slices ($P < 0.05$ and $P < 0.001$, respectively). Furthermore, the enhancement in LTP produced by MRK-016 was significantly greater than that produced by $\alpha 5\text{IA}$ ($P < 0.05$).

MRK-016 Occupancy in Rat

Rat brain benzodiazepine binding site MRK-016 occupancy was dose-dependent in the range from 0.3 to 10 mg/kg p.o.,

Table 1

Affinity of MRK-016 for the benzodiazepine site of recombinant human and native rat brain GABA_A receptors

Data shown are the mean \pm S.E. ($n = 3$ separate determinations).

K_i for Human Recombinant GABA _A Receptors Containing $\beta 3$, $\gamma 2$ Plus						K_i for Native Rat Brain Receptors	
$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 4$	$\alpha 5$	$\alpha 6$	Cerebellum	Spinal Cord
0.83 ± 0.08	0.85 ± 0.16	0.77 ± 0.10	400 ± 170	1.4 ± 0.4	4100 ± 900	0.95 ± 0.37	1.5 ± 0.5

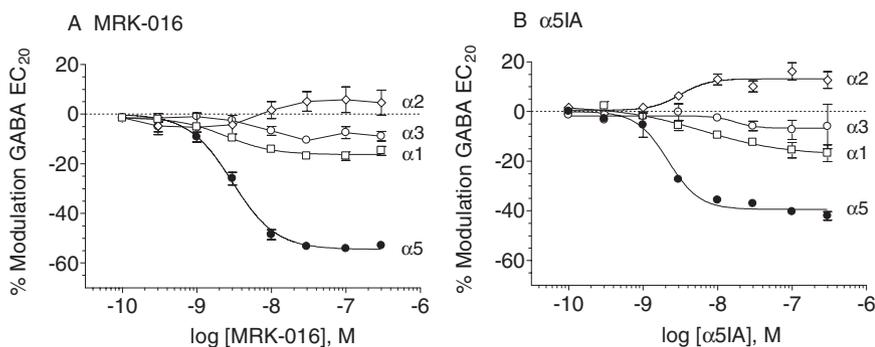


Fig. 2. Comparison of the intrinsic efficacies of MRK-016 and $\alpha 5\text{IA}$ at human recombinant GABA_A receptors. The ability of either compound to attenuate (negative values; inverse agonism or negative allosteric modulation) or potentiate (positive values; agonism or positive allosteric modulation) the current produced by an EC_{20} -equivalent concentration of GABA was measured by whole-cell patch-clamp electrophysiology in mouse fibroblast L-cells stably expressing human $\beta 3$, $\gamma 2$ and either $\alpha 1$, $\alpha 2$, $\alpha 3$, or $\alpha 5$ subunits. Values shown are mean \pm S.E.M. ($n = 4-7$). Data for $\alpha 5\text{IA}$ are modified from Dawson et al. (2006).

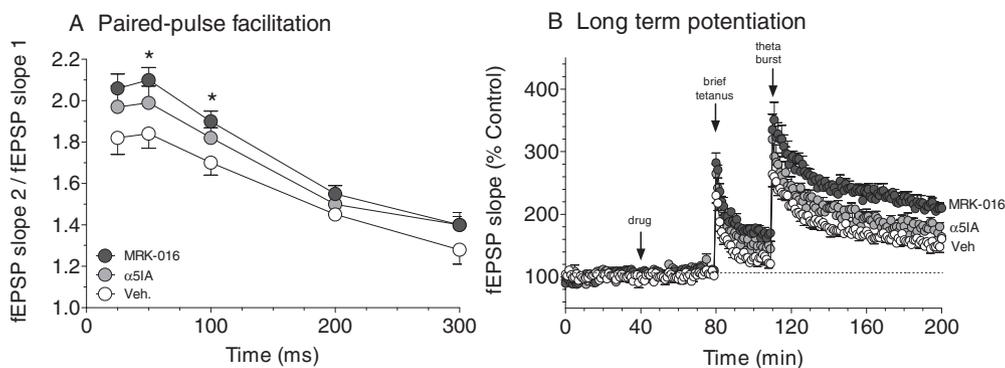


Fig. 3. Effects of MRK-016 and α 5IA on PPF and LTP in mouse hippocampal slices. A, a pair of equivalent pulses separated by an interval of either 25, 50, 100, 200, or 300 ms were delivered to the CA3 region of hippocampal slices incubated with vehicle (0.1% dimethyl sulfoxide) under baseline conditions or with MRK-016 (100 nM) or α 5IA (100 nM). The ratio of the size of the two fEPSP responses recorded from the CA1 was plotted as a function of pulse interval. Values shown are mean \pm S.E.M. ($n = 23$ – 24 /group). B, both a brief tetanus (10 stimuli at 100 Hz) and a θ -burst (four pulses at 100 Hz repeated 10 times at an interval of 200 ms) produced a long-lasting increase in the fEPSP slope values (i.e., LTP) that was potentiated in the presence of 100 nM MRK-016 and α 5IA. Values shown are mean \pm S.E.M. ($n = 13$ – 16 slices/treatment group). *, $P < 0.05$, significant difference between MRK-016- and vehicle-treated slices.

with the estimated Occ_{50} value measured 0.5 h after dosing being 0.39 mg/kg (Fig. 4A). For each dose of either 1, 3, or 10 mg/kg p.o., maximal occupancy was observed 0.5 h after dosing, at which time respective occupancies at 1, 3, and 10 mg/kg were 79, 81, and 91% (Fig. 4B). At later times, there were bigger differences between groups such that after 4 h, for example, respective occupancies at 1, 3, and 10 mg/kg were 20, 33, and 76%. After 8 h, there was no detectable occupancy after a dose of 1 mg/kg and only modest levels of occupancy at 3 and 10 mg/kg (17 and 34%, respectively). There was no measurable occupancy 24 h after dosing with either 1, 3, or 10 mg/kg (data not shown).

Plasma Concentration-Occupancy Relationships in Rat and Rhesus Monkey

Plasma was collected from rats used for the dose-response and time course studies described above (Fig. 4), and plasma MRK-016 concentrations were measured by LC-MS/MS. This permitted occupancy to be plotted as a function of plasma drug concentrations (Fig. 5A), and these data were fitted to a single-site model, with a Hill slope of 1.02. The plasma drug concentration required to occupy 50% of brain receptors was estimated to be 15 ng/ml (equivalent to a plasma concentration of 42 nM). The corresponding concentration of drug in the brain was 34 ng/g (data not shown), suggesting that at a level of occupancy of 50%, the brain/plasma ratio of MRK-016 is in the region of 2.3.

In the rhesus monkey PET imaging study, a bolus dose of MRK-016 (1 mg/kg) produced close to saturating receptor

occupancy ($\sim 90\%$) at 0.5 h after injection, with substantial occupancy ($\sim 50\%$) still present 3 to 4 h after dosing. Using administration protocols starting with a bolus injection followed by constant infusion of MRK-016, plasma steady-state drug concentrations were achieved in a series of rhesus monkey PET imaging studies with MRK-016 concentrations ranging between ~ 3 and ~ 200 ng/ml. From these collective data, a plasma concentration-occupancy curve was constructed, with the EC_{50} value being 21 ng/ml (Hill slope = 0.97; Fig. 5B).

MRK-016 Effects in Morris Water Maze

In the hippocampal-dependent, delayed-matching-to-position version of the Morris water maze, MRK-016 dose-dependently increased the difference between trials 1 and 2 (i.e., reduced the latency to find the platform in trial 2), and this improved performance was significantly greater than in vehicle-treated rats at doses of 1 and 3 mg/kg (Fig. 6A). There was no significant effect of MRK-016 on the time to locate the platform in trial 1, which is consistent with the lack of effect of the compound on general locomotor activity. Occupancy studies, conducted in parallel in a separate group of rats, showed that at the minimally effective dose of 1 mg/kg, the occupancy of rat brain GABA_A receptors 0.5 and 4.5 h after dosing (corresponding to the times of trials 1 and 2) was 72 and 30%, respectively (Fig. 6A). The 0.3-mg/kg dose of MRK-016 failed to produce a significant effect on the savings score, even though the receptor occupancies were estimated at 61%

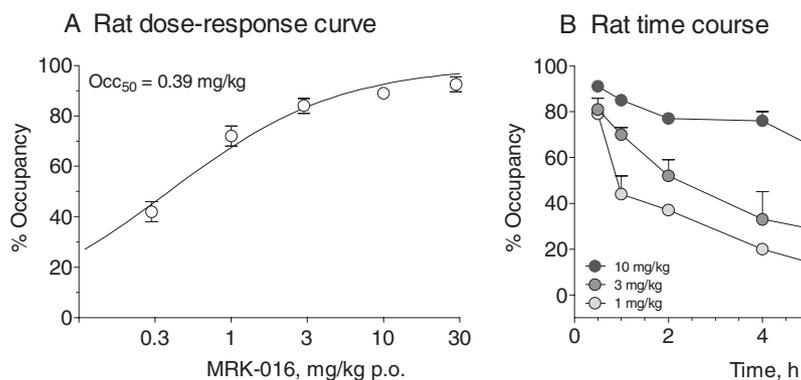


Fig. 4. Occupancy of Sprague-Dawley rat brain GABA_A receptors by MRK-016. A, rats were given either vehicle (0.5% methylcellulose) or MRK-016 (0.3–30 mg/kg) via the per os route, and occupancy was measured 0.5 h later using a [³H]flumazenil in vivo binding assay. B, rats were dosed with either vehicle (0.5% methylcellulose) or 1, 3, or 10 mg/kg MRK-016, and occupancy was then measured 0.5, 1, 2, 4, 8, or 24 h later using [³H]flumazenil in vivo binding. Occupancy was defined as the extent by which the specific binding of [³H]flumazenil was reduced in drug-treated relative to vehicle-treated rats. Values shown are mean \pm S.E.M. ($n = 4$ – 7 /group).

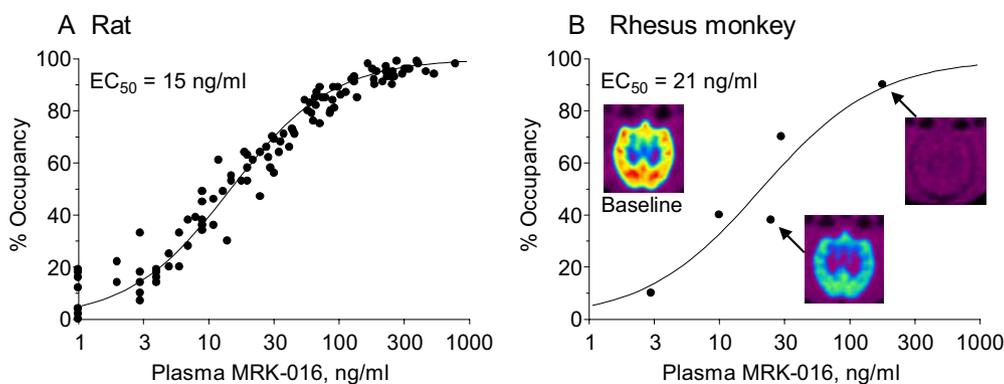


Fig. 5. Relationship between plasma MRK-016 concentrations and brain GABA_A receptor occupancy in rat and rhesus monkey. A, rat occupancy data are derived from the MRK-016 occupancy dose-response and time course data described in Fig. 4. B, rhesus monkey brain occupancy was measured using [¹¹C]flumazenil PET. Insets show representative pseudocolor images of the uptake of [¹¹C]flumazenil in a horizontal plane either before (baseline) or after administration of MRK-016.

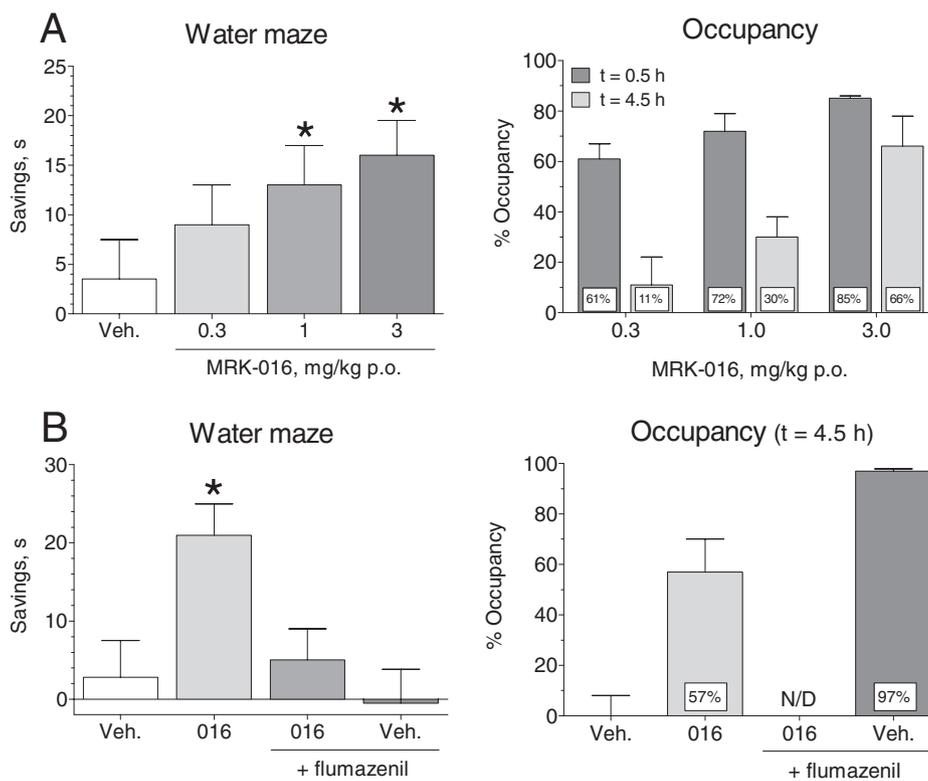


Fig. 6. Cognition-enhancing effects of MRK-016 on the delayed matching-to-position version of the Morris water maze. A, MRK-016 administered to hooded Lister rats 0.5 h before trial 1 produced a dose-dependent increase in the savings time (i.e., the difference between the latencies of trials 1 and 2 using a 4-h intertrial interval), which was significantly greater than vehicle (0.5% methylcellulose) at doses of 1 and 3 mg/kg. A separate group of animals were given either vehicle or MRK-016 (0.3, 1, or 3 mg/kg p.o.), and occupancy was measured 0.5 and 4.5 h later (times corresponding trial 1 and trial 2, respectively). Values shown are mean \pm S.E.M. ($n = 10$ /group or 4–6/group for water maze and occupancy experiments, respectively), with figures within the occupancy bar chart denoting the mean occupancy value. B, on each of 5 consecutive days, MRK-016 (3 mg/kg; $n = 20$) or vehicle (0.5% methylcellulose; $n = 20$) was administered per os 0.5 h before trial 1. Fifteen minutes before trial 2, half of the animals from each group received intraperitoneal injections of either flumazenil (10 mg/kg) or vehicle (0.5% methylcellulose). A satellite group of rats was treated in the same way, and at a time corresponding to the commencement of trial 2 (i.e., 4.5 h after dosing with MRK-016 or vehicle), MRK-016 or flumazenil occupancy was measured. Values shown are mean \pm S.E.M. ($n = 10$ /group or 5–6/group for water maze and occupancy experiments, respectively), with figures within the occupancy bar chart denoting the mean occupancy value. For the water maze data, the statistically significant differences between groups were determined using Newman-Keuls post hoc tests: *, $P < 0.05$ for MRK-016- versus vehicle-treated animals.

during trial 1. However, occupancy during trial 2 (4 h later) was 11%, suggesting that good receptor occupancy during recall may be an important factor in producing cognition enhancement.

By using the prototypic benzodiazepine antagonist flumazenil, it was possible to confirm that the effects of MRK-016 were mediated via the benzodiazepine binding site. Hence, when administered alone, flumazenil (10 mg/kg i.p.) did not affect performance (consistent with the antagonist efficacy and therefore minimal in vivo effects of this compound)

(Bonetti et al., 1982) but was able to block the cognition enhancing effects of 3 mg/kg MRK-016 (Fig. 6B).

Parentetically, in these studies the estimated Occ₅₀ value for MRK-016 0.5 h after dosing in the hooded Lister rats was 0.14 mg/kg (Fig. 6A), which differs from the Occ₅₀ value of 0.39 mg/kg in Sprague-Dawley rats (Fig. 4). This may, in part, be related to differences in strain but is more likely a consequence of day-to-day differences in occupancy measurements related variability in exposure. For example, in the two hooded Lister occupancy assays, occupancy measured

4.5 h after 3 mg/kg dosing was measured as either 66 or 57% (Fig. 6, A and B, respectively).

MRK-016 Is Not Proconvulsant and Does Not Produce Kindling in Mice

The dose of PTZ required to induce clonic or tonic convulsions was not significantly altered in mice pretreated with MRK-016 (1, 3, or 10 mg/kg i.p.) compared with vehicle-treated mice (Fig. 7, A and B), despite high levels of MRK-016 occupancy being achieved (98% at 10 mg/kg).

Before starting the mouse kindling study, occupancy was measured after a single dose of either MRK-016 (30 mg/kg p.o.) or FG-7142 (40 mg/kg i.p.). MRK-016 gave sustained and high receptor occupancy (Fig. 7C), with maximal occupancy of $94 \pm 2\%$ after 0.5 h and $14 \pm 6\%$ occupancy remaining 8 h after dosing. In contrast, FG-7142 gave relatively short-lived and modest occupancy. Thus, 0.5 h after dosing FG-7142 occupancy was $52 \pm 5\%$, with only $25 \pm 4\%$ occupancy after 1 h and no measurable occupancy after 2 h.

Throughout the 20-day period of the kindling experiment, animals treated with vehicle or MRK-016 (30 mg/kg p.o., once a day) showed no incidence of any type of convulsions (Fig. 7D). However, kindling was observed in FG-7142-treated animals, with seizure severity increasing as the experiment progressed. Specifically, Before the development of convulsant activity, the animals displayed marked hypolocomotion along with slit eyes and flattened ears. The initial convulsions were characterized during the first few occurrences by myoclonic jerks accompanied by Straub tail, and these were followed by clonic contraction of the front legs sometimes accompanied by vocalization. During the later stages of the experiment, convulsions became more frequent and the severity of the convulsion

increased, developing into tonic seizures with more intense myoclonic activity and vocalization.

Elevated Plus Maze and Mouse Rotarod

In the rat elevated plus maze assay, the nonselective partial inverse agonist FG-7142 (30 mg/kg i.p.) had anxiogenic-like activity because it significantly increased the percentage of time spent on the closed arms (Fig. 8A). In contrast, MRK-016 did not significantly alter the time spent on the closed arms, even at a dose (30 mg/kg p.o.) corresponding to 96% occupancy.

MRK-016 did not significantly alter performance on the mouse Rotarod assay, with all mice in each dose group completing the 2-min trial without falling off, even at the highest dose tested, 30 mg/kg p.o., which corresponded to occupancy of 97%. In contrast, a dose of diazepam (10 mg/kg p.o.) that produced 66% occupancy caused a significant impairment with mice lasting for only 32 ± 12 s before falling off the Rotarod.

Pharmacokinetics of MRK-016 in Rat, Dog, and Rhesus Monkey

A comparison of the intravenous and per os pharmacokinetics of MRK-016 in rat, dog, and rhesus monkey is shown in Fig. 9A, with key parameters derived from these data being presented in Table 2. In all three species, the half-life was short (≤ 0.5 h) as a consequence of the relatively high rates of clearance (27, 39, and 33 ml/min/kg in rat, dog, and rhesus monkey, respectively), the values of which approach liver blood flow in dog and rhesus monkey and low volumes of distribution (range, 0.6–1.6 l/kg; Table 2).

In rat, MRK-016 had good oral bioavailability at doses of 1 and 30 mg/kg, with respective bioavailability values of 52 and

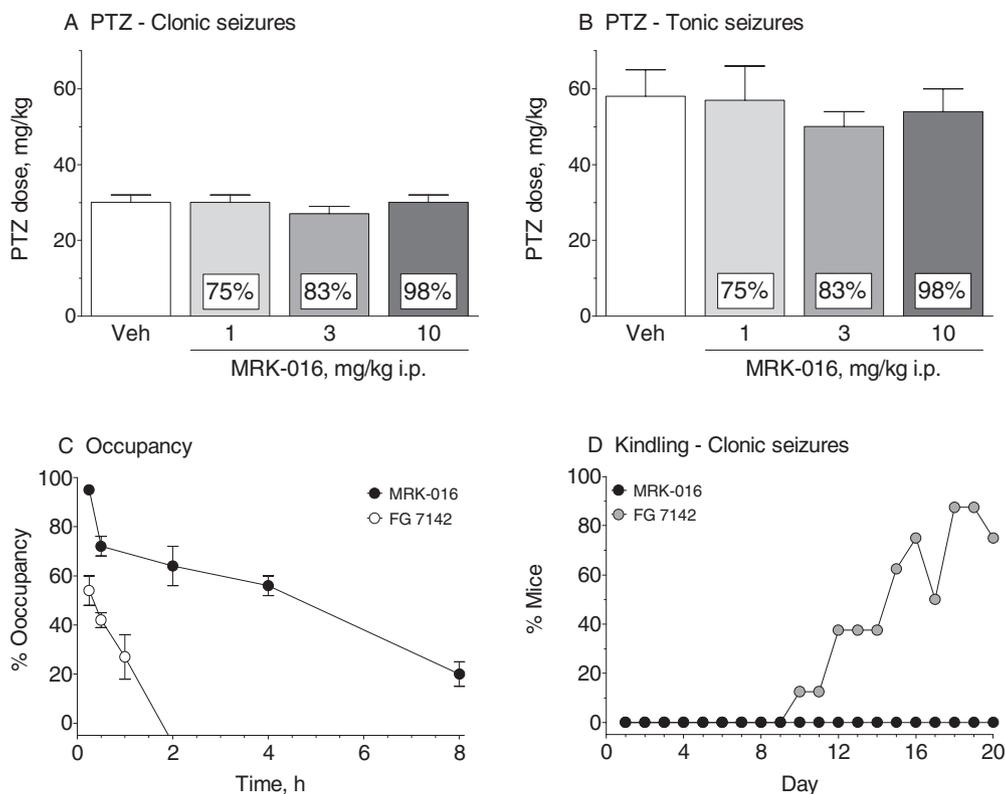


Fig. 7. MRK-016 is not proconvulsant nor does it produce kindling in mice. A and B, dose of pentylentetrazole required to produce clonic and tonic convulsions was measured in mice pretreated for 0.5 h with either vehicle (70% polyethylene glycol i.p.) or MRK-016 (1, 3, or 10 mg/kg i.p.). Figures within bars show the degree of receptor occupancy measured in a satellite group of mice 0.5 h after dosing ($n = 4-8$ /group). Bars represent the mean \pm S.E.M. ($n = 12-13$ /group), with figures within individual bars denoting the mean occupancy value measured in satellite groups of mice. C, comparison of the occupancy of mouse brain GABA_A receptors at various times after dosing with MRK-016 (30 mg/kg p.o. in 0.5% methylcellulose) or FG-7142 (40 mg/kg i.p. in 0.2% Tween 80). Values shown are mean \pm S.E.M. ($n = 5-8$ /group) D, percentage of mice developing clonic seizures ($n = 8$ /group) in a 45-min period after dosing on each day of a 20-day study. For clarity, data for vehicle-treated mice, none of which showed any signs of seizure activity throughout the duration of the study, have been omitted.

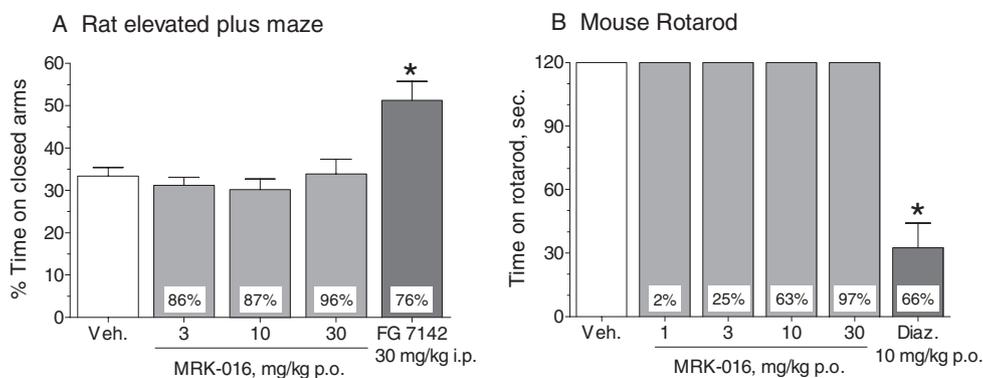


Fig. 8. MRK-016 was not anxiogenic in the rat elevated plus maze assay nor did it impair performance in mice on the Rotarod. A, rats were given either vehicle (0.5% methylcellulose), MRK-016 (3, 10, or 30 mg/kg p.o.), or FG-7142 (30 mg/kg i.p. in 70% polyethylene glycol 300), and 0.5 h later they were used in a 5-min trial on the elevated plus maze, and the time spent on the open closed arms was used as an index of anxiety. Data shown are mean \pm S.E.M. ($n = 17$ – 18 /group). Figures within individual bars denote the mean occupancy value measured in a subgroup of rats ($n = 9$ /group) immediately after completion of the plus maze trial. B, mice were pretreated for 0.5 h with either vehicle (0.5% methylcellulose), MRK-016 (1, 3, 10, or 30 mg/kg), or diazepam (10 mg/kg p.o.) after which they were given a 2-min trial on the Rotarod. Data shown are mean \pm S.E.M. ($n = 8$ /group). Figures within individual bars denote the mean occupancy value measured in mice immediately after completion of the Rotarod trial. In these studies, the OC_{50} value for MRK-016 in mouse brain was estimated at 6.6 mg/kg. *, $P < 0.05$ (using Newman-Keuls post hoc test for the elevated plus maze or Dunn's multiple comparison test following a Kruskal-Wallis nonparametric one-way ANOVA for the Rotarod assay).

A i.v. and p.o. kinetics

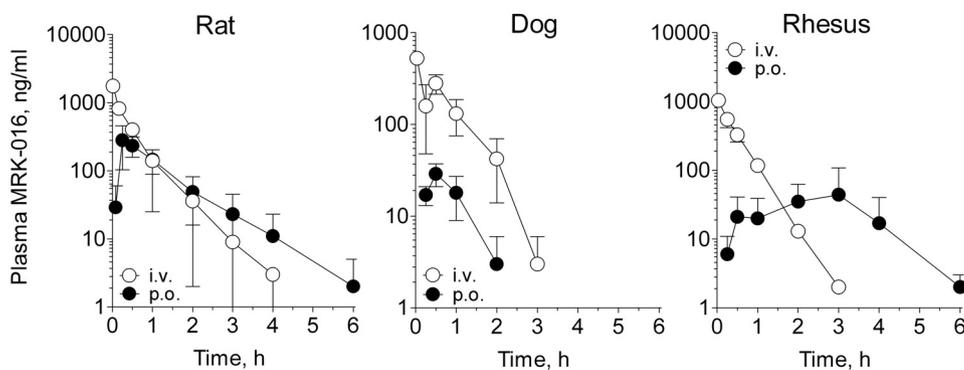
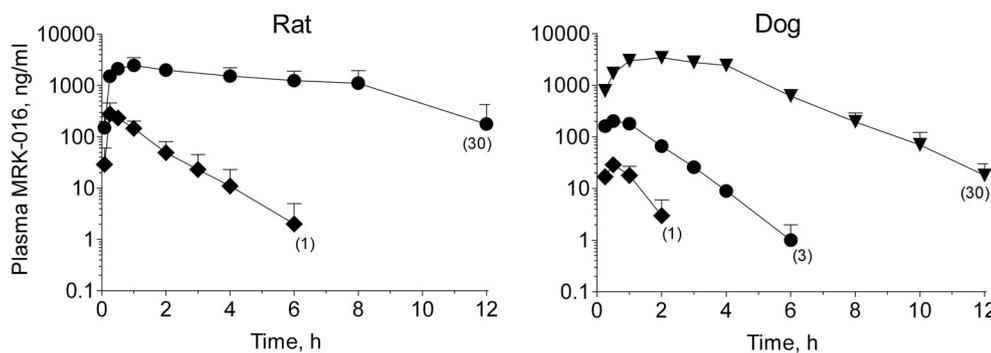


Fig. 9. Plasma pharmacokinetics of MRK-016 in male Sprague-Dawley rats, female beagle dogs, and male rhesus monkeys. A, comparison of intravenous and per os kinetics. Intravenous doses in rat, dog, and rhesus monkey were all 1 mg/kg, whereas the corresponding oral doses were 1, 1, and 30 mg/kg, with the latter dose being chosen for rhesus monkey because plasma concentrations at a dose of 1 mg/kg were all below the level of detection. Values shown are mean \pm S.E.M. ($n = 6$ for rat and 3 for dog and rhesus monkey). B, dose-dependent exposure of MRK-016 in male Sprague-Dawley rats (1 and 30 mg/kg p.o.) and female beagle dogs (1, 3, and 30 mg/kg p.o.). Values shown are mean \pm S.D. ($n = 6$ for rat; $n = 3$ for dog).

B Dose proportionality



75% and had an approximately dose-proportional increase in total exposure (AUC values at 1 and 30 mg/kg of 350 and 15,300 ng \cdot h/ml, respectively; Table 2). This dose proportionality was not solely related to the C_{max} value, which varied by less than 10-fold (respective C_{max} values of 306 and 2727 ng/ml at 1 and 30 mg/kg) but also made up a component due to a sustained absorption phase observed at the higher dose (Fig. 9B). The sustained absorption at 30 compared with 1 mg/kg is reflected by the T_{max} being later and more variable

at the higher compared with the lower dose (3 ± 3 and 0.4 ± 0.4 h, respectively). It is noteworthy that the systemic exposure and plasma clearance of MRK-016 in Sprague-Dawley rats was relatively variable, which may be a consequence of the variability in expression of aldehyde oxidase, which is partially responsible for its metabolism (Rashidi et al., 1997). In contrast, dog oral bioavailability varied considerably with dose, increasing from 8% at 1 mg/kg to 26 and 109% at 3 and 30 mg/kg, respectively, probably as a consequence of the

Table 2

Pharmacokinetic parameters of MRK-016 in rat, dog, and rhesus monkey

Parameter	Rat		Dog		Rhesus Monkey	
i.v. dosing						
Dose, mg/kg	1		1		1	
Clearance, ml/min/kg	27		39		33	
Volume of distribution, l/kg	0.6		1.6		0.9	
<i>t</i> _{1/2} , h	0.5		0.5		0.3	
AUC, ng · h/ml	679 ± 220		453 ± 136		502 ± 36	
p.o. dosing						
Dose, mg/kg	1	30	1	3	30	30
<i>T</i> _{max} , h	0.4	3	0.5	0.6	1.7	2
<i>C</i> _{max} , ng/ml	306	2727	29	220	3487	61
AUC, ng · h/ml	350 ± 120	15,300 ± 4100	37 ± 6	360 ± 42	14,800 ± 2500	132 ± 106
Oral bioavailability, %	52 ± 24	75 ± 32	8 ± 3	26 ± 9	109 ± 37	<2

saturation of first-pass metabolism with increasing doses (Fig. 9B). In rhesus monkey, MRK-016 oral bioavailability was negligible (<2%) at low and high doses (1 and 30 mg/kg p.o.), suggesting that the first-pass effect is not saturable in this species (whereas it was in dog), consistent with an approximately blood flow-dependent rate of clearance of 33 ml/min/kg.

In Vitro Metabolism of MRK-016

In rat, rhesus monkey, and human systems, the metabolism of MRK-016 was dominated by three oxidative pathways; a cytosolic, non-NADPH-dependent oxidation of the triazine core (metabolite M1 in Fig. 10) plus NADPH-dependent microsomal oxidation of the tertiary butyl and the isoxazole methyl groups (M2 and M3, respectively; Fig. 10). In dogs, in vitro metabolism studies showed that the NADPH-dependent microsomal-mediated M2 and M3 metabolites were produced but that the cytosolic aldehyde oxidase-mediated M1 metabolite was not (Jones et al., 2006). In addition to these three major metabolites, minor metabolites in rat, rhesus monkey, and human (but not dog) included the products of dual microsomal/cytosolic metabolism (e.g., aldehyde plus *tert*-butyl hydroxylation or aldehyde plus methyl isoxazole hydroxylation).

The three major metabolites had only GABA_A receptor binding activity in a panel of more than 130 receptor, en-

zyme, and transporter assays (MDS Pharma Servies, King of Prussia, PA). Relative to parent, the M1 and M2 metabolites had approximately 500- and 10- to 30-fold lower affinity, respectively, whereas the M3 metabolite had an affinity that was comparable with MRK-016 (Table 3). With respect to efficacy, the M2 metabolite was a nonselective partial inverse agonist, whereas the M3 metabolite showed α 5-selective inverse agonist efficacy. However, the brain-to-plasma ratios in rats (3 mg/kg i.p.) were ~0.05 and ~0.08 for M2 and M3, respectively, indicating low brain penetration for both metabolites compared with MRK-016 (brain/plasma ratio of 2.3; see above), consistent with MRK-016 being the primary mediator of in vivo efficacy.

Turnover of MRK-016 in Hepatocytes and Predicted Clearance Values

The rate of turnover of MRK-016 when incubated with hepatocytes from rat, dog, rhesus monkey, and human is illustrated in Fig. 11. The most striking feature of these data is that whereas MRK-016 is rapidly turned over by hepatocytes from rat, dog, and rhesus monkey, it was relatively stable when incubated with human hepatocytes. From these data, the predicted in vivo hepatic clearance values were calculated, and these values are compared with the measured in vivo plasma clearance values in Table 4. For dog and rhesus monkey, the predicted clearance values (both 31 ml/

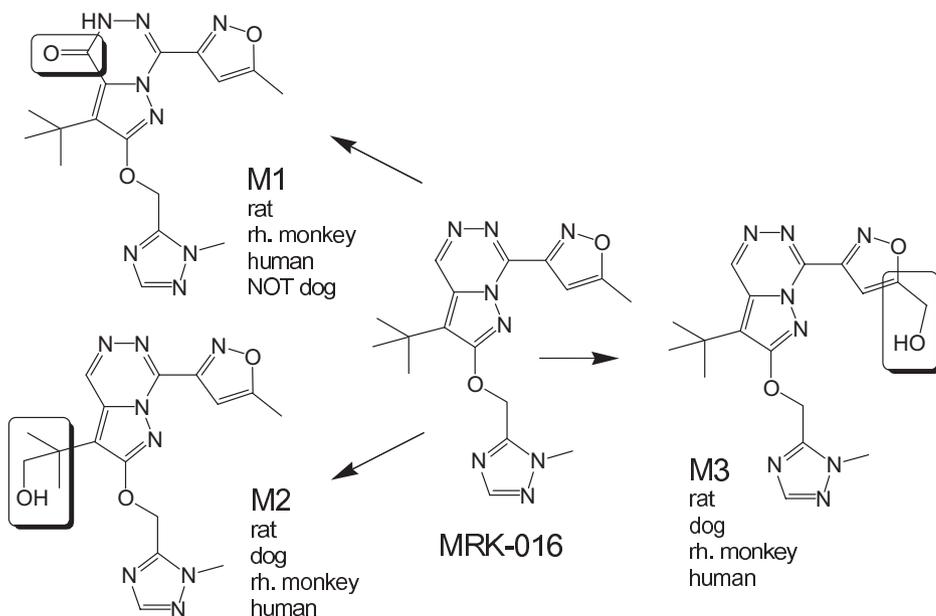


Fig. 10. Scheme summarizing the phase I metabolites produced in vitro by the incubation of MRK-016 with rat, dog, rhesus monkey, and human liver microsomes. The cytosolic, non-NADPH-dependent aldehyde oxidase-mediated oxidation of the triazine core produced metabolite M1 in rat, rhesus monkey, and human, but not dog, microsomes, whereas the products of NADPH-dependent microsomal oxidation of the tertiary butyl and the isoxazole methyl groups (M2 and M3, respectively) were produced by all four species.

Table 3
Affinity and efficacy of MRK-016 and major metabolites at GABA_A receptor subtypes

		Human Recombinant GABA _A Subtype			
		α1	α2	α3	α5
MRK-016 (parent)	Affinity, nM ^a	0.83	0.85	0.77	1.36
	Efficacy, % ^b	-16	6	-9	-55
M1 (aldehyde-aldehyde oxidase product)	Affinity, nM	372	N.D.	578	700
	Efficacy, %	1	0	-8	-39
M2 (<i>tert</i> -butyl hydroxylation)	Affinity, nM	26	N.D.	9.8	9.4
	Efficacy, %	-41	-23	-32	-40
M3 (isoxazole hydroxylation)	Affinity, nM	1.5	N.D.	0.69	1.1
	Efficacy, %	-11	-1	-15	-52

N.D., not determined.

^a Affinity was measured in human recombinant GABA_A receptors using a competition [³H]flumazenil radioligand binding assay as described in detail under *Materials and Methods*.

^b Efficacy was determined using whole-cell patch-clamp electrophysiology and was defined as the ability of a compound to decrease (negative values, inverse agonism) or increase (positive values, agonism) the current produced by the application of an EC₂₀-equivalent concentration of GABA.

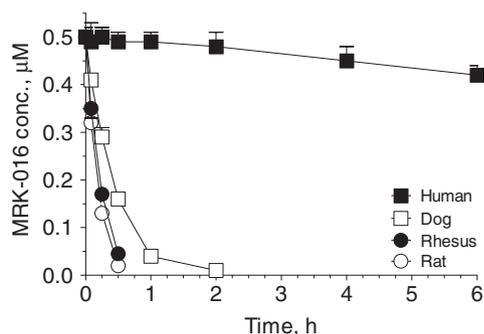


Fig. 11. Turnover of MRK-016 in hepatocytes prepared from single samples of rat, dog, rhesus monkey, and human liver. Samples were removed and analyzed for drug concentrations using high-performance LC-MS/MS. Values shown are mean ± S.D. of triplicate analyses.

Table 4
Predicted hepatic clearance values compared with those measured in vivo

Species	Intrinsic Clearance (Scaled)	Clearance	
		Predicted Hepatic ^a	In Vivo (Measured)
	<i>ml/min/mg</i>	<i>ml/min/kg</i>	
Rat	437	46	27 ± 11
Dog	210	31	39 ± 10
Rhesus monkey	255	31	33 ± 2
Human	<2	<2	N.D.

N.D., not determined.

^a The predicted hepatic clearance assumes that in vivo clearance is mediated solely via the hepatic route and was calculated assuming that MRK-016 was moderately bound to proteins from rat (69%), dog (49%), rhesus (57%), and human (69%) plasma and with in vitro blood-to-plasma partition ratios of 0.9 (rat), 0.9 (dog), rhesus (0.95), and 0.8 (human).

min/kg) are reasonably accurate estimates of the actual in vivo clearance values (39 and 33 ml/min/kg for dog and rhesus monkey, respectively). However, the predicted value for rat clearance (46 ml/min/kg) was a less accurate estimate of the actual value (27 ml/min/kg).

Human Studies

Three different placebo-controlled studies of the tolerability and pharmacokinetics of MRK-016 were performed in human; a single-ascending dose study in healthy young men, a multiple-dose study in healthy young men, and a single-dose study in healthy elderly men and women.

Tolerability

Healthy Young Subjects. In single-dose studies in healthy young male subjects, dose-limiting adverse events (AEs) of lightheaded/dizziness, tingling of fingers/lips, and nausea/vomiting were observed at a dose of 10 mg, with 5 mg being defined as the maximal tolerated dose (Table 5). In a multiple-dose study, a dose of 1 mg given every 6 h for 3.25 days to young healthy male subjects (a total of 13 doses) was generally well tolerated. However, 2 mg given every 6 h was not tolerated on the second day of dosing in three of eight subjects; therefore, all subjects were discontinued on day 2.

Healthy Elderly Subjects. In contrast to healthy young males, in elderly male and female subjects, MRK-016 was poorly tolerated at the initial dose of 0.5 mg, with severe nausea/vomiting being observed in a single male subject (Table 5). The difference in sensitivity of elderly compared with young subjects was not related to differences in pharmacokinetics because pharmacokinetic parameters were similar in young and elderly subjects (see below).

Pharmacokinetics

Healthy Young Subjects. In the single-dose studies, the pharmacokinetic profiles of MRK-016 in healthy young male subjects (Fig. 12A) showed that drug was rapidly absorbed, with a T_{max} value ~1 h. The half-life was in the region of 3 h, and there was a relatively linear relationship between dose and C_{max} value (Fig. 12A, inset) and total exposure (area under the curve; data not shown). The coefficient of variation ranged between 17 and 42% for the C_{max} and between 35 and 55% for the total exposure.

In the multiple-dose study, the C_{max} values after the initial 1- and 2-mg doses on day 1 (16 ± 6 and 32 ± 11 ng/ml, respectively) were similar to those observed in the single-dose study (14 ± 2 and 30 ± 13 ng/ml; Table 5). At the 1-mg dose, for which more data were available, there was no sign of MRK-016 accumulation on day 4 (respective day 1 and day 4 plasma C_{max} values of 16 ± 6 and 17 ± 11 ng/ml and AUC_{0-6h} of 60 ± 27 and 60 ± 50 ng · h/ml). Consequently, the poor tolerability in the 2-mg multiple-dose study does not seem to be related to drug accumulation.

Healthy Elderly Subjects. The pharmacokinetics of a single 0.5-mg dose of MRK-016 did not differ appreciably between healthy elderly male and female subjects (Fig. 12B; Table 5). Moreover, the pharmacokinetic profiles and the parameters derived from these data were comparable in the elderly and young male subjects. Hence, the T_{max} and $t_{1/2}$

Table 5

Summary of pharmacokinetic properties and blinded adverse events for MRK-016 when dosed to healthy human volunteers

 T_{max} and C_{max} values are expressed as mean ± S.D.

Dose	<i>n</i>	T_{max}	C_{max}	$T_{1/2}$	Lightheaded/ dizziness	Tingling of fingers/lips	Nausea/ vomiting	Headache	Warm feeling	Anxiety	Dysphagia	Urticaria	Neck Pain
		<i>h</i>	<i>ng/ml</i>	<i>h</i>									
Single dose, healthy young men													
0.2 mg	8(6) ^a	0.8 ± 0.3	3.2 ± 0.8	3.6	—	—	—	5	—	—	—	—	—
0.5 mg	8(6)	0.9 ± 0.2	6.7 ± 2.2	2.9	—	—	—	2	—	—	—	—	—
1 mg	8(6)	1.0 ± 0	14 ± 2	3.4	—	—	—	—	—	—	—	—	—
2 mg	8(6)	0.8 ± 0.3	30 ± 13	2.9	2	—	—	2	1	—	—	—	—
5 mg	8(6)	1.3 ± 0.5	69 ± 18	3.5	1	2	—	2	—	—	—	—	—
10 mg	8(6)	1.0 ± 0	154 ± 46	3.7	4 ^b	2 ^c	4 ^d	1	2 ^e	—	—	—	—
Healthy young—multiple dose study (QID dosing for 3¼ days—preliminary data)													
1 q.i.d.	8(6) ^f	1.0 ± 0	16 ± 6 ^g	2.3 ^h	1	—	—	3	—	—	—	—	—
2 q.i.d.	8(6)	1.2 ± 0.4	32 ± 11	N/D	5	—	3 ⁱ	2	—	3 ^j	1 ^k	—	—
Single dose, healthy elderly men and women													
0.5 mg (all)	16 ^l				1	—	1 ^m	5	—	—	—	1 ⁿ	1
0.5 mg (male)	6	1.1 ± 0.5	8.8 ± 3.2	3.3	—	—	—	—	—	—	—	—	—
0.5 mg (female)	6	0.9 ± 0.2	10.9 ± 4.0	2.6	—	—	—	—	—	—	—	—	—

N.D., not determined.

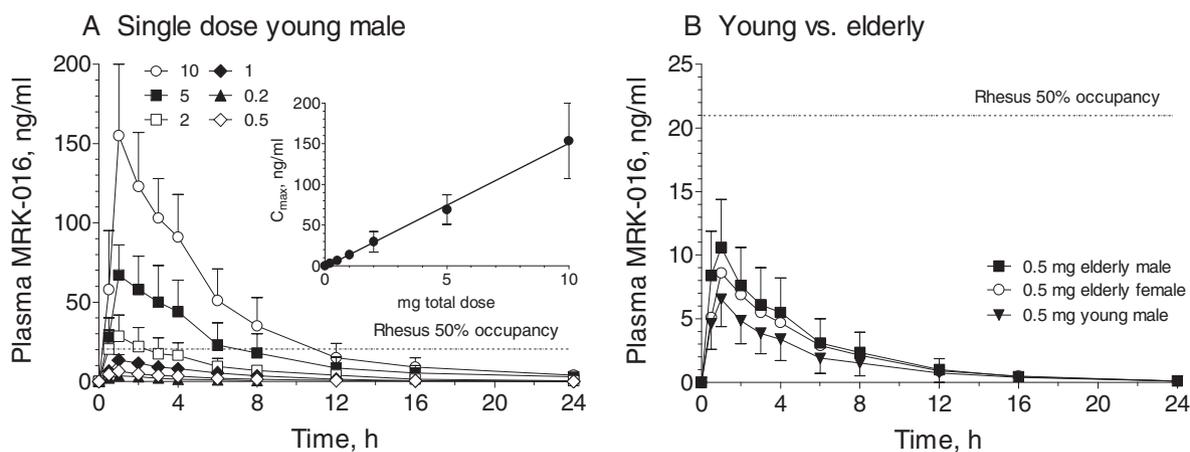
^a 8(6), *n* = 8 for blinded clinical AEs and *n* = 6 for pharmacokinetic analyses.^b 1, mild; 2, moderate; 1, severe.^c 1, moderate; 1, severe.^d 2, moderate; 2, severe.^e 2, moderate.^f 8(6), *n* = 8 for blinded clinical AEs and *n* = 6 for pharmacokinetic analyses.^g C_{max} data from Day 1; ^h, $T_{1/2}$ data calculated after final dose on Day 4;ⁱ 1 severe, resulting in discontinuation.^j 1 resulting in discontinuation.^k moderate resulting in discontinuation.^l *n* = 16 for blinded clinical AEs (of which 12 were given drug and 4 were given placebo).^m Severe, resulting in discontinuation.ⁿ Moderate, resulting in discontinuation.

Fig. 12. Pharmacokinetics of MRK-016 after single doses to healthy normal volunteers. A, plasma drug concentrations of MRK-016 as a function of time after single ascending doses (range, 0.2–10 mg) in healthy young male subjects (values are mean ± S.D.; *n* = 6). Inset shows the linearity of C_{max} as a function of dose. B, comparison of the pharmacokinetic profiles after single doses of MRK-016 (0.5 mg) to healthy young male, elderly male, or elderly female volunteers. Values shown are mean ± S.D. (*n* = 6). The dashed line illustrates the plasma concentration required to achieve 50% occupancy in rhesus monkey as measured using [¹¹C]flumazenil PET (see Fig. 5).

values in the elderly (1.1 and 3.6 h) were similar to corresponding values in young males (0.9 and 2.9 h; Table 5). However, the C_{max} and total exposure values in elderly males (8.8 ± 3.2 ng/ml and 49 ± 25 ng · h/ml, respectively) were slightly higher than the corresponding values in young males (6.7 ± 2.2 ng/ml and 36 ± 20 ng · h/ml). Nevertheless, the plasma exposure of MRK-016 at the poorly tolerated dose of 0.5 mg in the elderly (combined average male plus female $C_{max} = 10$ ng/ml; AUC = 52 ng · h/ml) was still considerably

below the maximal tolerated dose of 5 mg in young male subjects ($C_{max} = 69$ ng/ml; AUC = 436 ng · h/ml).

Interindividual Variability. In the healthy young male single-dose study, the coefficient of variation of the C_{max} and total exposure (AUC) at a dose of 0.5 mg was 33 and 55%, respectively. This variability was comparable in the elderly in which the corresponding coefficients of variation were 37 and 50%. The variability in the elderly is illustrated by comparing the pharmacokinetic profiles of

the two elderly females that had the highest and lowest exposures (Fig. 13A). Analysis of these data showed that there was an approximately 4-fold variation in the C_{\max} values (4.1 and 16 ng/ml) and an 8-fold difference in the total exposure (10 and 85 ng · h/ml).

In the 1-mg multiple-dose study, there was a modest variation in the day 1 C_{\max} and AUC_{0-6h} values (C_{\max} and total exposures ranging from 7.0 to 25 ng/ml and from 20 to 90 ng · h/ml), but by day 4 this variation had increased to an approximately 10-fold difference in minimal and maximal C_{\max} (3.4 and 33 ng/ml) and an 18-fold difference in the AUC_{0-6h} (7.8 and 139 ng · h/ml) (Fig. 13B).

Discussion

MRK-016 is a pyrazolotriazine with high affinity for the benzodiazepine binding site of recombinant human and native rat brain GABA_A receptors ($K_i = 0.8$ – 1.4 and 1.0 – 1.5 nM, respectively), for which it is highly selective because it had very low affinity (K_i or $IC_{50} > 1$ μM) in 19 enzyme and 128 receptor and transporter assays (MDS Pharma Services; data not shown). The affinity of MRK-016 for the benzodiazepine binding site is comparable with that of the triazolopyridazine α5IA (affinity at recombinant human and native rat GABA_A receptors of 0.6–1.2 nM; Dawson et al., 2006). Moreover, the intrinsic efficacy of these two compounds is qualitatively similar in that both are α5-selective inverse agonists. However, quantitatively, the inverse agonist efficacy of MRK-016 (–55%) is greater than that reported for α5IA (–40%; Dawson et al., 2006). This difference in inverse agonist efficacy of –15% is the same order of magnitude as the difference in efficacy of the full and partial inverse agonists DMCM and FG-7142 (range, 15–24%, depending on subtype; Dawson et al., 2006), the properties of which can clearly be distinguished both in vitro and in vivo. Likewise, the difference in α5 intrinsic efficacy of MRK-016 and α5IA manifested itself in vitro in that MRK-016 produced greater changes in PPF and LTP compared with α5IA. The role of the α5 subtype in mediating the MRK-016-induced enhancement of LTP was confirmed in separate experiments in hippocampal slices from α5H105R point-mutated mice in which MRK-016 did not alter LTP (data not shown). Furthermore, the intrinsic efficacy profile observed in stably expressed human recombi-

nant GABA_A receptors (i.e., pronounced α5 inverse agonism with weak, if any, efficacy at the α1, α2, and α3 subtypes) was similar to that observed in human recombinant GABA_A receptors transiently expressed in *Xenopus laevis* oocytes, which in turn was similar to rat recombinant receptors expressed in the same system (data not shown). It should be emphasized, however, that intrinsic efficacy data for MRK-016 were all generated in recombinant receptor systems, using modulation of peak current amplitude as the primary efficacy measure. Accordingly, it is uncertain to what extent such data reflect the efficacy at native receptors, especially when effects upon the rate of receptor activation, desensitization, and/or deactivation may also be important factors.

In the delayed-matching-to-position version of the Morris water maze, the minimal effective dose of MRK-016 was 1 mg/kg, which gave occupancy at the time of encoding (trial 1) of 72% and during recall (i.e., 4 h later at trial 2) of 30%. These data compare with a minimal effective dose of α5IA of 0.3 mg/kg, which gave occupancy of ~25% at trials 1 and 2 (Dawson et al., 2006). From a mechanistic view, it is not clear at which stage of the “memory” process, namely, encoding, consolidation, or recall, an α5-selective inverse agonist might exert its effects. However, when given before trial 2, the benzodiazepine antagonist flumazenil could block the performance-enhancing effects of MRK-016, suggesting that α5-selective inverse agonists might affect recall. A more systematic analysis was carried out using α5IA-II, which contains a pyridyl group in contrast to the methyl triazole found in α5IA. By varying the time at which α5IA-II was administered (i.e., before or after trial 1 or before trial 2), as well as by using flumazenil, it was shown that α5IA-II seemed to affect both the encoding and recall processes but did not appear to influence the consolidation phase (Collinson et al., 2006). It is therefore not clear whether a target occupancy for human studies with MRK-016 should be based upon the occupancy during encoding (72%) or recall (30%), although the work of Nutt et al. (2007) suggests that the effects of α5IA on the reversal of ethanol-induced impairments of word recall in human was as a consequence of modifying encoding rather than recall.

Assuming that the rhesus monkey plasma concentration-occupancy relationship is similar to that observed in human (which is not unreasonable given the similarity between the

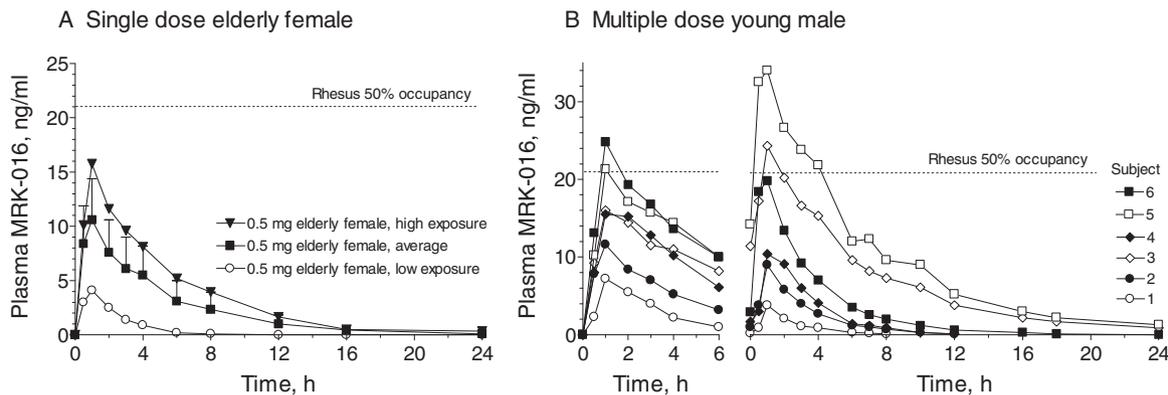


Fig. 13. Variability in human plasma MRK-016 concentrations. A, comparison of the interindividual variability in exposure in the 0.5-mg single-dose, elderly female study showing the mean value (also see Fig. 12B) as well as those individuals with the highest and lowest exposures. B, individual pharmacokinetic profiles for the six healthy young male subjects that received four doses/day of 1 mg MRK-016 as measured after the initial dose on day 1 and then after the first (and final) dose on day 4. The dashed line illustrates the plasma concentration required to achieve 50% occupancy in rhesus monkey as measured using [¹¹C]flumazenil PET (Fig. 5).

rat and rhesus monkey plasma EC₅₀ values of 15 and 21 ng/ml, respectively), then the C_{max} values at the maximal tolerated single and multiple doses of 5 mg (69 ng/ml) and 1 mg (16 ng/ml) would correspond to occupancy values of ~75 and ~45%, respectively. This suggests that in human it is possible to achieve the levels of occupancy predicted to be required for efficacy based on studies in rat. More specifically, efficacy in rat requires occupancy in the region of 30 to 72% for MRK-016 (Fig. 6), 25% for α 5IA (Dawson et al., 2006), or 30% for RO4938581 (Ballard et al., 2009). Clearly, the extrapolation between occupancy of an α 5-selective inverse agonist required for efficacy in the rat water maze and cognitive performance in human is tenuous. Nevertheless, consistent with the hypothesis that only moderate (~25–75%) levels of α 5 occupancy may be required for efficacy in human is the observation that the 4-mg dose of α 5IA that reversed the ethanol-induced impairment of word list learning in human (Nutt et al., 2007) corresponds to occupancy in the region of 50 to 60% (Atack, 2009).

The data showing that MRK-016 is not anxiogenic or proconvulsant and does not produce kindling are consistent with previous data obtained with α 5IA (Dawson et al., 2006). These data, taken together with those from studies conducted with the α 5 binding-selective compounds L-655,708 and RO4938581 (Atack et al., 2006a; Ballard et al., 2009), demonstrate that in preclinical species inverse agonism at the α 5 subtype is not associated with the liabilities associated with nonselective inverse agonists, such as FG-7142 (Dorow et al., 1983; Horowski and Dorow, 2002).

The half-life of MRK-016 was considerably longer in human (range, 2.3–3.7 h, depending upon the dose, age, and gender of subjects) than in preclinical species (0.3–0.5 h; Table 2), consistent with the observations in vitro that the turnover of compound was much lower in human compared with rat, dog, or rhesus monkey hepatocytes (Fig. 11). However, there were appreciable interindividual differences in exposure that increased upon multiple dosing (Fig. 13), and these may be related to differences in the expression of aldehyde oxidase (Rodrigues, 1994; Al-Salmly, 2001), an enzyme that is also involved in the metabolism of famciclovir, zaleplon, ziprasidone, and zonisamide (Kitamura et al., 2006; Strolin Benedetti et al., 2006). It is important to note that none of the major metabolites of MRK-016 were considered responsible for the pharmacological activity of MRK-016 as a consequence of either low affinity and/or efficacy as well as low brain penetration and occupancy (Table 3).

Regarding the tolerability of MRK-016 in human, there are several features worthy of comment. In the single-dose, young male studies, the dose-limiting unblinded AEs observed after a 10-mg dose were lightheadedness/dizziness, tingling of fingers/lips, and nausea/vomiting. At the poorly tolerated 10-mg dose and the maximal tolerated 5-mg dose, the plasma C_{max} values (154 \pm 46 and 69 \pm 18 ng/ml) correspond to occupancy values (estimated based upon the rhesus monkey plasma concentration-occupancy EC₅₀ of 21 ng/ml) of 89 and 76%, respectively. In a multiple-dose (four times/day) study, the 1-mg dose was well tolerated for the planned duration (3.25 days) of the study, but although the 2-mg dose was well tolerated on day 1 it was poorly tolerated on day 2. This lack of tolerability in the 2-mg multiple dose was not due to drug accumulation nor was it related to exposure per se because much higher plasma drug concen-

trations were achieved at maximal tolerated dose of 5 mg in the young (C_{max} values of 32 \pm 11 and 69 \pm 18 ng/ml in the 2-mg multiple-dose and 5-mg single-dose studies, respectively). The reason for the poor tolerability of MRK-016 in the 2-mg multiple-dose study is unclear but is presumably unrelated to the mechanism because there was no reduction in tolerability in the multiple- compared with single-dose studies of α 5IA (Atack, 2009). One of the unblinded AEs observed in the 2-mg multiple-dose study was anxiety, but this was not comparable with the waves of anxiety reported after dosing with the nonselective partial inverse agonist FG-7142 (Dorow et al., 1983; Horowski and Dorow, 2002). It is important to note that anxiety was not reported as an AE in either of the single-dose studies performed with MRK-016 in young or elderly volunteers, and neither was it observed in the single- or multiple-dose studies of α 5IA carried out in young and elderly volunteers (Atack, 2009).

Although MRK-016 was well tolerated in young males, with the maximal tolerated single dose being 5 mg, it was poorly tolerated in the elderly, even at the relatively low dose of 0.5 mg. This pronounced difference in tolerability was not related to pharmacokinetics, because the drug had a similar C_{max} and half-life in elderly and young subjects (Table 5) and so is presumably a pharmacodynamic response because it is generally accepted that elderly subjects have an increased sensitivity to central nervous system-active drugs (Turnheim, 2003; EIDesoky, 2007).

In summary, MRK-016 is an α 5-selective GABA_A receptor inverse agonist with an improved intrinsic efficacy profile relative to α 5IA (Dawson et al., 2006). It enhanced PPF and LTP in a mouse hippocampal slice model and had improved performance in the delayed matching-to-position version of the water maze but was not anxiogenic or proconvulsant. It was progressed into human where, as predicted, it had a longer half-life than in preclinical species but despite being well tolerated in healthy young male subjects was poorly tolerated in the elderly, an effect that was not a pharmacokinetic effect but was rather due to increased sensitivity to central nervous system-related adverse events in the elderly. This, along with the interindividual variability in pharmacokinetics that was possibly related to variations in aldehyde oxidase activity, resulted in the termination of the development of this compound. Moreover, although an α 5-selective inverse agonist has been shown to enhance a pharmacologically (ethanol)-induced cognitive deficit (Nutt et al., 2007), the hypothesis that such a compound might improve performance in a clinically impaired population (e.g., Alzheimer's disease or schizophrenia) remains to be evaluated, although the novel pharmacological properties of compounds such as MRK-016, α 5IA (Dawson et al., 2006), PWZ-029 (Savić et al., 2008), and RO4938581 (Ballard et al., 2009) should encourage further studies in this area.

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