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TITLE PAGE

NS11394 ([3'-[5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitrile]), a unique subtype-selective $GABA_A$ receptor positive allosteric modulator: *In vitro* actions, pharmacokinetic properties and *in-vivo* anxiolytic efficacy

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Running title page: NS11394, a subtype-selective $GABA_A$ receptor modulator

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Abbreviations: CNS, central nervous system; GABA, gamma-amino-butyric acid; CER, conditioned emotional response.

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Abstract

The novel positive allosteric modulator NS11394 [3'-[5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitrile] possesses a functional selectivity profile at $GABA_A$ receptors of: $\alpha_5>\alpha_3>\alpha_2>\alpha_1$ based on oocyte electrophysiology with human $GABA_A$ receptors. Compared to other subtype-selective ligands, NS11394 is unique in having superior efficacy at $GABA_A$-α_3 receptors while maintaining low efficacy at $GABA_A$-α_1 receptors. NS11394 has an excellent pharmacokinetic profile, which correlates with pharmacodynamic endpoints (CNS receptor occupancy) yielding a high level of confidence in deriving *in-vivo* conclusions anchored to an *in-vitro* selectivity profile, and allowing for translation to higher species. Specifically, we show that NS11394 is potent and highly effective in rodent anxiety models. The anxiolytic efficacy of NS11394 is most likely mediated through its high efficacy at $GABA_A$-α_3 receptors, although a contributory role of $GABA_A$-α_2 receptors cannot be excluded. Compared to benzodiazepines, NS11394 has a significantly reduced side effect profile in rat (sedation, ataxia and ethanol interaction) and mouse (sedation) even at full CNS receptor occupancy. We attribute this benign side-effect profile to very low efficacy of NS11394 at $GABA_A$-α_1 receptors and an overall partial agonist profile across receptor subtypes. However, NS11394 impairs memory in both rats and mice, which is possibly attributable to its efficacy at $GABA_A$-α_5 receptors, albeit activity at this receptor might be relevant to its anti-nociceptive effects (Munro et al., 2008). In conclusion, NS11394 has a unique subtype-selective $GABA_A$ receptor profile and represents an excellent pharmacological tool to further our understanding on the relative contributions of $GABA_A$ receptor subtypes in various therapeutic areas.

Introduction

Preclinical studies using genetically modified mice suggest that $GABA_A$-α_1 (Rudolph *et al.*, 1999; McKernan *et al.*, 2000), α_2 (Low *et al.*, 2000) and α_5 (Collinson et al., 2002; Crestani et al., 2002) containing receptors mediate the sedative/motor-impairing, anxiolytic, and memory impairing effects of benzodiazepines, respectively. Pharmacologically, various non-benzodiazepine compounds have been described that show either selective affinity (e.g., zolpidem and indiplon) or selective efficacy (e.g., L838,417, SL651498, TP003, TPA023) for subtypes of $GABA_A$ receptors (Sanger et al., 1987; McKernan et al., 2000; Griebel et al., 2001; Dias et al., 2005; Atack et al., 2006). Such selectivity profiles have been argued to be the basis for these compounds' selective behavioural profiles in rodents and in some cases man. For example, zolpidem is selective for $GABA_A$-α_1 containing receptors, has a preferential sedative-hypnotic profile in animals and is marketed as a sleep aid (ambien®). Although additional compounds with various selectivity profiles have emerged, limited clinical data in patient populations exists to date (but see Basile et al., 2006; de Haas et al., 2007; Nutt et al., 2007).

One major area of interest has been to develop compounds with selectivity for $GABA_A$-α_2 and/or α_3 over $GABA_A$-α_1 receptors, with the prospect of bringing a non-sedative anxiolytic to the market for the treatment of anxiety disorders (Atack, 2003). In this field, the only relevant clinical data, on a well characterised preclinical molecule, that has been forthcoming is on the $\alpha_{2/3}$ selective molecule TPA023, which was compared to lorazepam in human volunteers in a double-blind, double-dummy cross-over study (de Haas et al., 2007). TPA023 had a reduced liability to induce sedation and impair cognition in human volunteers compared to lorazepam. These clinical observations are consistent with this compounds lack of efficacy at human $GABA_A$-α_1 and α_5 receptors as determined by *in-vitro* electrophysiology (Atack et al., 2006). However, no data confirming that TPA023 has anxiolytic efficacy in man at doses not engendering side effects in human volunteers is available, so that to date there is no translation of this preclinical profile to a meaningful therapeutic

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outcome. Thus, at this point, the increased preclinical understanding of the roles of some of the major types of $GABA_A$ receptors has not been tested clinically. For this reason we would emphasize the need for new molecules with novel selectivity profiles, which are amenable for testing in man, to aid in driving preclinical as well as clinical research.

In the current study, we introduce the novel compound 3'-[5-(1-Hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitrile (NS11394, Figure 1) which binds with subnanomolar potency to human $GABA_A$ receptors but which shows differential efficacy at these receptors, as determined by *in-vitro* electrophysiology. NS11394 has a unique profile compared to other recently described compounds, adding to the armamentarium of novel subtype-selective ligand's available to understand the role of $GABA_A$ receptor subtypes in disease models and propensity to engender side-effects. Specifically, NS11394 shows a selectivity profile in the order of $GABA_A$- $\alpha_5 > \alpha_3 > \alpha_2 > \alpha_1$ containing receptors, with notably higher $GABA_A$-α_3 receptor efficacy compared to the majority of subtype-selective molecules recently described.

Given this unique profile we have made efforts to determine the efficacy of NS11394 in animal models covering various disease areas including anxiety and pain, making comparison with relevant reference compounds. In this, the first of two articles, we introduce NS11394 as a novel subtype-selective $GABA_A$ receptor positive allosteric modulator and highlight: (i) its *in-vitro* binding and electrophysiology profile at human $GABA_A$ receptors, as well as its selectivity over other targets; (ii) its excellent pharmacokinetic profile in rodents; (iii) the close correlation observed between pharmacokinetic and pharmacodynamic (receptor occupancy) properties; (iv) its *in-vivo* profile in animal models of anxiety-like behaviour, in addition to its *in-vivo* side-effect profile in comparison to various benzodiazepine site positive modulators. We chose to compare NS11394 variously to alprazolam, diazepam or chlordiazepoxide in the efficacy and side effect models, since it is clear that benzodiazepines differ considerably on various parameters, including potency, half-life,

selectivity, metabolite formation etc (Chouinard, 2004; Mirza and Nielsen, 2006). Given these differences we were keen not to restrict ourselves to comparing NS11394 to a single representative for the entire benzodiazepine class, albeit at the expense of not being exhaustive in profiling all three benzodiazepines in all models. Finally, in the accompanying paper (Munro et al., 2008) we focus exclusively on NS11394's profile in pain, with comparative data to a range of $GABA_A$ receptor modulators in rat models of acute, inflammatory and neuropathic pain, supplemented by supportive *in-vitro* spinal cord electrophysiological data.

Methods

Animals

Male rats (PVG: Harlan, Netherlands; Sprague Dawley or Wistar: Taconic M&B, Denmark) and female mice (NMRI, Taconic M&B) were housed and habituated for at least 7 days prior to experimental procedures in Macrolon III cages (20 x 40 x 18 cm; 2 rats/cage or 8 mice/cage). Food (Altromin®) and water were available *ad libitum*, with the exception of restricted water in the conditioned emotional response procedure described below. Animals were allowed a minimum of seven days of acclimatization in the animal facility prior to testing. Animals were in a temperature-controlled environment with a light-dark cycle of 13:11 h (lights on at 06.00 hours and off at 19.00 hours). All testing procedures were in accordance with "Methods and Welfare Considerations in Behavioral Research with Animals" (NIH Publication No. 02-5083, March 2002) and licensed by the Animal Experiments Inspectorate, The Danish Ministry of Justice.

Compounds

NS11394 [3'-[5-(1-Hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitrile] and zolpidem were synthesized at NeuroSearch A/S, Medicinal Chemistry Department, whereas paroxetine, chlordiazepoxide, Ro 15-4513 (Sigma-Aldrich A/S, Vallensbæk Strand, Denmark), diazepam (Nomeco A/S, Copenhagen, Denmark), alprazolam (Cambrex Corporation, Charles City, IA) and clonazepam (Roche) were purchased from commercial sources. For all in-vivo studies in mice and rats, NS11394 was dissolved in 5% Tween80/milliQ water and administered perorally (p.o.) in a dosing volume of 5 ml/kg for rat and 10 ml/kg for mouse. Diazepam, alprazolam and chlordiazepoxide were dissolved in 5% cremophor (BASF, Ludwigshaden, Germany) and administered intraperitoneally (i.p.), whereas paroxetine was dissolved in saline and also administered i.p. All compounds were administered 30 min prior to behavioural procedures, unless otherwise stated. Doses are expressed as the mg salt weight per kg body weight.

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Cloning of cDNA and cRNA preparation.

cDNAs for the $GABA_A$ receptor subunits α_{1-6}, β_2, β_3 and γ_{2S} were cloned from human hippocampus poly A^+ mRNA (Clontech) using PCR. Briefly, first strand cDNA was obtained using oligo dT primer and MMLV reverse transcriptase (Pharmacia). Full-length cDNA sequences were amplified by PCR reactions using 100 ng first strand cDNA per reaction, Expand HF polymerase (Boehringer Mannheim) and gene specific primer sets (MWG Biotech). PCR conditions were: 1) 94 °C 60s; 2) 15 times (94 °C 60s, 55°C 60s, 72°C 120s); 3) 20 times (94 °C 60s, 55°C 60s, 72°C 180s); 4) 72°C 10 min using a Robocycler (Stratagene). Amplified products were polished with *pfu* polymerase (Stratagene), purified on a QIAquick column (Qiagen) and cloned into pCRScript (Stratagene) or pSwas. Several positive clones were sequenced bi-directionally and verified clones were subcloned into either a pNS1 or pNS3 vector. pSws and pNS1/pNS3 are custom designed vectors derived from the InVitrogen vectors pZErO-1 or pcDNA3, respectively. For cRNA production plasmids were linearized using a unique downstream polylinker enzyme (NotI, XhoI or XbaI). cRNA was prepared and capped from the linearized cDNAs using the mMESSAGE mMACHINE T7 Transcription kit (Ambion). RNA was purified using the Rneasy mini kit (Qiagen), adjusted to a concentration of 0.5 μg/μl and stored at -80 ºC until use. For establishment of stable cells lines, β subunits mutated to have a cation permeable selectivity segment were used (Jensen et al., 2002).

Cell culture and stable transfections

HEK-293 (ATCC1573) cell lines were propagated in culture flasks (Nunc) at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. The growth medium consisted of Dulbecco's Modified Eagle Medium (Lonza BE12-604/U1) supplemented with 10% fetal bovine serum (InVitrogen). HEK-293 cells, seeded in a T12.5 culture flask (Nunc) and cultured to 50-70 % confluency, were transfected with a total of 1 μg of expression plasmids pNS3n(α_{1-6}), pNS3z($\beta_{2/3}$) and pNS3h(γ_{2s})

("n", "z" and "h" denotes NeoR, ZeoR and HygR genes in the plasmids, respectively) using Lipofectamine Plus (Life Technologies) according to manufactures protocol. 24 h past transfection cells were detached using trypsin/EDTA (Life Technologies) and seeded in a T75 culture flask (Nunc) with a tear-off lid. For stable expression cells were selected in medium supplemented with 0.5 mg/ml G 418 (Sigma, A1720) and 0.125 mg/ml Zeocin (InVitrogen, 450430) and 0.15 mg/ml Hygromycin (Roche, 10843555001). Single clones were picked and propagated in selection media until sufficient cells for freezing were available; thereafter the cells were cultured in regular culture media with one or more selection agents.

Preparation of rat cerebral cortical membranes

Wistar rat cerebral cortices were removed rapidly after decapitation, homogenized for 5 to 10 s in 10 volumes of Tris-HCl buffer (30 mM, pH 7.4), and pelleted by centrifugation at 27,000*g* for 15 min. All procedures were performed at 0 to 4°C unless otherwise indicated. After washing three times by re-suspension in 10 volumes of ice-cold buffer and centrifugation at 27,000*g* for 10 min, the pellet was re-suspended and homogenized in Tris-HCl buffer, incubated on a water bath at 37°C for 30 min, and then pelleted at 27,000*g* for 10 min. Following one more wash, the pellet was re-suspended in 10 volumes buffer, and stored at -20°C until use. On the day of the experiment, the membrane preparation was thawed, pelleted at 27,000*g* for 10 min and washed twice by re-suspension in Tris-citrate buffer (50 mM, pH 7.1) and centrifugation at 27,000*g* for 10 min. The final pellet was re-suspended in Tris-citrate buffer (500 ml of buffer/g of original tissue).

Preparation of cell line membranes

Cell culture medium was removed from confluent cell cultures, cells were rinsed once with DPBS (Dulbecco's Phosphate Buffered Saline), harvested into a small volume of DPBS by gently scraping the cells off the bottom of the culture flask and pelleted by centrifugation at 1550*g* for 10 min. All procedures were performed at 0 to 4°C unless otherwise indicated. The cell pellet was gently

washed once in 15 ml Tris-HCl or Tris-citrate buffer (50 mM, pH 7.1), re-suspended in the same buffer, homogenized using an Ultra-Turrax homogenizer and centrifuged at 27,000*g* for 10 min. The pellet was re-suspended in 15 ml Tris-HCl or Tris-citrate buffer and stored at -80°C. On the day of the experiment, the membranes were thawed, pelleted at 27,000*g* for 10 min, and re-suspended in Tris-citrate buffer (50 mM, pH 7.1, α_1, α_2, α_3 and α_5, containing cell lines) or KH_2PO_4 buffer (20 mM, pH 7.4, α_4 and α_6 containing cell lines) containing 100 mM KCl.

***In-vitro* [^{3}H]flunitrazepam, [^{3}H]Ro 15-1788 and [^{3}H]Ro 15-4513 binding**

Aliquots of 500 µl of rat cortical membranes or cell suspension (30 - 150 µg protein per assay) were added to 25 µl of test compound and 25 µl (1-5 nM, final concentration) of [^{3}H]flunitrazepam (88 Ci/mmol, GE Healthcare UK Limited, Little Chalfont, UK), [^{3}H]Ro 15-1788 (87 Ci/mmol, PerkinElmer Life and Analytical Sciences, Boston, MA) or [^{3}H]Ro 15-4513 (28 Ci/mmol, PerkinElmer Life and Analytical Sciences), mixed, and incubated for 40 or 90 min (α_4 and α_6 containing cell lines) at 2°C. Non-specific binding was determined in the presence of 1 µM Clonazepam or 10 µM Ro 15-4513 (α_4 and α_6). Compounds were tested at 5-10 concentrations ranging from 0.01 nM to 30 µM. Binding was terminated by rapid filtration over Whatman GF/C glass fiber filters (Whatman), and the amount of radioactivity on the filters was determined by conventional liquid scintillation counting using a Tri-Carb^{TM} counter (PerkinElmer Life and Analytical Sciences).

***In-vivo* [^{3}H]Flunitrazepam binding in mouse or rat brain**

Groups of three female NMRI mice (25–28 g) or three male Wistar rats (180 g) were administered either p.o. with NS11394 or i.p. with alprazolam, chlordiazepoxide or diazepam. Twenty min before decapitation, the animals were injected i.v. (tail vein) with 5.0 µCi [^{3}H]flunitrazepam in 0.2 ml of saline (mice) or 20 µCi [^{3}Hflunitrazepam in 0.4 ml of saline (rats). Following decapitation, mice forebrains were rapidly excised and homogenized in 12 ml of ice-cold Tris-citrate buffer (50 mM,

pH 7.1) using an Ultra-Turrax homogenizer. For rats, only half of the forebrain was homogenized in 15 ml of ice-cold buffer. Three aliquots of 1 ml (mice) or six aliquots of 1.5 ml (rats) were filtered through Whatman GF/C glass fiber filters and washed with 2 x 5 ml of ice-cold buffer. Groups of vehicle-treated animals served as control for estimation of total binding. Non-specific binding was determined by animal groups administered 3.0 mg/kg i.p. clonazepam (mice) or 10 mg/kg i.p. clonazepam (rats) 10 min before [^{3}H]flunitrazepam injection. Radioactivity on the filters was determined by conventional liquid scintillation counting using a Tri-Carb^{TM} counter.

Bioavailability

Wistar rats were dosed with 3 mg/kg NS11394 either as an i.v. bolus (N=3, 1.5 mg/ml in Tween80, clear solution) or p.o. (N=3, 1.5 mg/ml in Tween80, clear solution). At specified time-points after i.v. (0.08, 0.5, 1, 2, 4, 6 and 24 h) or p.o. (0.5, 1, 2, 3, 4, and 6 h) administration, blood samples were collected in EDTA-K^+ tubes (Milian), mixed and kept on ice. Plasma was prepared by centrifugation at 1000g for 20 min and stored at –18°C prior to analysis. Plasma proteins were precipitated by adding 3 volumes acetonitrile containing 100 ng/ml of an internal standard and centrifugation at 16,000 g for 25 min at 5°C after which the supernatant was transferred to a fresh tube and diluted with 1 volume of water. Samples were subsequently analysed by liquid chromatography HPLC (Waters, Alliance 2795) in combination with a triple quadrupole mass spectrometer (Micromass, Quattro Ultima). Detection of NS11394 and the internal standard was performed by selected reaction monitoring (SRM) in electrospray positive ion mode, fragmenting protonated parent ion to a specific prominent product ion. Quantification of NS11394 was performed using quadratic regression (weighted 1/x). The calibration range was 10-5000 ng/ml in plasma and samples above the upper limit were diluted 10 times with water and re-analysed.

Pharmacokinetic-Pharmacodynamic relationship

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Animals were administered NS11394, [^{3}H]flunitrazepam and clonazepam as detailed in the *in-vivo* binding experiments above and decapitated at fixed time points. Blood samples for plasma isolated from NS11394 dosed animals were treated as described in the bioavailability section. Furthermore, from each animal, half of the forebrain was transferred to a small plastic bag, frozen on dry ice and stored at –20°C for later analysis, whereas the other forebrain half was used for *in-vivo* binding. For analysis of brain concentrations of NS11394, brain tissue (0.1 g) was homogenised with 1 mm zirconia beads in 1000 µl acetonitrile:water (80:20), containing 100 ng/ml internal standard, using a bead beater (Biospec Products, Inc., Minibeadbeater, 96+). The tissue homogenates were centrifuged at 16,000g for 25 min at 5°C after which the supernatant was transferred to a fresh tube and diluted with 1 volume of water. Samples were analysed as described in the rat bioavailability section. Quantification of NS11394 was performed using linear regression (unweighted fit).

Microsomal stability

Microsomes were supplied by BD Biosciences (NJ, U.S.). In each of four tubes 2.5 µl microsomes (20 mg/ml protein), 87.5 µl COMIX and 10 µl test solution (10 µM compound in 20/80 MeCN/water), were mixed. The reaction in two of the tubes was immediately stopped with 20 µl ice cold MeCN (5% acetic acid). The supernatant was taken for later analyses by LC-MS. In the two remaining tubes the reaction was allowed to continue for 60 min and at this timepoint stopped with 20 µl ice cold MeCN (5% acetic acid). Microsomal stability was calculated as the percentage of compound in the t=60 sample relative to the t=0 sample according to the formula:

$$(1)\ \text{Stability}\% = \frac{\text{Area}_{t60}}{\text{Area}_{t0}} * 100\%$$

Isolation and injection of *Xenopus laevis* oocytes

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Adult female *X. laevis* (Nasco) were anaesthetized with Tricane 0.28% (Sigma, A5040) in ice-cold water and lobes of ovaries were removed surgically after which the frogs were killed. Following removal the lobes were placed in a glass petri dish in Mod. Barth's solution and cut into small pieces with surgical knifes. Lobular pieces were transferred to a 50 ml tube (Nunc) and oocytes were dispersed as well as defolicated using 0.2% collagenase (Sigma, C-9891) in Low-Ca Barth's (Mod. Barth's without $CaCl_2$) using gentle agitation. After dispersal for 1-2 h, oocytes were washed 5 times in Lo-Ca Barths followed by 5 times in Mod. Barth's and then placed in a glass petri dish. Stage V and VI oocytes were selected and transferred to a clean glass petri dish and maintained at 18 degrees Celsius in Mod. Barth's. For injection, the oocytes were placed in a custom designed chamber in Mod. Barth's and injected with 25-50 nl of cRNA mixture using a Pico Pump (WPI). The cRNA mixture contained $GABA_AR$ subunits α_x, β_2, and γ_{2s} in the ratio of 1:1:3 and in a total concentration of 0.5 μg/μl. Following injection, oocytes were maintained at 18° C in Mod. Barth's for 1-5 days.

Two-electrode voltage clamp

Electrophysiological responses from *X. laevis* oocytes were measured using the two-electrode-voltage clamp technique. Single oocytes were placed in custom designed recording chambers that were continuously perfused with > 2 ml/min OR2. Recording electrodes were fabricated from borosilicate glass tubings with filament (Sutter BF150-110-10) using a DMZ-Universal puller (Zeitz Instrument), backfilled with 2 M KCl and when submerged into OR2 solution the electrode resistances were in the range of 0.5-1 MΩ. The oocyte was impaled using manual micro manipulators and allowed to equilibrate at a holding potential of -50 mV to -80 mV for at least 1 min to ensure a maximal leak current of 100 nA before the experiment was initiated. Currents were amplified by a Geneclamp 500B amplifier (Axon), low-pass filtered at 20Hz, digitized at 200 Hz by a Digidata 1322A (Axon) and then recorded as well as analyzed by a PC (Compaq Evo) using the pClamp9 suite (Axon).

Drug solutions were applied through a capillary tube, with an inner diameter of 1.5 mm (Modulohm 214813), placed approximately 2 mm from the oocyte and connected through Teflon tubing to a Gilson 233XL autosampler. Gilson 735 software suite was used to control all the Gilson equipment (233XL autosampler, 402 diluter and Minipuls 3 pumps) and to trigger recording by pCLAMP9. A flow rate of 2.5 ml/min through the capillary tube during applications ensured a rapid exchange of liquid surrounding the oocyte (in the order of few s). The application length was set to last 60 s which was sufficient to obtain peak currents. The time interval between recordings was 5 min, during which the oocyte was perfused with OR2 through the capillary tube as well.

For each experimental set, GABA was freshly dissolved in OR2 in a concentration known to give rise to EC_5-EC_{25} elicited currents for a given $GABA_AR$ subtype combination (0.5-5μM) and this solution was then used for controls as well as a stock solution for dissolving the compounds to test in the experiment. A complete experimental set contained 4 control traces of GABA, a reference 0.5μM diazepam trace, 10 GABA control traces and finally 8 test traces of a compound in increasing concentrations. The oocyte was discarded after one experimental set. Modulatory effects of diazepam were calculated by comparing the diazepam trace to the control trace immediately before. Likewise, modulatory effects of the compound in the test traces were obtained by comparing to the control immediately before the test traces. Oocytes with receptor expression levels giving rise to a control GABA evoked whole-cell currents either below or above the range of 250 nA to 2000 nA were discarded. Also if the diazepam potentiation was below 70% or above 400% the experiment was discarded. To enable comparison of effects of a compound between individual oocytes, all compound potentiations were normalized to the control diazepam potentiation on the same oocyte.

Rat conditioned emotional response (CER) test

The CER procedure was the same as that recently described by Mathiasen et al. (2007). Briefly, eight standard operant chambers, equipped with a 3 watt house light (centre of ceiling), an operant lever (2 cm above the grid floor) and a valve-operated water spout on one wall, were used (ENV-008: 32x25x25 cm, MED Associates Inc.). Water-deprived male PVG rats were trained to associate lever pressing with water reward. During 30 min sessions, rats were initially trained to make 10 lever presses to receive 1 reward (fixed ratio 10, FR10). Thereafter animals were trained to maintain a high level of lever pressing by increasing the requirements culminating in a variable interval (VI) 60 s reward schedule. The house light remained on throughout all training sessions. Thereafter conditioned emotional response (CER) training commenced. In the sequence L1, D1, L2, D2, each session consisted of four alternating periods during which the house light was either on (L, 5 min) or off (D, 2.5 min). After the second D period there was a third 5 min L period, although response rate data in this final period was not considered further and was a mean to ensure animals maintained baseline responding prior to the next day's session. During the D periods, a scrambled 0.4 mA, 0.1 s scrambled footshock was applied according to a VI20 s schedule. The VI60 s reward schedule was maintained throughout both L and D periods. Response rates during the L and D periods were used to calculate a suppression ratio (SR) according to the following formula:

$$(2)\ SR = \frac{\text{response rate in D}}{\text{response rate in L} + \text{response rate in D}}$$

A suppression ratio of 0 indicates that the D has evoked conditioned fear and has completely suppressed lever pressing relative to the L. By contrast, an SR of 0.5 would indicate that the response rate during the L and D periods were equivalent, i.e., no fear to the D cue. Drug testing commenced only when a stable SR below 0.05 had been established. Of the 8 animals that commenced training 7 reached this level of proficiency and were used in subsequent drug tests. A within-subject design was used in these studies, such that each individual animal was tested with all

doses of NS11394 or alprazolam in a pseudo-randomized order. Test sessions were identical to training sessions with the exception that no foot-shocks were delivered. Drug testing was conducted on Tuesdays and Fridays with other days serving as baseline days to determine if animals maintained normal performance levels between drug test days.

Four-plate testing (FPT, based on the method of Aron et al. 1971)

This apparatus consisted of a non-transparent plexiglas™ box (25×18×16 cm) with a transparent lid constructed at NeuroSearch. The floor of the chamber consisted of four identical rectangular metal plates (11×8 cm) which were separated by a 4 mm gap, and through which an electric foot shock (0.6 mA; 0.5 s) could be delivered manually using a foot pedal (BioSeb). Female NMRI mice were placed individually in the chamber and after a 15 s habituation period, the animal was given an electrical foot shock whenever crossing from one plate to the other. The definition of crossing between two metal plates was that the mouse had two paws on one plate and two on an adjacent plate. After the delivery of a foot-shock, there was a 3 second pause before another foot shock was delivered manually. The number of punished crossings an animal made was determined by the experimenter, blind to the treatment, over a 1 min period. An anxiolytic-like effect of drug treatment was inferred from an increase in the number of punished crossings made under drug treatment compared to the number of punished crossings made by control animals.

Marble burying

Female NMRI mice were placed for 1 h in novel cages (1 mouse per cage, 20 x 30 cm) in which there were 20 glass marbles (15 mm in diameter) situated in 4 rows of 5 on top of 5 cm of sawdust. The mean number of glass marbles buried between 10-60 min was taken as an index of "anxiety", i.e. the more marbles buried the more anxious the mouse (Broekampp et al., 1986). A marble was classified as buried by an experimenter blind to treatment, when at least two-thirds was covered by sawdust.

Locomotor activity in non-habituated mice or rats

Mice or rats (Sprague dawley) were placed individually in transparent cages (30 x 20 x 25 cm) within activity frames (TSE Systems) and activity levels determined over 30 mins. The activity frames were equipped with 12 (6 x 2) infrared sensors. Locomotor activity was monitored automatically in the chambers and measured as the interruption of two consecutive infrared sensors. Interruptions were detected by a control unit and registered by a computer running ActiMot software (TSE Systems). All data are presented as mean distance (m) travelled ± SEM.

Sensorimotor function in the rat assessed using the rotarod: influence of ethanol

The relative level of motor incoordination/ataxia in rats engendered by either NS11394 or diazepam (0.3-3 mg/kg, i.p) in the presence/absence of ethanol (0.8 g/kg), was determined by assessing the ability of uninjured rats (Sprague Dawley) to maintain balance on an accelerating rotarod (Ugo Basile, Italy). The rotarod speed was increased from 3-30 rpm over a period of 180 s, so that the maximum time animals could maintain balance on the rotarod was 180 s and the minimum time 0 s. Initially, rats received two training trials (separated by 3-4 h) on two separate days to acclimatise to the task. The dose of ethanol (0.8 g/kg) chosen for the interaction studies was selected based on prior reference dose response curves indicating that this dose was subthreshold for impairing rat rotarod performance.

Memory impairment in mice and rats

Passive avoidance. Mice were tested in a step-through passive avoidance task using 4 two compartment chambers (MED Associates Inc): one compartment lit (10 cm x 12 cm) and the other dark (16 cm x 20 cm) separated by a shutter with a small doorway. The floor of the box consisted of metal grid steel bars. The mice were handled on the day before training. On the training day mice were introduced individually to the lit compartment and allowed to habituate for 60 s before access to the dark compartment was granted when the central door was raised. When a mouse completely entered the dark compartment (all 4 paws), the latency to enter was recorded and two mild foot-shocks (0.6 mA, 0.5 s, 5 s between foot-shocks) delivered through the grid floor. The animals remained in the dark compartment for 60 s after which they were removed and returned to their home cage. Twenty-four h after this training session, the mouse was again introduced to the lit compartment for 60 s, and thereafter the latency to enter the dark chamber was taken as a measure of memory retention. If a mouse had not crossed to the dark compartment within 180 s, it was assigned this value and removed from the apparatus.

Fear conditioning in rats. Fear conditioning was assessed in eight ventilated, sound-attenuated chambers (LxWxH, inner dimensions: 320x320x320 mm; TSE Startle Response, TSE systems, D-61350), each consisting of a nonrestrictive plexiglas™ cage (LxWxH: 100x60x70 mm) with a 9-rod grid floor (rod = 4 mm; distance between centre of adjacent rods = 8.9 mm) inside which rats were placed. Two high-linearity speakers situated 6 cm on either side of the plexiglas™ cage delivered background noise and tone-cues. Sound intensities were measured using a Bruel and Kjaer (Denmark) sound level meter (model, 2238) in conjunction with a microphone placed within the chamber (model, 4188). The movement of animals in the cages was recorded using a strain gage load cell mounted directly below the animal. The output signal from the load cell was converted to arbitrary units using an analog-to-digital converter using appropriate software (TSE Systems). A calibration system (TSE Systems) was used to ensure comparable sensitivity between the eight systems. On the day prior to conditioning, animals were habituated to the conditioning chambers for

ten minutes after which they were returned to their home cages. During this 'habituation' session rats were exposed to a constant background noise (65 dB, white noise). On the subsequent day the animals were conditioned to associate a brief tone cue (10 s, 5 kHz, 80 dB) with a single foot-shock (0.6 mA, 0.5 s) delivered through the grid floor and which co-terminated with the tone. The 10-s tone cue and associated shock were presented ~3 minutes after the animals had been placed in the cages. After this single tone-shock pairing animals remained in the chambers for another 2 minutes. The entire conditioning session lasted ~5 min with background noise (65dB, white noise) throughout the session. Twenty-four h after the conditioning session rats were re-exposed to the chambers and all details were identical with the exception that no foot-shock was delivered through the grid floor. The movement of the animals in the period prior to tone presentation (i.e., context fear conditioning) was recorded in 4 ms bins and averaged over ~3 min. Thereafter, movement was recorded in 4 ms bins during the tone and averaged over the entire 10 s tone presentation (i.e., cue fear conditioning). Data is presented as average movement (arbitary units) ± SEM in each of these periods. When assessing the effects of vehicle, NS11394 and alprazolam on fear conditioning, an additional vehicle control group was run which was exposed to the chambers and tone cue but which did not receive a foot-shock. This latter, 'no-shock' group was a control to ensure that we had robust context and cure fear conditioning on the test day in the 'vehicle-shock' group.

Data analysis

For *in vitro* binding studies, IC_{50} values were determined based on the equation

$$(3)\ B = 100 - (100 \cdot C^n/(IC_{50}^n + C^n)),$$

where B is the binding in percent of total specific binding; C the concentration of test compound; and n the Hill coefficient. Estimates of binding parameters were calculated with the nonlinear curve-fitting program GraphPad Prism™ (version 4.03; GraphPad Software, Inc., San Diego, CA).

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K_i values were calculated from IC_{50} values using the Cheng and Prusoff equation (1973). All results are given as mean ± SEM. For two-electrode voltage clamp experiments compound concentration-response curves were fitted to a sigmoidal dose-response curve using GraphPad Prism™. For all behavioral studies ANOVA was used to analyse overall effects of treatments. When the *F* value was significant this was followed by Dunnett's *post hoc* text with a significance level of $p < 0.05$ (Sigmastat 2.03, SPSS Inc., Chicago, ILL.).

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Results

In-Vitro Binding

The affinity of NS11394 for the benzodiazepine site of $hGABA_A$ receptors containing the α_1, α_2, α_3 or α_5 subunits together with β_3 and γ_{2s} stably expressed in HEK-293 cells, ranged from 0.1-0.8 nM. Diazepam, was approximately 10-fold less potent with a range of 5-20 nM. Neither NS11394 nor diazepam showed affinity selectivity for any of these four receptor combinations. The affinity of NS11394 and diazepam at a mixed population of $rGABA_A$ receptors in rat cortical membranes and these four cloned $hGABA_A$ receptors expressed in cell lines was approximately the same, indicating no obvious species difference with respect to binding affinity (Table 1). Contrary to the potent binding at α_1, α_2, α_3 and α_5 containing receptors, NS11394 had between 600 and 2000 times lower affinity for α_4 and α_6 containing $hGABA_A$ receptors. Likewise, diazepam was considerably less potent at $hGABA_A$ receptors containing either α_4 or α_6 subunits (Table 1).

NS11394 was also tested at 10 μM in a MDS Pharma Services (Bothell, WA, USA) LeadProfilingScreen for binding to a broad range of over 60 different receptors, transporters and ion channels. In addition to confirming high affinity for rat $GABA_A$ benzodiazepine binding sites labelled by [^{3}H]flunitrazepam, the screen also indicated a high selectivity relative to other targets. The only targets for which greater than 50% inhibition was noted were the human opiate κ (62% inhibition, [^{3}H]diprenorphine), human adenosine A_3 (61% inhibition, [^{125}I]AB-MECA), and the rat sodium channel site 2 (84%, [^{3}H]batrachotoxin). In follow-up studies it was shown that the NS11394 had a K_i of 9.6 and 2.7 μM at adenosine A_3 and opiate κ receptors, respectively. In-house patch clamp studies showed that NS11394 inhibited Na^+ channels in rat embryonic dorsal root ganglion cells with a IC_{50} of 16 μM (n=2). No other significant off-target activities were seen (data not shown).

In-Vitro Efficacy

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NS11394-induced potentiation of GABA evoked currents at human α_1, α_2, α_3 or α_5 containing receptors expressed in combination with β_2 and γ_{2s} subunits in *Xenopus laevis* oocytes can be seen in Figure 2. A GABA concentration giving rise to approximately 5-25% of maximal GABA current (EC_{5-25}) was chosen as testing concentration for each receptor combination and all datasets are indexed relative to the potentiating effects of 0.5 μM diazepam on the same oocytes. As seen, NS11394 modulated GABA responses at all four receptor combinations with greatest efficacy at α_3 and α_5 containing receptors at which NS11394 engendered a maximum 52% and 78% potentiation relative to diazepam, respectively. NS11394 had less effect at α_2 containing receptors with a maximal potentiation of 26% relative to diazepam. By contrast, NS11394 only weakly potentiated the GABA responses at α_1 containing receptors with a maximal potentiation of 8% relative to diazepam. All data points are obtained from 5-25 individual oocytes (74 oocytes used in total). As expected from the binding experiments no apparent selectivity could be observed with respect to functional potencies (see Table accompanying Figure 2).

***In-Vivo* Binding**

NS11394 dose-dependently displaced [^{3}H]flunitrazepam binding to mouse brain benzodiazepine receptors when administered 30 min prior to culling. The dose of NS11394 estimated to inhibit 50% of [^{3}H]flunitrazepam binding to mouse forebrain was 0.38 mg/kg. This study was repeated with mice culled at 120 min after NS11394 administration, with NS11394 inhibiting 50% of [^{3}H]flunitrazepam binding at 0.49 mg/kg (Figure 3a). In studies in rats NS11394 inhibited 50% of [^{3}H]flunitrazepam binding to rat brain benzodiazepine receptors at 1.3 and 0.69 mg/kg p.o., when administered 30 and 120 min prior to culling, respectively (Figure 3b). In the rat, an additional time-course study showed that NS11394 (3 mg/kg, p.o.) inhibition of [^{3}H]flunitrazepam binding to brain benzodiazepine receptors was long lasting with occupancies of 70%, 100% and 97% at 30, 180 and 360 min, respectively (data not shown). To support the in-vivo efficacy and side-effect studies described below we also determined the in-vivo receptor occupancy in mice treated i.p. with various doses of alprazolam, chlordiazepoxide and diazepam, the three benzodiazepines used as comparators. From Figure 3c it is clear that at the doses tested the three

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benzodiazepines attained receptor exposure on a par with that seen with NS11394 in the mouse. Alprazolam, chlordiazepoxide and diazepam inhibited 50% of [^{3}H]flunitrazepam binding to mouse forebrain at 0.17, 10 and 2.2 mg/kg, respectively.

Pharmacokinetic-Pharmacodynamic relationship in mouse and rat

The relationship between plasma concentration, brain concentration and CNS *in-vivo* receptor occupancy after oral administration of NS11394 was determined in mice and rats. NS11394 was dosed to separate animal groups (0.1-3 mg/kg, p.o.) and plasma and brain samples taken at a single time-point 30 min post dosing. What is evident from Figure 4 is that in both the mouse and rat there is a strong relationship between plasma and brain concentrations, and that both these parameters correlate with CNS *in-vivo* receptor occupancy, a pharmacodynamic marker. However, at equivalent doses in both species brain and plasma concentrations are higher in mice compared to rat. From Figure 4 it is clear that in both species the brain/plasma is ~1 at all doses indicating rapid equilibration between plasma and brain at the 30 min time-point.

Profile of NS11394 in rodent models of anxiety-like behaviour

To evaluate the potential anxiolytic properties of NS11394, the compound was tested in three *in-vivo* models using either alprazolam, chlordiazepoxide or diazepam as reference comparators.

Rat conditioned emotional response (CER). Figure 5a shows that NS11394 significantly affected the response rate of rats in the dark anxiety-provoking periods ($F_{4,24}$ = 11.5, P<0.001) of a CER session, with all doses of NS11394 significantly increasing response rate compared to vehicle treatment (P<0.01). Interestingly, NS11394 also affected response rate in the light periods ($F_{4,24}$ = 3.1, P<0.03), however, this effect is not clearly dose related, with a significant increase (P<0.05) compared to control animals at 0.3, 1 and 10 mg/kg NS11394, with no effect at 3 mg/kg. Figure 5b shows the main effect of NS11394 treatment on the suppression ratio parameter ($F_{4,24}$ = 15.5, P<0.001), with all four doses engendering an increase in suppression ratio compared to vehicle treatment (P<0.01). For comparative purposes the effect

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of the benzodiazepine alprazolam at doses of 0.3-3 mg/kg are shown in Figures 5c and 5d. It is clear that alprazolam is also anxiolytic, as expected, with a significant effect at 1-3 mg/kg on both dark period response rate ($F_{5,15}$ = 6.5, P<0.01) and suppression ratio ($F_{3,15}$ = 7.7, P<0.01) parameters. However, contrary to NS11394, alprazolam at the higher dose also significantly reduces baseline response rate during the light period ($F_{3,15}$ = 5.0, P<0.03), an effect clearly due to sedation/motor impairment based on visual observation.

Mouse four plate test (FPT). Treatment with NS11394 significantly and dose-dependently increased the number of punished crossings made by mice ($F_{4,49}$ = 10.6, P<0.001, Figure 6a). All doses of NS11394 significantly increased the number of punished crossing's made by mice compared to vehicle treated mice (P<0.01). For comparative purposes we tested the benzodiazepine chlordiazepoxide which also significantly increased punished crossings ($F_{3,33}$ = 7.3, P<0.003, Figure 6b) with mice dosed with10-20 mg/kg differing significantly from vehicle treated mice (P<0.01) .

Mouse marble burying test (MBT). In the MBT, NS11394 treatment dose-dependently reduced the number of marbles mice buried ($F_{4,34}$ = 13.0, P<0.001, Figure 6c). All doses significantly affected the burying behaviour of mice compared to vehicle treated animals (P<0.01). The efficacy of NS11394 at 1 mg/kg p.o. was similar to the efficacy seen with paroxetine 10 mg/kg i.p. run in the same study (Figure 6c, right-hand bar). In addition to using paroxetine as a positive control, we also assessed the effect of the benzodiazepine diazepam in the mouse marble burying test. Like NS11394, diazepam significantly reduced burying behaviour ($F_{4,35}$ = 9.2, P<0.001). All doses of diazepam reduced the burying behaviour of mice compared to vehicle treated control mice, (P<0.01, Figure 6d), despite animals dosed with 3 mg/kg diazepam clearly showing signs of sedation.

Side-effect profile of NS11394 relative to benzodiazepines in rodents

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Since classical benzodiazepines are known to have a range of side effects coupled to their mechanism of action NS11394 was evaluated in several *in-vivo* models known to reveal different side effects, and compared with either diazepam, chlordiazepoxide or alprazolam.

Mouse and rat motor activity. NS11394 had no significant effect on mouse motility ($F_{3,26}$ = 1.5, ns) over a 30 min period up to a dose of 100 mg/kg p.o., although at the lowest dose tested there was a tendency toward an increase in motility relative to the control group (Figure 7a). By contrast, diazepam dose-dependently and significantly reduced motility ($F_{3,26}$ = 27.4, P<0.001). *Post hoc* Dunnett's test showed that diazepam at all doses significantly (P<0.01) reduced motility in mice compared to vehicle treatment (Figure 7b).

In an equivalent study in rats, NS11394 was initially tested at doses of 3-30 mg/kg p.o. with no significant effect on motility in novel cages (data not shown). Therefore, NS11394 was re-tested at doses of 30-120 mg/kg p.o. In this case there was a significant effect of NS11394 ($F_{3,27}$= 3.5, P<0.03), with both 60 and 120 mg/kg p.o. engendering a significant (P<0.01) reduction in motility compared to control animals (Figure 7c). As seen in the mouse study, diazepam significantly and dose dependently reduced motility in rats ($F_{3,27}$ = 25, P<0.001) with all tested doses reaching significance compared to vehicle treated animals (P<0.01, Figure 7d).

Rat rotarod - ataxia and ethanol interaction. Separate groups of rats were pretreated with either vehicle or a subthreshold dose of ethanol (i.e., a dose that does not in itself induce ataxia) in combination with various doses of NS11394 30 min prior to the rotarod test (Figure 8a). Analysis of the data showed a significant main overall effect of NS11394 treatment ($F_{5,84}$ = 6.1, P<0.01). Specifically, 120 mg/kg NS11394 significantly reduced time on the rotarod compared to control animals (P<0.05). However, analysis indicated no significant NS11394 × ethanol interaction ($F_{5,84}$ = 1.0, ns) indicating no propensity for synergy between NS11394 and ethanol with respect to rotarod performance, although from Figure 8a it would appear that some interaction was apparent at the 120 mg/kg dose.

Diazepam dose-dependently impaired rotarod performance in the absence of ethanol ($F_{3,56}$ = 48, P<0.001), with 3 mg/kg diazepam engendering a significant impairment compared to the control group (P<0.001, Figure 8b). Furthermore, there was a significant diazepam × ethanol interaction ($F_{3,56}$ = 6.1, P<0.001), indicating that the effects of diazepam were altered in the presence of ethanol. Further analysis indicated that there was a significant interaction between diazepam and ethanol at all three doses of diazepam tested (P<0.001, Figure 8b).

Mouse passive avoidance test (PA). Mice were trained to associate an environmental context with an aversive foot-shock. Memory for the strength of this association was demonstrated in a test 24 h later where animals typically show an increased latency to enter the same context in which they previously received the foot-shock. In separate groups of mice administered different doses of NS11394 prior to the training session, there was a significant dose-dependent impairment in 24 h recall performance ($F_{5,71}$ = 7.3, P<0.001), indicated by the reduction in latency to cross to the dark compartment compared to control animals (Figure 9a). At doses from 10 mg/kg, NS11394 significantly impaired memory compared to vehicle treated animals (P<0.01). There was also a significant impairment in 24 h recall in mice treated with chlordiazepoxide ($F_{3,44}$ = 10, P<0.001), with *post hoc* tests indicating a significant reduction in cross-over latency in mice treated with 20 mg/kg chlordiazepoxide as compared to vehicle treated mice (Figure 9b).

Rat fear conditioning test (FC). In the rat FC animals were trained to associate a context and a short auditory tone-cue with an aversive foot-shock. Animals treated with vehicle and exposed to a context and a tone cue associated with foot-shock (vehicle shock group) froze more when exposed to the same conditions 24 h later in the absence of foot-shock, compared to vehicle treated animals exposed to context and tone-cue but never shock (vehicle no-shock group). In the studies described here the differences between the vehicle no-shock and vehicle shock groups were as follows for: (i) context fear conditioning: 46.7 ± 3.1 & 34.8 ± 3.3, respectively (P<0.01); and (ii) cue fear conditioning: 40.7 ± 4.2 & 18.5 ± 1.8,

respectively (P<0.001). In the study of NS11394 and alprazolam described below drug effects are shown relative to the vehicle shock group only (Figures 9c- 9f).

Separate groups of rats were administered various doses of NS11394 or alprazolam, in addition to a vehicle treated control group, 30 min prior to training for context-shock and cue-shock conditioning. When tested 24 h later there was for both compounds a near significant effect of treatment on context fear conditioning ($F_{6,57}$ = 2.1, P<0.06; Figure 9c and 9d), and a clear significant effect of treatment on cue fear conditioning ($F_{6,57}$ = 3.7, P<0.003; Figure 9e and 9f). Further analysis indicated that the 3 mg/kg dose of both NS11394 and alprazolam significantly impaired cue fear conditioning compared to the vehicle control group. Nonetheless, from Figures 9c and 9d it is evident that both NS11394 and alprazolam both also showed a dose-dependent trend to impair context fear conditioning.

Pharmacokinetic profile of NS11394

Microsomal stability. When NS11394 was incubated with liver microsomes derived from mouse or rat at a concentration of 1 μM for 1 h approximately 90% of parent compound was still present in the media after this time. These data indicate that NS11394 is relatively stable with respect to first pass metabolism and support the bioavailability data described below.

Bioavailability in the rat. Intravenous administration of 3 mg/kg NS11394 indicated that it had a low-moderate clearance rate of 0.14 l / h / kg and an elimination half-life of 2.8 h. After oral administration, 3 mg/kg NS11394 was well absorbed with a bioavailability of 82% and a prolonged mean plasma concentration level of ~1200 ng/ml between 30 min – 6 h (Figure 10), giving an $AUC_{0\text{-}24\ h}$ of 17160 h*ng/ml and a half-life of > 6 h. Since Tmax was reached at 30 min, it is unlikely that the prolonged exposure was due to slow absorption. The volume of distribution of 0.8 L/kg is similar to body water (~0.71 l / kg) indicating fast equilibration between plasma and other body organs. A summary of this data is given in the Table accompanying Figure 10.

Summary

To give an overall visual snap-shot of the therapeutic index for NS11394, and to aid in orientating the discussion, in Figure 11 we show the separation between plasma concentrations at minimum effective doses of NS11394 engendering efficacy in rodent anxiety models relative to plasma concentrations attained at doses inducing side-effects. At high doses assessed in side-effect models for which measured plasma concentrations of NS11394 were not determined, we estimated the concentrations based on linear regression analysis of mouse and rat data in Figure 4 (r^2=0.99, P<0.0005). Figure 11 also depicts the in-vitro K_i from binding studies (Table 1) and the functional potency (EC_{50}) of NS11394 at $GABA_A$-α_3 from in-vitro electrophysiology studies (Fig. 2).

Discussion

NS11394 represents a novel subtype-selective $GABA_A$ receptor positive allosteric modulator, with a unique profile compared to other $GABA_A$ receptor molecules published to date. In particular, NS11394 has substantially greater $hGABA_A$-α_3 efficacy compared to several recently described subtype-selective molecules (McKernan et al., 2000; Dias et al., 2005; Atack et al., 2006) whilst maintaining low $hGABA_A$-α_1 efficacy (Griebel et al., 2001). Similar to recently described subtype-selective $GABA_A$ modulators, NS11394 shows no affinity selectivity between $hGABA_A$-α_1, α_2, α_3, α_5 containing receptors; although NS11394 has lower affinity for $hGABA_A$-α_4 or α_6 containing receptors. Furthermore, the affinity of NS11394 for rat (cortex) and $hGABA_A$-α_1, α_2, α_3, α_5 containing receptors is equivalent, an important consideration in cross-species translation. Functionally, NS11394's selectivity profile is dependent upon the α subunit present in the $hGABA_A$ receptor complex with an efficacy order of: $\alpha_5 > \alpha_3 > \alpha_2 > \alpha_1$. However, functional efficacy data of this sort are somewhat tricky to obtain since the modulatory effects are highly dependent on the GABA concentration used. Within a given receptor combination the GABA EC_{50} can vary significantly between individual cells giving rise to large variation in modulator efficacy. When comparing data for different receptor combinations this problem is exacerbated, since it is difficult to ensure that exactly the same EC_x concentration of GABA is used for each combination. We therefore chose to index NS11394's modulatory actions to diazepam. Diazepam is described as being equally effective at the four $GABA_A$ receptor subtypes at which we tested (Sieghart, 1995) although this may be an approximation as we consistently see modulation of GABA responses in the order of $\alpha_3 \geq \alpha_2 > \alpha_1 > \alpha_5$. Thus, NS11394 has the order of efficacies seen in Figure 2 relative to diazepam but relative to GABA the order would be somewhat different in particular the α_5 efficacy would in effect be lower.

The relationship between NS11394's pharmacokinetic (plasma and brain concentrations) and pharmacodynamic (CNS receptor occupancy) properties was an important consideration in driving our *in-vivo* studies. There was a linear increase in plasma and brain concentrations with increasing NS11394

dose and a brain/plasma ratio of ~1, indicating that NS11394 readily crossed the blood brain barrier. Once in the brain NS11394 readily occupied benzodiazepine receptors, and there was a dose-linear increase in receptor occupancy paralleling the increases in plasma and brain concentrations. In rat, NS11394 had a bioavailability of ~80% and long plasma exposure, not dependent on absorption, consistent with long receptor exposure seen for up to 6 h. *In-vitro* stability data were excellent since NS11394 showed >90% stability when incubated for 1 h with rat/mouse microsomes, indicating limited first pass metabolism and supporting the bioavailability data. In summary, NS11394 has outstanding pharmacokinetic and pharmacodynamic properties making it a highly useful compound for *in-vitro* and *in-vivo* studies since data generated can confidently be attributed to the parent compound.

In the rat CER test NS11394 induced a robust anxiolytic-like effect, which is unlikely to be secondary to any analgesic effect since: (i) no footshock is delivered on drug test days and, further, (ii) in the companion paper we demonstrate no effect of NS11394 in the tail flick or hot plate models of acute nociceptive pain (Munro et al., 2008). Although it could be argued that NS11394 had greater efficacy in CER compared to alprazolam, it is clear that alprazolam's efficacy was limited by motoric impairment at higher doses. However, NS11394 generally increased baseline response rate, a disinhibitory effect also described with low doses of benzodiazepines (Mathiasen et al., 2007). NS11394 was also effective in two mouse models of anxiety. Overall, the minimum significantly effective dose of NS11394 in rodent anxiety models was 0.1-0.3 mg/kg, equating to receptor occupancy in mouse/rat of ~20% (Figure 11). By contrast, NS11394 only reduced locomotor activity at 60-120 mg in rat with no effect in mice up to 100 mg/kg, marginally affected rat rotarod performance at 120 mg/kg, and did not *significantly* interact with ethanol in rat up to 120 mg/kg (Figure 11). Thus, NS11394 has a benign side-effect profile even at doses supra-threshold for inducing full receptor occupancy in forebrain. By contrast, diazepam significantly reduced locomotor activity in mouse and rat, impaired rat rotarod performance and engendered considerable ethanol interaction at doses (0.3-3 mg/kg) that could readily be ascribed to occupancy at $GABA_A$ receptors (Mirza and Nielsen, 2006). The doses of diazepam engendering side-effects clearly

overlap with doses effective in animal models of anxiety (e.g., Griebel et al., 1999a; Mathiasen et al., 2007). The basis for the effect of NS11394 on rat motility at doses supra-threshold for inducing full receptor occupancy are unclear but might feasibly be related to: (i) NS11394 acting at $GABA_A$-α_4 or α_6 containing receptors at high doses, since activation of these receptor subtypes induces motoric impairment (Ebert et al., 2006). Although we would be cautious about this interpretation as we tested NS11394 at $GABA_A$-α_4 or α_6 containing receptors which contain a γ2 subunit, whereas current literature suggests that α_4 and notably α_6 subunits combine preferentially with a δ subunit (Ebert et al., 2006); or (ii) the result of off-target effects, although these were few and follow-up on specific targets indicated weak potency compared to $GABA_A$ receptors.

Although the therapeutic index for NS11394 is exceptional with respects to sedation, ataxia and ethanol interaction, a different pattern emerges when cognition is considered. NS11394 impaired memory for aversive events in both the mouse (PA) and rat (FC). NS11394's high efficacy at $GABA_A$-α5 receptors possibly explains these effects since positive modulation of $GABA_A$-α5 receptor is an important determinant of benzodiazepine-induced memory impairment (Maubach, 2003). However, what is surprising is the apparent better therapeutic index for NS11394 compared to alprazolam and chlordiazepoxide. When comparing separation between anxiolytic potency and potency to impair cognition for chlordiazpoxide (FPT versus PA) and alprazolam (CER versus FC) this is ~2-3 fold. For NS11394 this separation is 100-fold in mouse (MB versus PA) and 10-fold in rat (CER versus FC) (Figure 11). Although overall the therapeutic index of NS11394 is less impressive with respect to memory impairment it is still noteworthy that whereas anxiolytic efficacy is seen at ~20% brain occupancy in both mouse and rat, memory impairments are apparent at much higher brain receptor occupancy (Figure 11).

Based on our data what do we believe is responsible for the excellent *in-vivo* anxiolytic efficacy and low side effect profile of NS11394? With respect to *in-vivo* anxiolytic efficacy there are several possibilities:

(i) substantial efficacy at $GABA_A$-α_3 receptors; (ii) sufficient efficacy at $GABA_A$-α_2 receptors; (iii) a combination of efficacy at α_2 and α_3 receptors. Although compounds with efficacy at $GABA_A$-α_1 receptors are often very efficient in anxiety models such as CER (Mathiasen et al., 2007), knowledge from our in-house programme indicates that compounds with no efficacy at $GABA_A$-α_1 receptors can still demonstrate efficacy in CER as long as they demonstrate sufficient efficacy at $GABA_A$-α_3 receptors (>30% using our electrophysiology methods). Furthermore, in our hands non-selective weak partial agonists with efficacy of ~15% at all receptor subtypes are rarely active in CER (data not shown). Thus, we believe it unlikely that the low efficacy of NS11394 at $GABA_A$-α_1 receptors contributes significantly to its anxiolytic effects. Regarding NS11394's low side-effect profile compared to benzodiazepines, two factors probably work in synergy – firstly, NS11394 is a partial agonist at all subtypes, a profile known to impart an improved side-effect profile (Haefely et al., 1990), and secondly, the efficacy at $GABA_A$-α_1 receptors is low.

Having discussed what we believe explains NS11394's excellent efficacy versus side-effect profile, it is fair to say that the *in-vitro* electrophysiology profile for NS11394 is somewhat inconsistent with conclusions derived from work with gene knock-in mice (Rudolph et al., 1999) implicating $GABA_A$-α_2 receptors as most important in the anxiolytic effect of benzodiazepines. However, pharmacological studies with $GABA_A$-α_1 selective antagonists are also inconsistent with gene knock-in mouse data in implicating $GABA_A$-α_1 receptors in anxiolytic efficacy (Shannon et al., 1984; Griebel et al., 1999b; Paronis et al., 2001; Rowlett et al., 2005; Huang et al., 1999). Indeed, ocinaplon an arguably $GABA_A$-α1 selective molecule demonstrates anxiolytic efficacy in GAD patients without engendering sedation (Basile et al., 2006). Further, based on data with weak positive and negative $GABA_A$-α_3 subtype-selective modulators, others like us advocate that this receptor subtype is sufficient to engender an anxiolytic response in rodents (Dias et al., 2005; Atack et al., 2005). Thus these discrepancies and our data with

NS11394 suggest that results generated with gene knock-in animals should perhaps not be considered pivotal but rather parallel datasets.

When comparing NS11394 to other subtype-selective ligands described in the literature it is important to appreciate that functional selectivity is a rather loose term. For example, as stated earlier in our hands diazepam is a somewhat more effective modulator at $GABA_A$-$\alpha_{2/3}$ over $GABA_A$-$\alpha_{1/5}$ receptors. Also Adipiplon/NG2-73 described as a α_3-*preferring* $GABA_A$ partial agonist (www.neurogen.com), using our methods is a non-selective partial agonist (~40% at α_1 and α_3 containing receptors, unpublished data). Another point to consider is that many subtype-selective compounds described may not necessarily be good *in-vivo* tools. For example, whilst anxiolytic efficacy for L838,417 has been described in the mouse (McKernan et al., 2000; van Bogaert et al., 2006), its pharmacokinetic profile in this species is poor after peroral administration (Scott-Stevens et al., 2005), and we see only 10% of parent compound remaining in our rodent microsomal stability assays (data not shown), making it reasonable to query conclusions from *in-vivo* studies with this molecule.

In conclusion, NS11394 represents a potent and novel subtype-selective $GABA_A$ receptor modulator with a unique selectivity profile compared to other recently described molecules. We believe this profile largely explains NS11394's potency in animal models of anxiety and excellent therapeutic index. NS11394 also shows excellent pharmacokinetic properties which correlate with pharmacodynamic endpoints allowing for a powerful translational approach. Given the confidence we have in attributing *in-vivo* effects to NS11394 *per se*, this makes it an invaluable tool in posing questions as to the relevant $GABA_A$ subtype selectivity profile necessary for efficacy in emerging therapeutic areas such as pain (see Munro et al., 2008; Knabl et al., 2008).

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Legends for Figures

Figure 1: Chemical structure of NS11394: [3'-[5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl] biphenyl-2-carbonitrile]

Figure 2: *In-vitro* efficacy of NS11394 in two-electrode voltage clamp experiments at human recombinant $GABA_A$ receptors expressed in *Xenopus laevis* oocytes. GABA (EC_5-EC_{25}) evoked currents were recorded from oocytes in absence and presence of NS11394 and the percent of GABA modulation was calculated. NS11394 modulation was then indexed to the modulation of 0.5 μM Diazepam on the same oocytes and termed "% modulation (rel. DZ)". Each datapoint are the result of testing at n=5-25 individual oocytes (74 oocytes in total) and the data were fitted to a sigmoidal dose-response equation using GraphPad Prism 4. The Table below the Figure quantifies the maximum efficacy (E_{Max}, %) of NS11394 at each receptor subtype as well as its potency (EC_{50}, nM).

Figure 3: Inhibition of in-vivo [^{3}H]flunitrazepam binding with NS11394 in (a) mouse and (b) rat forebrain: open (O) and closed (●) symbols represent binding assessed either 30 or 120 min, respectively, after p.o. NS11394 administration. Figure (c) shows inhibition of in-vivo [^{3}H]flunitrazepam binding to mouse forebrain 30 min after i.p. administration with alprazolam (open circle), chlordiazepoxide (closed triangle) or diazepam (closed circle). Data are means ± S.E.M. of 3 independent experiments.

Figure 4: Relationship between plasma concentration (dotted line), brain concentration (solid line) and CNS in-vivo receptor occupancy (columns) in (a) mice and (b) rats after dosing animals with NS11394 (0.1-3 mg/kg, p.o., n=3 per dose level) and taking relevant plasma and brain samples at a single 30 min time-point post dosing. Data are means ± S.E.M.

Figure 5: Upper panel: effect of NS11394 (0.3-10 mg/kg, p.o., n=7) on rat CER performance. Figures (a) and (b) show mean light/dark and suppression ratio data, respectively. Lower panel: effect of alprazolam

(0.3-3 mg/kg, i.p., n=6) on rat CER performance. Figures (c) and (d) show mean light/dark and suppression ratio data, respectively. Data are means ± S.E.M. * P<0.05 compared to vehicle control.

Figure 6: Upper panel: effect of (a) NS11394 (0.3-10 mg/kg, p.o., n=10) and (b) chlordiazepoxide (5-20 mg/kg, i.p., n=8-10) in the mouse four plate test. Lower panel: effect of (c) NS11394 (0.1-1 mg/kg, p.o., n=8) and paroxetine (PRX, 10 mg/kg, i.p., n=7) and (d) diazepam (0.1-3 mg/kg, i.p., n=8) in the mouse marble burying test. Data are means ± S.E.M. * P<0.05 compared to vehicle control.

Figure 7: Upper panel: effect of (a) NS11394 (10-100 mg/kg, p.o., n=6-7) and (b) diazepam (0.3-3 mg/kg, i.p., n=6-7) on motility in non-habituated mice. Lower panel: effect of (c) NS11394 (30-120 mg/kg, p.o., n=7) and (d) diazepam (0.3-3 mg/kg, i.p., n=7) on motility in non-habituated rats. Data are means ± S.E.M. * P<0.05 compared to vehicle control.

Figure 8: Effect of (a) NS11394 (1-120 mg/kg, p.o., n=8) and (b) diazepam (0.3-3 mg/kg, i.p., n=8) on rat rotarod performance in the absence (white bars) and presence (black bars) of a dose of ethanol (0.8 g/kg, i.p.) subthreshold for inducing ataxia. Data are means ± S.E.M. * P<0.05 compared to appropriate control group.

Figure 9: Upper panel: effect of (a) NS11394 (1-100 mg/kg, p.o., n=8-20) and (b) chlordiazepoxide (5-20 mg/kg, i.p., n=12) in the mouse passive avoidance test. Middle panel: effect of (c) NS11394 (0.3-3 mg/kg, p.o., n=8) and (d) alprazolam (0.3-3 mg/kg, i.p., n=8) on context fear conditioning in the rat. Lower panel: effect of (e) NS11394 (0.3-3 mg/kg, p.o., n=8) and (f) alprazolam (0.3-3 mg/kg, i.p., n=8) on cue fear conditioning in the rat. The white bar to the left in Figure c/d and e/f indicates the level of freezing to context and cue, respectively, in vehicle treated rats. Data are means ± S.E.M. * P<0.05 compared to vehicle control.

Figure 10: Plasma pharmacokinetics of NS11394 in rats following intravenous (3 mg/kg, i.v., n=3, open circles) and oral administration (3 mg/kg, p.o., n=3, closed circles). Data are means ± SD. The Table below the Figure quantifies the key data.

Figure 11: Therapeutic index for NS11394. Measured/estimated plasma concentrations at minimum effective doses of NS11394 in rodent anxiety models relative to concentrations attained at doses inducing side-effects (above line). Below the line the receptor occupancy/plasma concentrations attained at 0.1 and 3 mg/kg in both mouse and rat are shown. Also depicted are the in-vitro K_i for binding to the $GABA_A$ receptor and functional potency (EC_{50}) at $GABA_A$-α_3 receptors. The prefix 'm' and 'r' refer to mouse and rat, respectively. All other abbreviations are as used in the text, except rMOT and rROT which refer to rat motility and rotarod, respectively.

Table 1 Affinity of NS11394 and diazepam at $GABA_A$ receptors in rat cortical membranes and human α_1-α_6 containing $GABA_A$ receptors expressed in HEK293 cells. Data are expressed as mean K_i, (nM) ± S.E.M. of 3-4 independent experiments in triplicate.

Compound	[^{3}H]FNM	[^{3}H]Ro 15-1788				[^{3}H]Ro 15-4513	
	rat cortex	$\alpha_1\beta_{3*}\gamma_{2S}$	$\alpha_2\beta_{3*}\gamma_{2S}$	$\alpha_3\beta_{3*}\gamma_{2S}$	$\alpha_5\beta_{3*}\gamma_{2S}$	$\alpha_4\beta_{3*}\gamma_{2S}$	$\alpha_6\beta_{3*}\gamma_{2S}$
NS11394	0.423 ± 0.083	0.41 ± 0.12	0.84 ± 0.10	0.497 ± 0.046	0.119 ± 0.029	324 ± 24	1009 ± 160
Diazepam	11.3 ± 3.1	10.1 ± 1.8	7.5 ± 1.4	19.3 ± 3.6	5.33 ± 0.53	>15,000	>15,000

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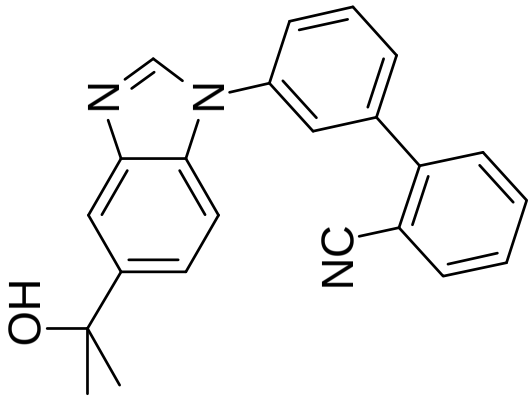
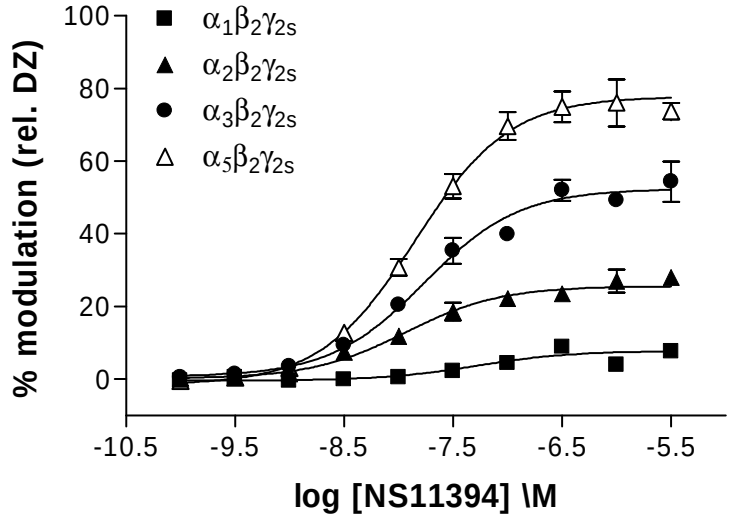
Figure 1



NS11394



	$\alpha_1\beta_2\gamma_{2s}$	$\alpha_2\beta_2\gamma_{2s}$	$\alpha_3\beta_2\gamma_{2s}$	$\alpha_5\beta_2\gamma_{2s}$
E_{Max} \%	7.8	26.0	52.0	78.0
EC_{50} \nM	52.0	12.0	17.0	14.0

Figure 2

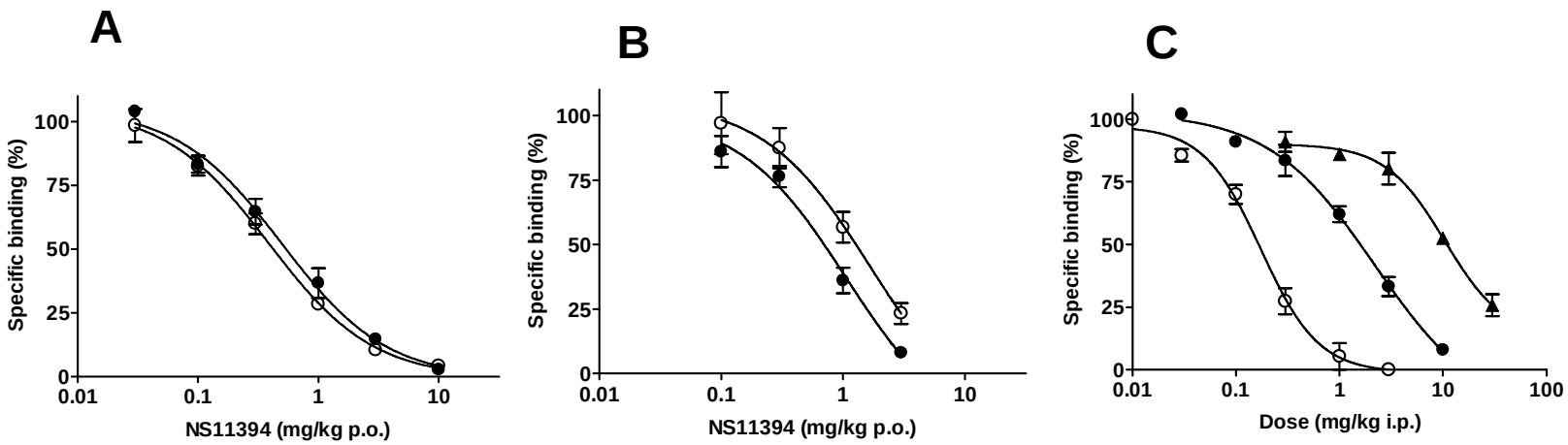
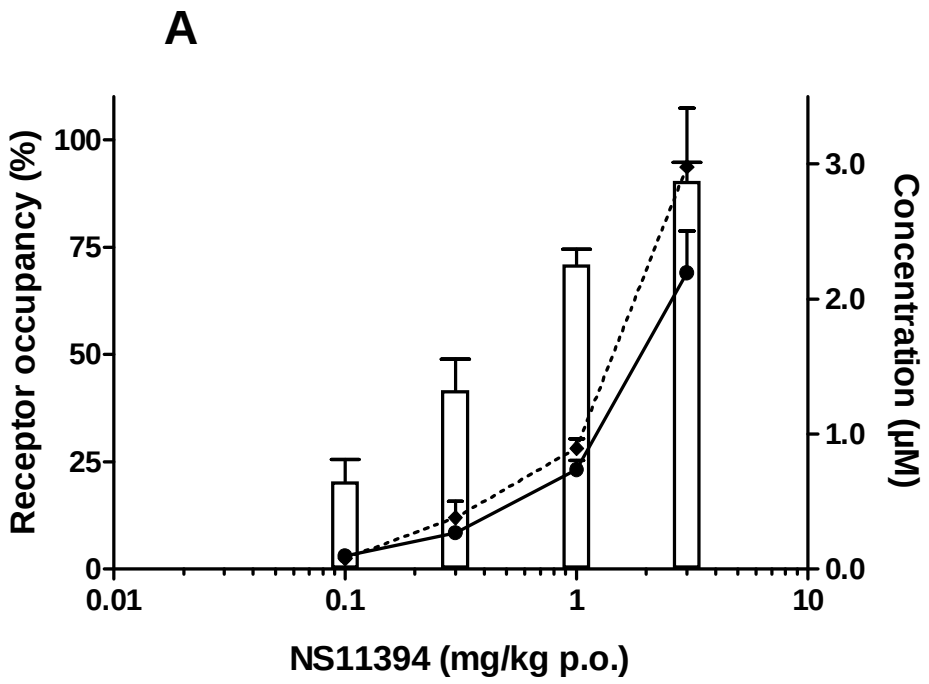


Figure 3



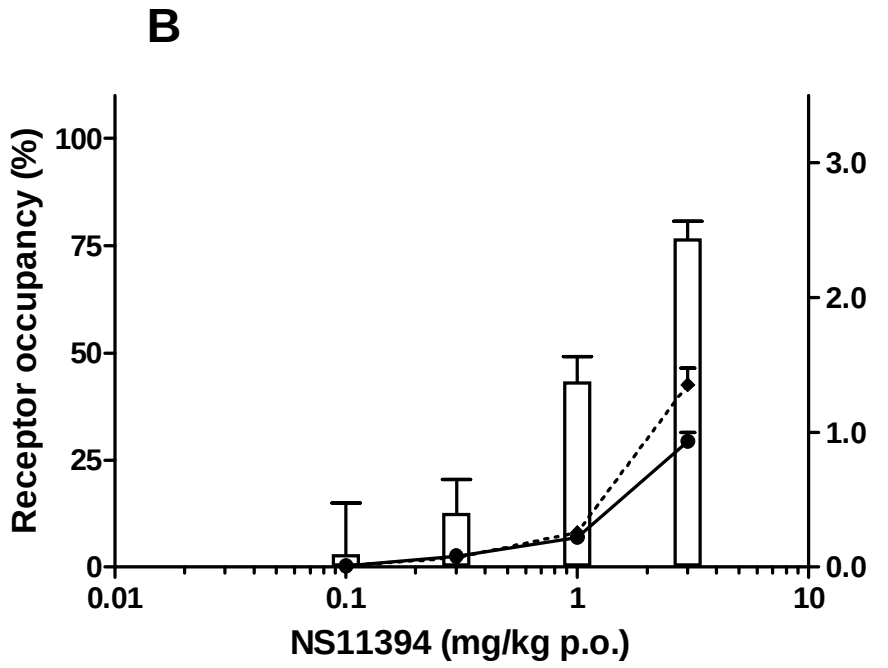


Figure 4

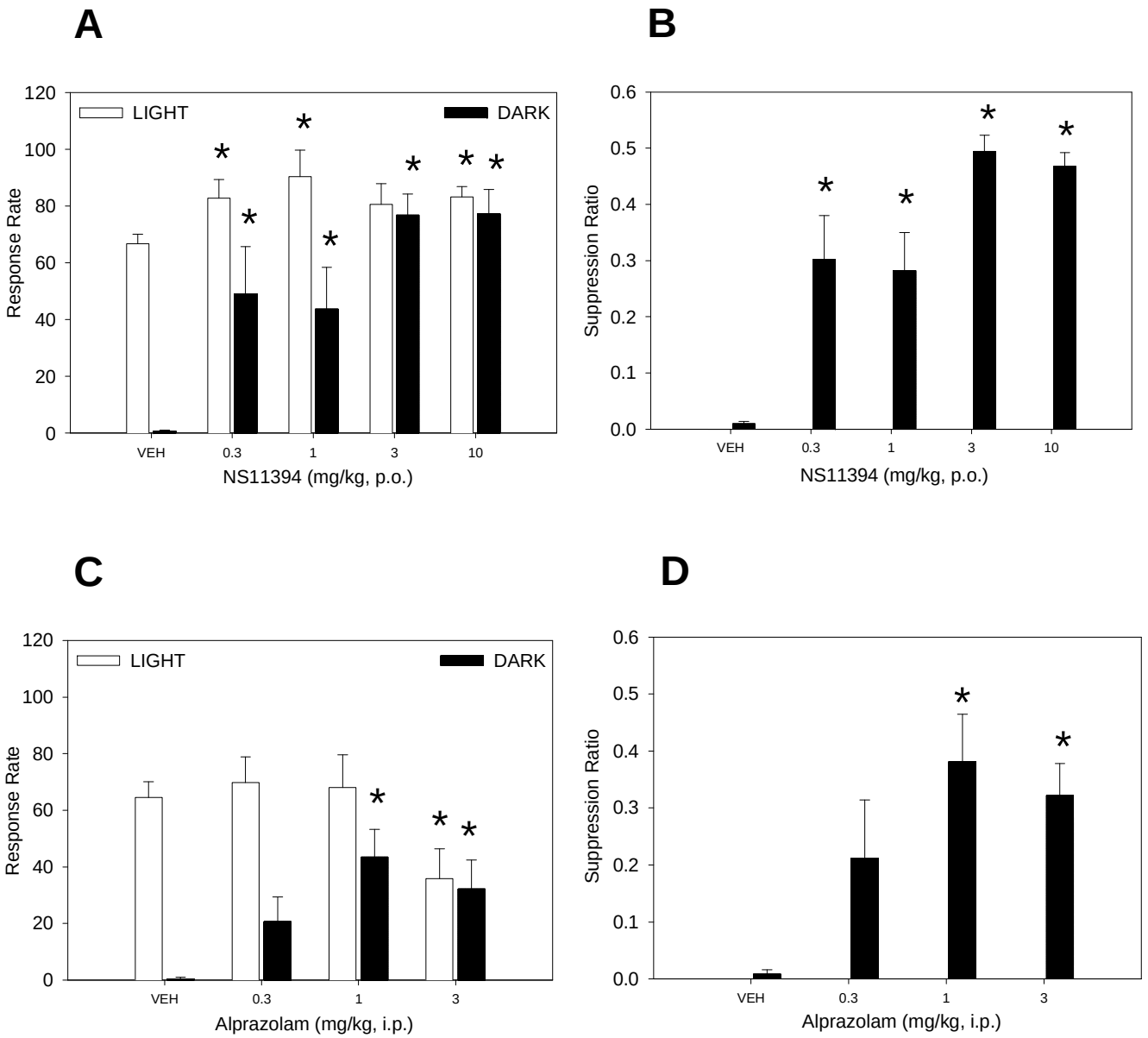


Figure 5

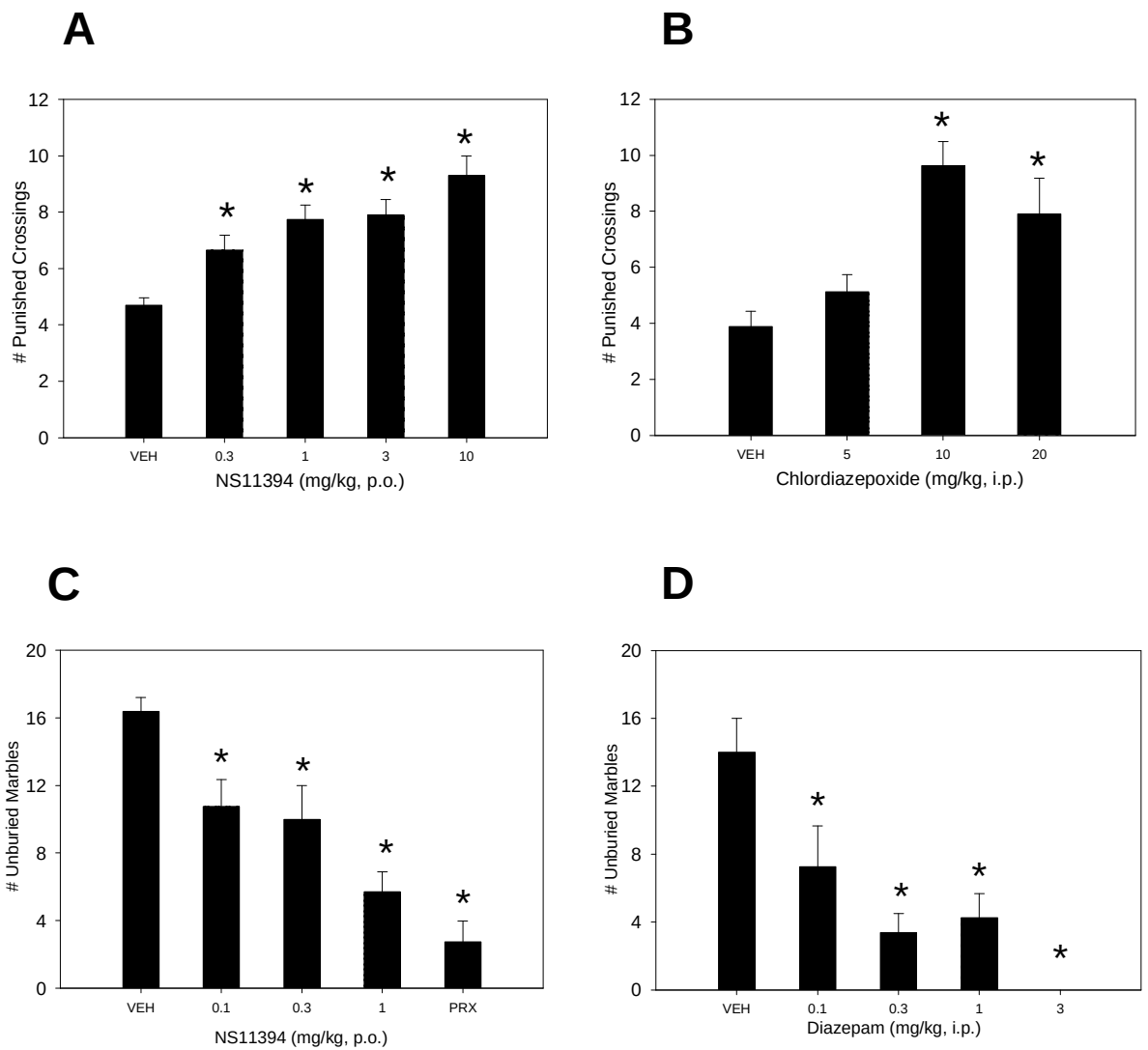


Figure 6

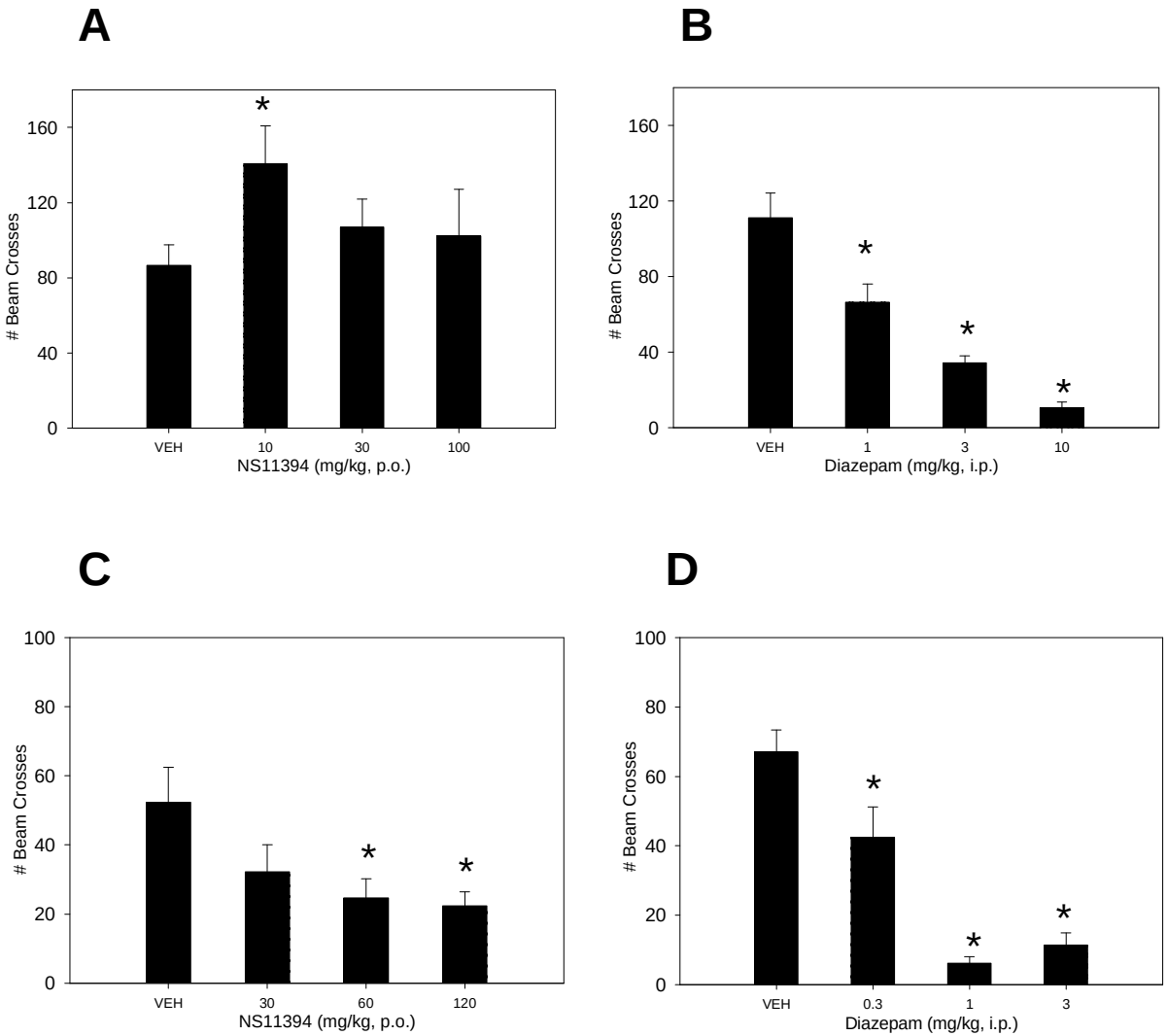


Figure 7

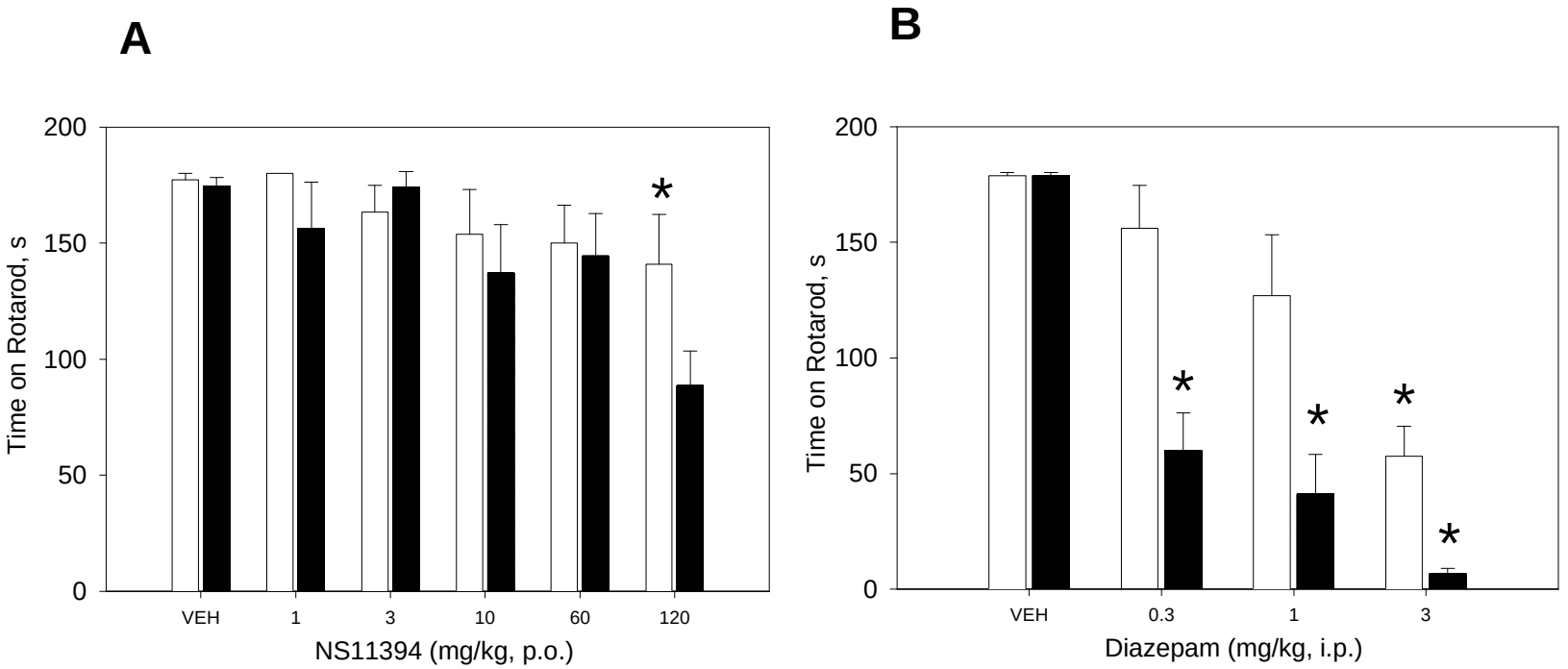


Figure 8

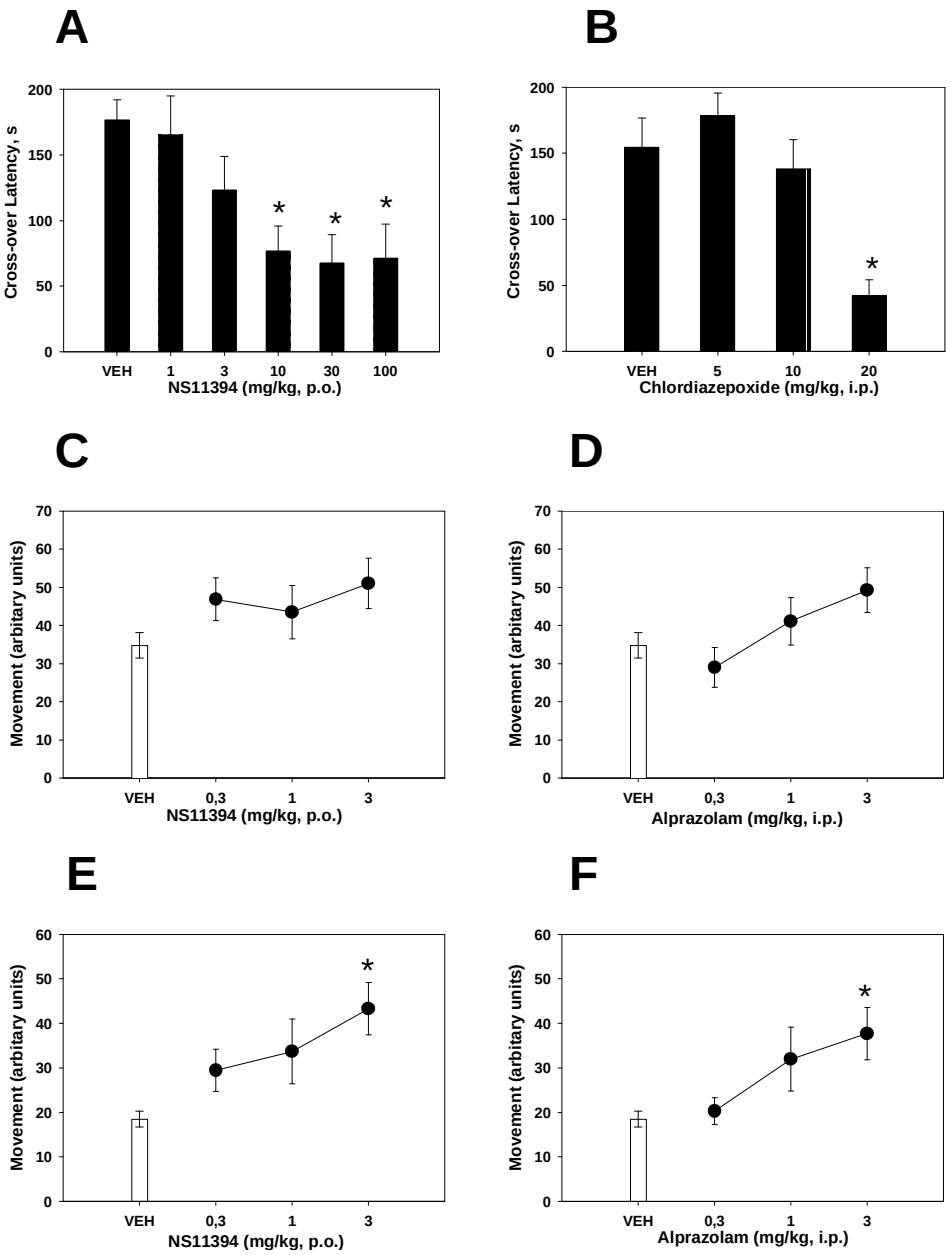
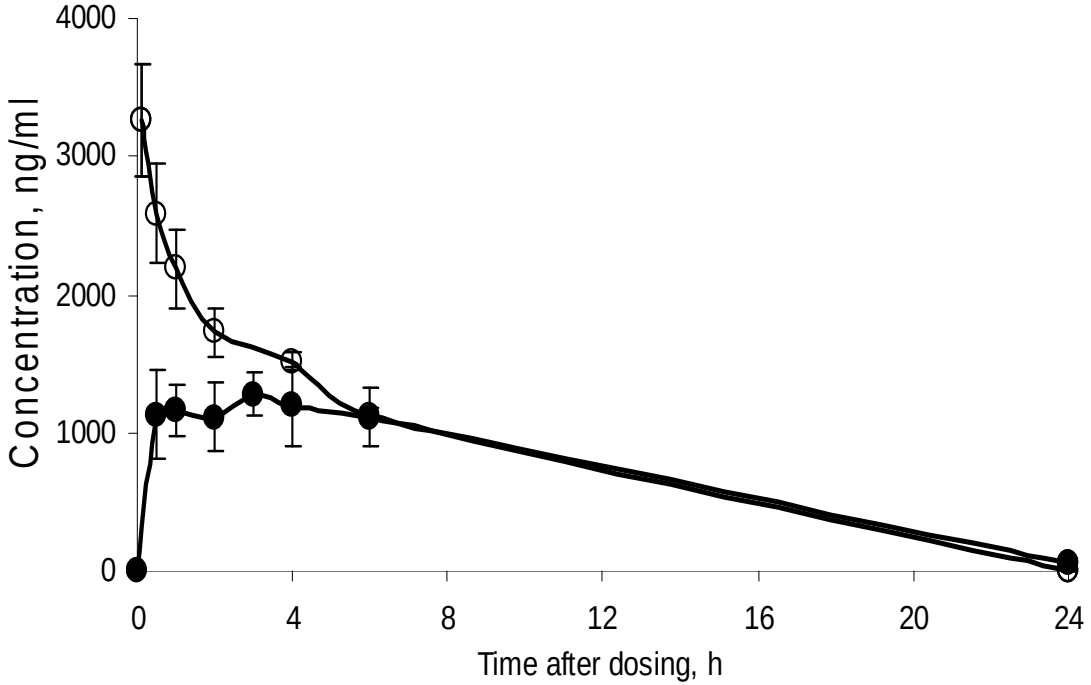


Figure 9



Route	F%	Cmax (ng/ml)	AUC_{0-6} (h*ng/ml)	T½ (h)	CL (L/h/kg)	Vd (l/kg)
IV	-	3593 (C_0)	20820	2.8	0.14	0.8
PO	82 %	1200	17160	NA	-	-

Figure 10

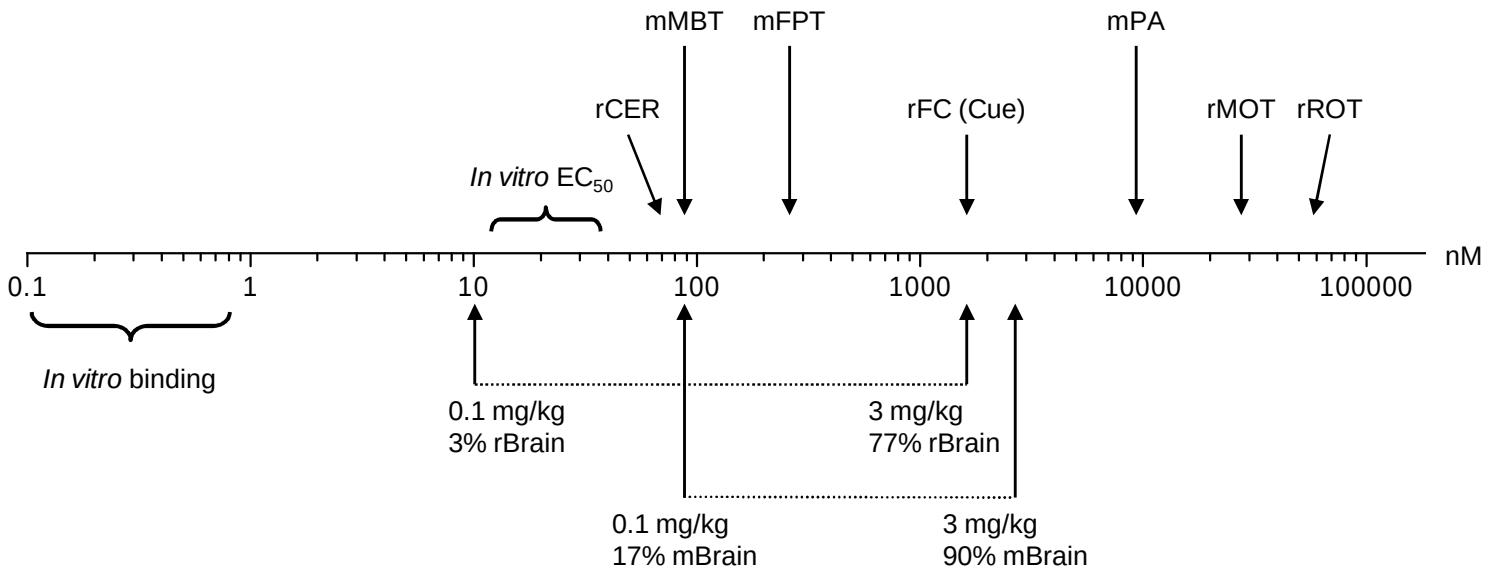


Figure 11