

The in vivo properties of pagoclone in rat are most likely mediated by 5'-hydroxy pagoclone

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Abstract

The cyclopyrrolone pagoclone binds with roughly equivalent high affinity (0.7–9.1 nM) to the benzodiazepine binding site of human recombinant GABA_A receptors containing either an $\alpha 1$, $\alpha 2$, $\alpha 3$ or $\alpha 5$ subunit. However, whereas it was a partial agonist at $\alpha 1$ -, $\alpha 2$ - and $\alpha 5$ -containing GABA_A receptors, pagoclone was a full agonist at receptors containing an $\alpha 3$ subunit. In the rat elevated plus maze assay pagoclone (3 mg/kg) had significant anxiolytic-like activity but at all three doses tested (0.3, 1 and 3 mg/kg p.o.) it produced a significant reduction in the total distance travelled. This sedative-like effect was confirmed in rat chain-pulling and spontaneous locomotor assays. Surprisingly, in the plasma and brain samples derived from the elevated plus maze assay, the major metabolite of pagoclone, 5'-hydroxy pagoclone, was present at 10–20-fold higher concentrations relative to the parent compound. In order to establish whether this metabolite might have pharmacological activity, we measured its affinity and efficacy profile and found that both were comparable to those of pagoclone with the exception that efficacy at the $\alpha 1$ subtype was considerably greater for 5'-hydroxy pagoclone compared with the parent. This metabolite had significant anxiolytic-like activity in the elevated plus maze but at these same doses (0.3–3 mg/kg p.o.) also produced sedation. It is therefore likely that in rats 5'-hydroxy pagoclone mediates the majority of the pharmacological actions following pagoclone administration.

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1. Introduction

Since their introduction in the early 1960s, the 1,4-benzodiazepines, typified by diazepam, have been extensively used in the clinic due to their anxiolytic, sedative/hypnotic, myorelaxant, cognitive impairing and anticonvulsant activities (Sternbach, 1979). The diverse pharmacology of these compounds is now understood to be a consequence of modulation of GABA_A receptors containing β and $\gamma 2$ subunits and either an $\alpha 1$, $\alpha 2$, $\alpha 3$ or $\alpha 5$ subunit at a binding site that is physically

distinct from, but allosterically coupled to, the agonist (GABA) binding site (Sieghart, 1995; Sieghart and Sperk, 2002). The classical benzodiazepines interact with these different GABA_A receptor subtypes with equivalent affinity and efficacy and are therefore non-selective GABA_A receptor modulators.

Depending upon the clinical setting, the various pharmacological attributes of the benzodiazepines may be either beneficial or a liability. For example, the myorelaxant and cognitive impairing properties may be beneficial when benzodiazepines are employed as premedication (Williams and Bowie, 1999; Buffett-Jerrott and Stewart, 2002) but are clearly liabilities for everyday living when given for other indications. Similarly,

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2.3. *In vitro* binding

The inhibition of the binding of 1.8 nM [³H]Ro 15-1788 to GABA_A receptors containing β₃, γ₂ plus either α₁, α₂, α₃ or α₅ subunits or 8.0 nM [³H]Ro 15-4513 to GABA_A receptors containing β₃, γ₂ plus either α₄ or α₆ subunits was performed in membranes of L(tk⁻) stably expressing receptors according to methods described previously (Hadingham et al., 1993, 1996; Wafford et al., 1996). In these assays, radioligand and cell membranes were incubated under equilibrium conditions (90 min at room temperature) with compound, dissolved in DMSO and diluted in half-log concentration units (to give a final DMSO concentration in the assay of 1% or less). Non-specific binding was defined using flunitrazepam (10 μM) for the [³H]Ro 15-1788 assay and Ro 15-4513 (10 μM) for the [³H]Ro 15-4513 assay. The inhibition of binding was plotted as a function of concentration and from these data the IC₅₀ values were calculated (XLfit, IDBS, Guildford, UK) and then converted to K_i values according to the equation of Cheng and Prusoff (1973): $K_i = IC_{50}/(1 + ([L]/K_D))$ where [L] = concentration of radioligand used and K_D = the affinity of the radioligand for each individual receptor subtype. Respective K_D values for [³H]Ro 15-1788 at α₁-, α₂-, α₃- or α₅-containing receptors were 0.92, 1.05, 0.58 and 0.45 nM and for [³H]Ro 15-4513 binding to α₄- and α₆-containing receptors were 5.0 and 6.5 nM.

2.4. *In vitro* efficacy

The intrinsic efficacy of compounds was assessed using whole cell patch clamping to mouse fibroblast L(tk⁻) cells using the same recombinant human GABA_A receptor expression system used for the *in vitro* binding studies (Hadingham et al., 1993, 1996; Wafford et al., 1996). Whole cell patch clamping was carried out 24 h after induction of GABA_A receptor expression (using 25 nM dexamethasone) according to previously published methods (Brown et al., 2002) and involved growing a monolayer of cells on a microscope coverslip which was placed in a chamber on the stage of a Nikon Diaphot inverted microscope and viewed using phase-contrast optics. Cells were perfused continuously with artificial cerebrospinal fluid (aCSF) and a triple-barrelled pipette was used to form cell membrane patches of around 4 MΩ resistance with cells being voltage-clamped at -20 mV using an Axon 200B amplifier (Axon Instr., Foster City, CA). Drug solutions were applied via a multi-barrel drug delivery system, which could pivot the barrels into place using a stepping motor, ensuring rapid application and washout of drugs. GABA was applied to the cell for 5 s with a 30 s washout period between applications. Pagoclone or 5'-hydroxy pagoclone were preapplied for 30 s prior to coapplication with GABA. Responses were plotted as a function of concentration and curves were fitted to the data using a nonlinear least square-fitting program to the equation $f(x) = B_{max}/[1 + (EC_{50}/x)^n]$, where x is the drug concentration, EC₅₀ is the concentration of drug eliciting a half-maximal response, and n is the Hill coefficient. Allosteric potentiation of GABA_A receptors was measured relative to a GABA EC₂₀ determined for each cell to account for differences in GABA affinity.

2.5. *Elevated plus maze*

The anxiolytic effects of pagoclone and 5'-hydroxy pagoclone were evaluated using the elevated plus maze (Dawson and Tricklebank, 1995). Male Sprague–Dawley rats (240–280 g; Charles River, UK, $n = 18$ /group) received either vehicle (0.5% methyl cellulose; 1 mL/kg p.o.), the full, non-selective agonist chlordiazepoxide (5 mg/kg i.p. in saline) as a positive control and, in separate experiments either pagoclone (0.3, 1 and 3 mg/kg p.o.) or 5'-hydroxy pagoclone (0.3, 1 and 3 mg/kg p.o.). Following a pretreatment time of 30 min, rats were placed in the centre of the elevated plus maze (each arm measuring 10 × 50 cm, with a central area of 10 × 10 cm) for 5 min and their movement was tracked using a closed circuit TV and analysed using Flexible Maze Software (HVS Image, Hampton, Middlesex, UK). From these data, the time spent in the open arms as well as the total distance moved during the 5 min trial were calculated. Treatment effects across groups were made using an ANOVA followed by post hoc Dunnett's *t*-tests to compare each treatment group to the vehicle control group.

The plus maze experiments were run over a 2-day period and rat from Day 1 were taken following completion of the trial and receptor occupancy measured using the [³H]Ro 15-1788 *in vivo* binding method described below.

2.6. *Rat chain-pulling test*

The rat chain-pulling assay is a test for sedation which has been described in detail elsewhere (Bayley et al., 1996) and which uses PVG rats due to the relative ease with which they can be trained in this operant task relative to Sprague–Dawley rats. In brief, food-deprived PVG rats (250–300 g, Harlan, UK, $n = 12$ /group) were trained on a random probability interval schedule of 60 s following which animals were given doses of either vehicle (0.5% methyl cellulose, 1 mL/kg p.o.), pagoclone (1, 3 or 10 mg/kg p.o.) or, as a positive control, diazepam (10 mg/kg p.o.). Immediately after dosing, animals were placed in the operant box and the rates at which rats pulled a chain for a food reward were recorded over a 60 min period. Data was expressed as a mean percentage of the baseline chain pulls per minute and then analysed using an ANOVA followed by Dunnett's *t*-tests to compare each treatment group to the vehicle control group.

2.7. *Rat locomotor assay*

In order to directly compare the sedative-like properties of pagoclone and 5'-hydroxy pagoclone, the effects on locomotor activity of both compounds were measured in male Sprague–Dawley rats (250–300 g Charles River, $n = 6$ –8/group) using Benwick AM1053 activity chambers (Linton Instruments, Norfolk, UK) measuring 46 × 25 × 20 cm. Ideally, 5'-hydroxy pagoclone would have been assessed in the chain-pulling assay but by the time the metabolite had been identified, synthesised and then characterized *in vitro*, a cohort of rats trained in the operant, chain-pulling procedure and of an appropriate size (250–300 g) were no longer available.

Activity chambers comprise clear Perspex cages containing sawdust bedding and infrared beams that are arranged as two independent matrices, each consisting of 24 beams. The activity of rats was measured as beam breaks, with data being recorded by computer using Amlogger software (Linton Instruments). Rats administered with vehicle (0.5% methylcellulose, 1 mL/kg p.o.), 0.3, 1 or 3 mg/kg (p.o.) of either pagoclone or 5'-hydroxy pagoclone or chlordiazepoxide (10 mg/kg i.p.) 30 min before being placed into the activity chambers. Horizontal activity was recorded for 20 min. The data are expressed as the total number of beam breaks and analysed using a one-way ANOVA followed by Dunnett's *t*-tests to compare each treatment group to the vehicle control group.

2.8. *In vivo* binding

The occupancy of the benzodiazepine binding site of rat brain GABA_A receptors was measured using the inhibition of the *in vivo* binding of [³H]Ro 15-1788 (Atack et al., 1999). Briefly, after rats had completed their 5 min trial on the elevated plus maze, they received an i.v. injection of [³H]Ro 15-1788 (diluted 1:150 with saline; 1 μL/g) via a tail vein. Three minutes later (i.e., ca. 40 min after dosing with either vehicle, pagoclone, 5'-hydroxy pagoclone or chlordiazepoxide), animals were killed by stunning and decapitation and trunk blood collected into heparinized tubes. Brains were rapidly removed, weighed and homogenized in 10 volumes of ice-cold buffer (10 mM phosphate buffer/100 mM KCl, pH 7.4) and 300 μL aliquots were filtered and washed in 10 mL buffer over Whatman GF/B filters. A satellite group of animals which had not been tested on the plus maze received bretazenil (5 mg/kg i.p. in 100% PEG 300) which occupies all benzodiazepine binding sites and thereby allows the level of non-specific [³H]Ro 15-1788 binding to be defined. Aliquots of brain homogenate and plasma samples were frozen on dry ice and stored at -80 °C for subsequent analysis of drug concentrations.

The radioactivity retained on the filters (i.e., membrane-bound radioactivity) was counted on a Beckman LS6500 scintillation counter and % occupancy was defined as the extent by which the specific binding in vehicle-treated animals (ca. 2000 dpm) was reduced by prior drug treatment.

2.9. Analysis of plasma and brain drug concentrations

Aliquots of plasma (100 μ L) or brain homogenate (250 μ L) derived from the [3 H]Ro 15-1788 *in vivo* binding assay were extracted in twice the volume of acetonitrile which contained 0.1% heptafluorobutyric acid plus an internal standard (500 ng/mL of 2-(7-chloro-[1,8]naphthyridin-2-yl)-3-(5-methyl-hexyl)-2,3-dihydro-isindol-1-one) by vortex mixing and then centrifugation. The resulting supernatants were analysed using a LC-MS/MS system consisting of an Agilent 1100 series binary pump, CTC PAL autosampler and a Quattro Micro mass spectrometer (Micromass, Manchester, UK).

The chromatography system used a Ultracarb C18 column (150 \times 3.2 mm; Hichrom LTD, Theale, UK) and compounds were eluted using a mobile phase of 25 mM ammonium formate (pH 3.0) and acetonitrile/0.1% heptafluorobutyric acid at a flow of 0.4 mL/min. A gradient system was used of 75% acetonitrile for 1 min increasing to 95% at 4 min and remaining at 95% until 7.5 min. The desolvation gas temperature was 350 $^{\circ}$ C. The compounds were detected using SRM transitions of 408.2 > 293.9 for pagoclone and 406.2 > 293.9 for 5'-hydroxy pagoclone (this compound eliminates water in the source and is observed as the M-H₂O + H⁺ ion) and 394.0 > 83.0 for the internal standard.

3. Results

3.1. *In vitro* properties of pagoclone

Table 1 shows the affinity of the racemate RP 59037 and its separate enantiomers, of which the (+)-enantiomer is pagoclone, for the benzodiazepine binding site of human recombinant GABA_A receptors containing different α subunits. RP 59037 has high and approximately equivalent affinity (K_i values = 1.4–10.5 nM) for recombinant human GABA_A receptors containing either an α 1, α 2, α 3 or α 5 subunit (Table 1). These affinities are consistent with those seen against native rat brain receptors (K_i = 0.4–1.6 nM; Piot et al., 1990, 1992; Doble et al., 1992, 1993). Moreover, the lack of GABA_A receptor subtype selective affinity is in agreement with the fact that RP 59037 does not demonstrate binding selectivity for the so-called rat brain BZ1 (α 1) or BZ2 (α 2, α 3 and α 5) subtypes (Doble et al., 1993). The lack of GABA_A subtype binding selectivity shown by RP 59037 is also demonstrated by pagoclone (the (+) isomer; Table 1) since it possesses comparable binding affinity (0.7–9.1 nM) across human recombinant GABA_A receptor subtypes.

In addition, RP 59037 (and its single enantiomers) had very low affinity for GABA_A receptors containing either an α 4 or α 6 subunit (K_i > 1 μ M). These receptor subtypes constitute the so-called diazepam-insensitive GABA_A receptors and consistent with the present data, RP 59037 has previously been reported to have very low affinity for rat brain diazepam-insensitive receptors (Wong et al., 1995).

With respect to efficacy, pagoclone has significant agonist activity at all four diazepam-sensitive GABA_A receptor subtypes, being essentially a full agonist at the α 3 subtype and partial agonist at the α 1, α 2 and α 5 subtypes (Fig. 2 and Table 2). Interestingly, despite its much lower affinity relative to pagoclone, the (–)-enantiomer retained efficacy (α 1 and α 3 efficacy = 84% and 151%, respectively; data not shown), suggesting that despite its importance to binding affinity, the configuration around the chiral centre does not dramatically affect efficacy. The functional affinity (EC₅₀) of pagoclone was similar at the different GABA_A receptor subtypes (ranging from 3.1 to 6.6 nM: Table 2), consistent with the lack of subtype selectivity in the binding assay (Table 1).

3.2. *In vivo* properties of pagoclone

3.2.1. Pagoclone has anxiolytic-like activity in the rat elevated plus maze

The % time spent on the open arms of the elevated plus maze is used as a standard index of anxiolytic-like activity. There was a significant effect of treatment on time spent on the open arms of the elevated plus maze ($F(4,85) = 6.06$, $P < 0.0001$; Fig. 3A) with more specific analyses showing that whereas vehicle-treated rats spent 16 \pm 2% of their time on the open arms, the corresponding time for chlordiazepoxide-treated animals, 42 \pm 4%, was significantly greater ($P < 0.05$, Dunnett's *t*-test), consistent with the known anxiolytic properties of this compound. Pagoclone produced a dose-dependent increase in time spent on the open arms but this only achieved significance at the highest dose tested (3 mg/kg), at which dose the % time on the open arms was 34 \pm 6%.

Using the total distance travelled during the 5 min plus maze trial as an indirect index of sedation, there was also a significant effect of treatment on this parameter ($F(4,85) = 32.1$, $P < 0.0001$; Fig. 3B). Hence, compared with vehicle-treated rats which travelled on average a distance of 19 \pm 1 m, chlordiazepoxide significantly ($P < 0.05$, Dunnett's *t*-test) increased the distance travelled to 23 \pm 2 m (Fig. 3B). This is consistent with the known stimulation of exploration by chlordiazepoxide at doses below those at which sedation is observed (Dawson et al., 1995). On the other hand, pagoclone was associated with a significant decrease in total distance travelled at all doses tested, suggesting a degree of sedation even at doses (0.3 and 1 mg/kg) below that at which a significant anxiolytic-like effect was observed (3 mg/kg).

A subgroup of rats were taken after completion of their plus maze trial and occupancy from these animals is shown in

Table 1
Affinity of the racemate RP 59037 and its enantiomers for human recombinant GABA_A receptors containing different α subunits

	K_i (nM) at human recombinant GABA _A receptors containing β 3, γ 2 plus					
	α 1	α 2	α 3	α 4	α 5	α 6
RP 59037 ^a	1.5 \pm 0.4	4.1 \pm 1.0	1.4 \pm 0.4	>1000	10.5 \pm 2.4	>1000
Pagoclone	0.9 \pm 0.7	2.8 \pm 1.1	0.7 \pm 0.2	>1000	9.1 \pm 2.4	>1000
(–)-enantiomer	92 \pm 10	N.D.	93 \pm 23	>1000	249 \pm 16	>1000

Values shown are mean \pm S.E.M. ($n = 4$ – 8 separate determinations). N.D., not determined.

^a RP 59037 = racemic pagoclone of which the (+)-enantiomer is pagoclone (also known as RP 62955, CI-1043; IP-456).

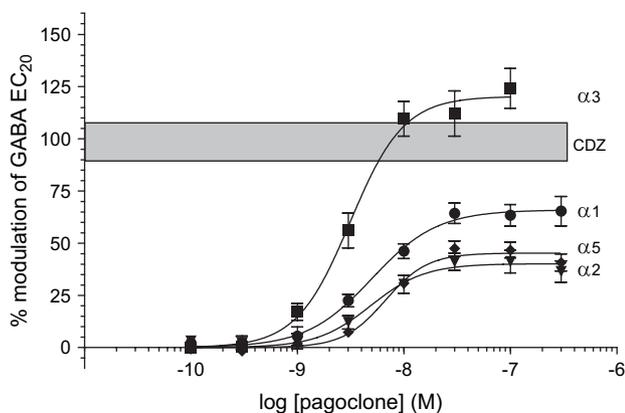


Fig. 2. Efficacy of pagoclone as measured at human recombinant GABA_A receptors containing $\beta 3$ and $\gamma 2$ subunits plus either an $\alpha 1$, $\alpha 2$, $\alpha 3$ or $\alpha 5$ subunit using whole cell patch clamping. Values shown are mean \pm S.E.M. of recordings from 5–6 individual cells. The shaded area represents the range of the % modulation (90–107%) produced by the non-selective full agonist chlordiazepoxide (CDZ) at the different GABA_A receptor subtypes.

Fig. 3C. Thus, there was a dose-dependent increase in occupancy with pagoclone such that doses of 0.3, 1 and 3 mg/kg occupied $35 \pm 4\%$, $52 \pm 5\%$ and $66 \pm 3\%$ of rat brain benzodiazepine binding sites, respectively, to give an ID₅₀ value of 0.9 mg/kg. In contrast, the significant anxiolytic effects of 5 mg/kg i.p. chlordiazepoxide were achieved at a much lower level of receptor occupancy ($23 \pm 3\%$).

The potency of pagoclone for inhibiting in vivo [³H]Ro 15-1788 binding in rat (ID₅₀ = 0.9 mg/kg p.o.) and mouse (0.4 mg/kg p.o.; data not shown) is slightly greater than previous data in which RP 59037 gave ID₅₀ values (following p.o. dosing) in the range of 2.1–6.3 mg/kg for rats and 0.5–2.0 mg/kg for mice (Piot et al., 1990, 1992; Doble et al., 1993). These modest discrepancies are presumably related to the use of the single, active enantiomer in the present study whereas previously the racemate of the active and less active enantiomers (RP 59037) was used (Piot et al., 1990, 1992; Doble et al., 1993).

3.2.2. Pagoclone impairs performance in the rat chain-pulling test

The lack of separation between anxiolytic-like and sedative doses of pagoclone on the elevated plus maze was a surprise given previous reports of a distinct separation (Doble et al.,

1993). Accordingly, we evaluated pagoclone in the rat chain-pulling assay, an operant procedure used previously to determine the extent of behavioural disruption produced by sedative compounds (Bayley et al., 1996).

In Fig. 4, the rate of responding (chain-pulling) in the response sensitivity test is shown for pagoclone in comparison with vehicle and diazepam, expressed either as the cumulative response rate over the 60 min trial period (Fig. 4A) or divided into 10 min intervals (Fig. 4B). In vehicle-treated animals, the overall rate of responding was $77 \pm 7\%$ of baseline (Fig. 4A). The fact that performance was less than 100% is primarily because during the 60 min total trial period performance drops off due to satiation (Fig. 4B). There was a significant effect of treatment on the cumulative chain-pulling response ($F(4,55) = 13.9$, $P < 0.0001$) with the response rate produced by diazepam (10 mg/kg p.o.), $25 \pm 6\%$, being significantly lower ($P < 0.05$, Dunnett's *t*-test) than vehicle as was the response rate of all doses of pagoclone tested, with 1, 3 and 10 mg/kg producing response rates of $54 \pm 8\%$, $40 \pm 6\%$ and $22 \pm 3\%$, respectively. These data clearly demonstrate that pagoclone impaired performance in this task.

3.2.3. Pagoclone is metabolized to 5'-hydroxy pagoclone in vivo

From the rats taken for occupancy measurements following the plus maze experiment, trunk blood was collected and aliquots of plasma and brain homogenates analysed for pagoclone and its major metabolite, 5'-hydroxy pagoclone. These data from a single time point (ca. 40 min post dosing) are summarized in Fig. 5, from which it can be seen that in both brain and plasma there are dose-dependent increases in pagoclone and metabolite concentrations. However, these increases were not linear (i.e., there was not a 10-fold increase in plasma or brain concentrations between the 0.3 and 3 mg/kg doses), but this may be related to the kinetics of absorption and hence a more detailed pharmacokinetic study with multiple time-points would be required to examine this further. The most striking feature of these drug measurements is that in both brain and plasma, the concentration of metabolite exceeds that of parent by 10–20-fold. For example at a dose of 3 mg/kg, the respective concentrations of pagoclone and 5'-hydroxy pagoclone were 2.2 and 26 ng/mL in plasma and 2.5 and 49 ng/g in brain.

3.3. In vitro properties of 5'-hydroxy pagoclone

Given the large excess of 5'-hydroxy pagoclone over pagoclone itself in the pagoclone elevated plus maze experiment, it was important to establish the pharmacological properties of this metabolite since if it possessed sufficient affinity and efficacy for GABA_A receptors, then the majority of the in vivo effects observed after pagoclone administration could be due to the metabolite, rather than the parent compound itself. On the other hand, if the metabolite proved to have minimal affinity and/or efficacy for GABA_A receptors, then it was unlikely to contribute the pharmacological effects of pagoclone.

Table 2
Efficacy of pagoclone at various subtypes of the human GABA_A receptor

	Human recombinant GABA _A receptors containing $\beta 3$, $\gamma 2$ plus			
	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 5$
Max. % modulation ^a	66 ± 6 (6)	42 ± 5 (6)	118 ± 10 (5)	45 ± 4 (6)
EC ₅₀ , nM ^b	4.6	4.9	3.1	6.6

^a Mean \pm S.E.M. of modulation observed in each individual cell (figures in parentheses = *n*).

^b Calculated from the curve fitted through the mean data.

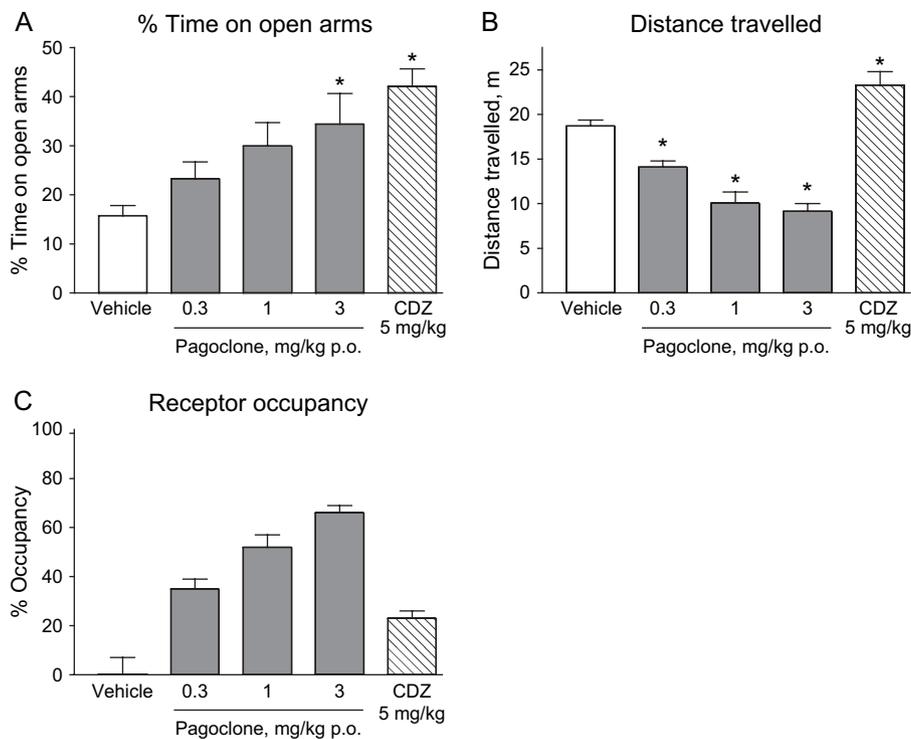


Fig. 3. Performance on the elevated plus maze and benzodiazepine binding site occupancy in rats dosed p.o. with pagoclone. (A) Effects of pagoclone on the % time spent on the open arms of the elevated plus maze expressed as a percentage of the total time (5 min). Pagoclone produced a dose-dependent increase in time spent on the open arms, but this was only significantly different from vehicle (0.5% methyl cellulose) at a dose of 3 mg/kg. The positive control chlordiazepoxide (CDZ, 5 mg/kg i.p.) produced a robust increase in time spent on the open arms. Values shown are mean \pm S.E.M. ($n = 18$ /group). *Significantly different from vehicle using an analysis of variance followed by Dunnett's post hoc t -tests. (B) Total distance travelled during the 5 min plus maze trial. Whereas chlordiazepoxide (CDZ) produced a significant increase in the total distance travelled, all three doses of pagoclone showed a significant decrease. Values shown are mean \pm S.E.M. ($n = 18$ /group). *Significantly different from vehicle using an analysis of variance followed by Dunnett's post hoc t -tests. (C) Occupancy of rat brain GABA_A receptor benzodiazepine binding sites in a subgroup of animals taken after completion of their plus maze trial. Values shown are mean \pm S.E.M. ($n = 7$ – 9 /group).

In order to characterize the metabolite more fully we examined the electrophysiological profile of 5'-hydroxy pagoclone in human L(tk⁻) cell lines expressing various combinations of GABA_A receptor subunits. Table 3 shows that 5'-hydroxy pagoclone binds to recombinant human GABA_A receptors with an affinity (0.6–3.6 nM) marginally higher than pagoclone itself (0.7–9.1 nM; Table 1).

Like pagoclone, 5'-hydroxy pagoclone was a full agonist at the $\alpha 3$ (modulation = $147 \pm 16\%$) and a partial agonist at the $\alpha 2$ and $\alpha 5$ GABA_A receptor subtypes ($46 \pm 7\%$ and $60 \pm 6\%$, respectively; Fig. 6 and Table 3). However, in contrast to pagoclone, which had partial agonist efficacy at the $\alpha 1$ subtype ($66 \pm 6\%$), 5'-hydroxy pagoclone demonstrated very high efficacy at this receptor ($150 \pm 19\%$).

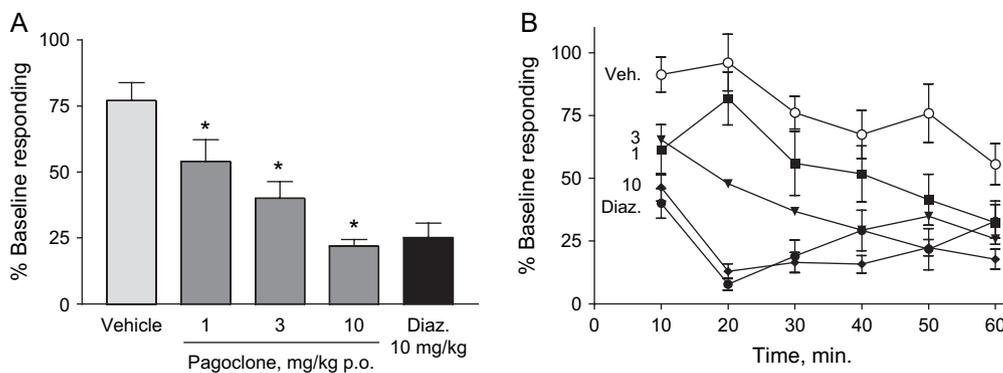


Fig. 4. Effects of pagoclone in the rat chain-pulling assay of sedation. Rats were dosed p.o. with either vehicle (0.5% methyl cellulose), pagoclone (1, 3 or 10 mg/kg) or diazepam (diaz., 10 mg/kg). A. Chain-pulling response rate averaged over the 60 min trial period as expressed as a % of the baseline (i.e., pre-treatment) response rates. (B) Data divided into 10 min time bins. Values shown are mean \pm S.E.M. ($n = 12$ /group). *Significantly different from vehicle using an analysis of variance followed by Dunnett's post hoc t -tests.

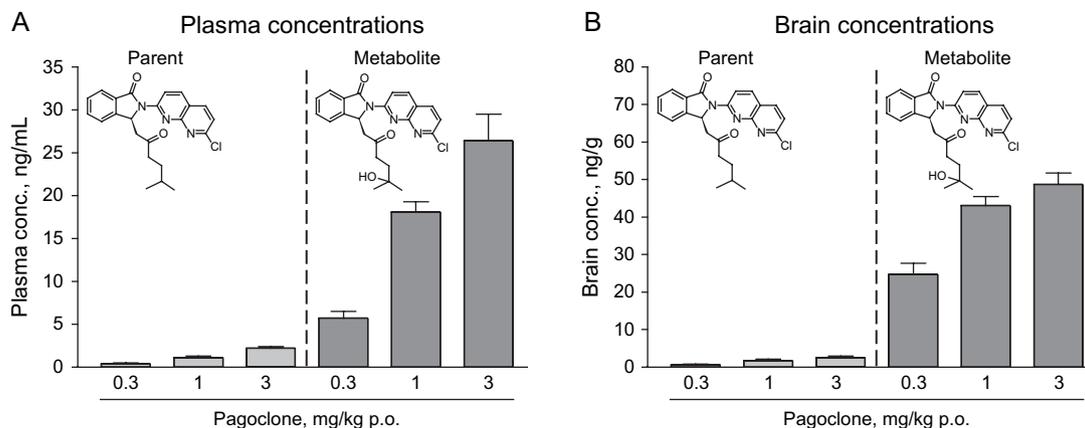


Fig. 5. Concentration of pagoclone and its major metabolite 5'-hydroxy pagoclone in (A) plasma and (B) brain of rats used for occupancy measurements following the elevated plus maze trial (i.e., ca. 40 min post dose). Values shown are mean \pm S.E.M. ($n = 7-9$ /group). Not surprisingly, plasma and brain metabolite concentrations mirrored those of the parent compound, albeit that at each dose, the concentrations of 5'-hydroxy pagoclone were much greater those of pagoclone. Plasma pagoclone (and consequently 5'-hydroxy pagoclone) concentrations were dose-dependent but not linear, with plasma concentrations of pagoclone being 0.4 ± 0.1 , 1.1 ± 0.2 and 2.2 ± 0.2 ng/mL, respectively at 0.3, 1 and 3 mg/kg p.o. doses (corresponding 5'-hydroxy pagoclone concentrations were 5.7 ± 0.8 , 18 ± 1 and 26 ± 3 ng/mL). At each dose, the amounts of pagoclone or 5'-hydroxy pagoclone in the brain (ng/g) were greater than the corresponding levels in the plasma (ng/mL), suggesting that both compounds have good brain penetration (i.e., brain:plasma ratio of >1).

3.4. *In vivo* properties of 5'-hydroxy pagoclone

3.4.1. 5'-Hydroxy pagoclone has anxiolytic-like activity in the rat elevated plus maze

There was a significant effect of treatment on the time spent on the open arms of the elevated plus maze ($F(4,85) = 8.34$, $P < 0.0001$) with all three doses of 5'-hydroxy pagoclone tested (0.3, 1 and 3 mg/kg), significantly ($P < 0.05$, Dunnett's *t*-test) increasing the open arm time ($25 \pm 3\%$, $34 \pm 4\%$ and $40 \pm 5\%$, respectively) relative to vehicle-treated animals ($12 \pm 2\%$; Fig. 7A). Similarly, the positive control chlordiazepoxide also significantly increased the time spent in the open arms ($33 \pm 4\%$). However, there was also an effect of treatment on the total distance travelled ($F(4,85) = 16.1$, $P < 0.0001$) with all doses of 5'-hydroxy pagoclone (0.3, 1

and 3 mg/kg p.o.) reducing, and chlordiazepoxide increasing, the total distance travelled ($P < 0.05$, Dunnett's *t*-test; Fig. 7B), thereby indicating 5'-hydroxy pagoclone produced a sedative-like effect. The levels of receptor occupancy of 5'-hydroxy pagoclone (Fig. 7C) were dose-dependent with doses of 0.3, 1 and 3 mg/kg producing occupancies of $40 \pm 2\%$, $67 \pm 3\%$ and $87 \pm 2\%$, respectively which corresponded to an ID_{50} of 0.5 mg/kg. In this particular experiment, chlordiazepoxide gave $33 \pm 3\%$ occupancy.

3.4.2. 5'-Hydroxy pagoclone and pagoclone reduce locomotor activity in rats

In order to directly compare their sedating properties in the same assay, the effects of pagoclone and 5'-hydroxy pagoclone on spontaneous locomotor activity were measured (Fig. 8). There was a significant effect of treatment on performance in this assay ($F(7,48) = 9.83$, $P < 0.0001$) and both compounds produced dose-dependent decreases in activity that were significantly ($P < 0.05$, Dunnett's *t*-test) different from vehicle-treated animals at all doses tested (0.3, 1 and 3 mg/kg p.o.). Chlordiazepoxide (10 mg/kg i.p.) was included as a positive control and it also produced a significant reduction in locomotor activity over the 20 min period of the trial. This is in marked contrast to a dose of 5 mg/kg i.p. which on the elevated plus maze produced a locomotor-stimulatory effect (Figs. 3 and 7) and aside from the different doses used may, in part, reflect differences in a fear-based (elevated plus maze) versus a more ethological (spontaneous locomotor activity) assay.

The effects of pagoclone and 5'-hydroxy pagoclone on the distance travelled during the elevated plus maze trials (Figs. 3 and 7) as well as locomotor activity (Fig. 8) were plotted as a function of benzodiazepine site occupancy (Fig. 9), the latter of which was derived from the plus maze experiments. These data show that whereas at comparable levels of occupancy, pagoclone appeared more sedating than 5'-hydroxy pagoclone on

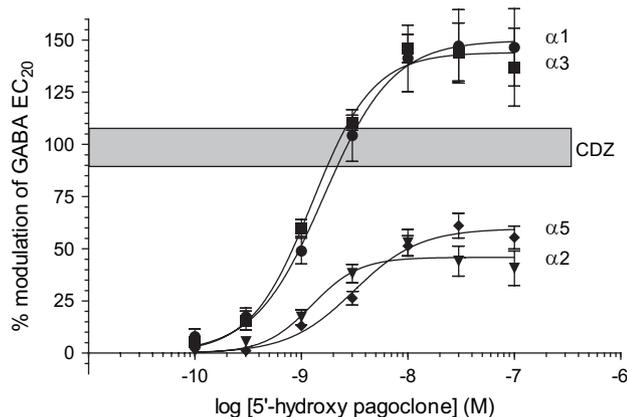


Fig. 6. Efficacy of 5'-hydroxy pagoclone as measured at human recombinant GABA_A receptors containing $\beta 3$ and $\gamma 2$ subunits plus either an $\alpha 1$, $\alpha 2$, $\alpha 3$ or $\alpha 5$ subunit using whole cell patch clamping. Values shown are mean \pm S.E.M. of recordings from 4–8 individual cells. The shaded area represents the range of the % modulation (90–107%) produced by the non-selective full agonist chlordiazepoxide (CDZ) at the different GABA_A receptor subtypes.

Table 3
Binding affinity and efficacy of 5'-hydroxy pagoclone at various subtypes of the human GABA_A receptor

	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 4$	$\alpha 5$	$\alpha 6$
K_i , nM ^a	0.6 ± 0.2	1.1 ± 0.5	0.6 ± 0.2	>1000	3.6 ± 0.6	>1000
Max. % modulation ^b	150 ± 19 (4)	46 ± 7 (6)	147 ± 16 (5)	N/A	60 ± 6 (8)	N/A
EC ₅₀ , nM ^c	1.6	1.2	1.3		3.1	

N/A, not assayed.

^a Mean ± S.E.M. of 5–7 separate determinations.

^b Mean ± S.E.M. of modulation observed in each individual cell (figures in parentheses = *n*).

^c Calculated from the curve fitted through the mean data.

the elevated plus maze, the converse was true in relation to spontaneous locomotor activity. Whether or not these differences reflect inherent intra-assay variability or are due to the different experimental paradigms (for instance, stress-induced locomotor activity on the elevated plus maze compared to a more ethological, spontaneous locomotor activity) these data are not inconsistent with the sedative-like activity observed following pagoclone dosing being due to 5'-hydroxy pagoclone.

4. Discussion

Pagoclone has attracted attention since it is reported to be anxiolytic, in that, unlike existing clinically used

benzodiazepines, there is a considerable separation between the doses required to cause sedation and those required to produce anxiolysis in preclinical species (Piot et al., 1990, 1992; Doble et al., 1993; Kinsora et al., 2000). Such a separation between anxiolysis and sedation was also described as occurring in man in Phase I studies in healthy normal volunteers (Sandford et al., 2001). However, the basis of this novel profile is uncertain since whilst selectivity of pagoclone for GABA_A receptor subtypes associated with anxiolysis rather than sedation would be an obvious explanation (McKernan et al., 2000; Rudolph and Möhler, 2004), no such data has been reported. Consequently, the initial purpose of this study was to define the affinity and efficacy of pagoclone at different GABA_A receptor subtypes.

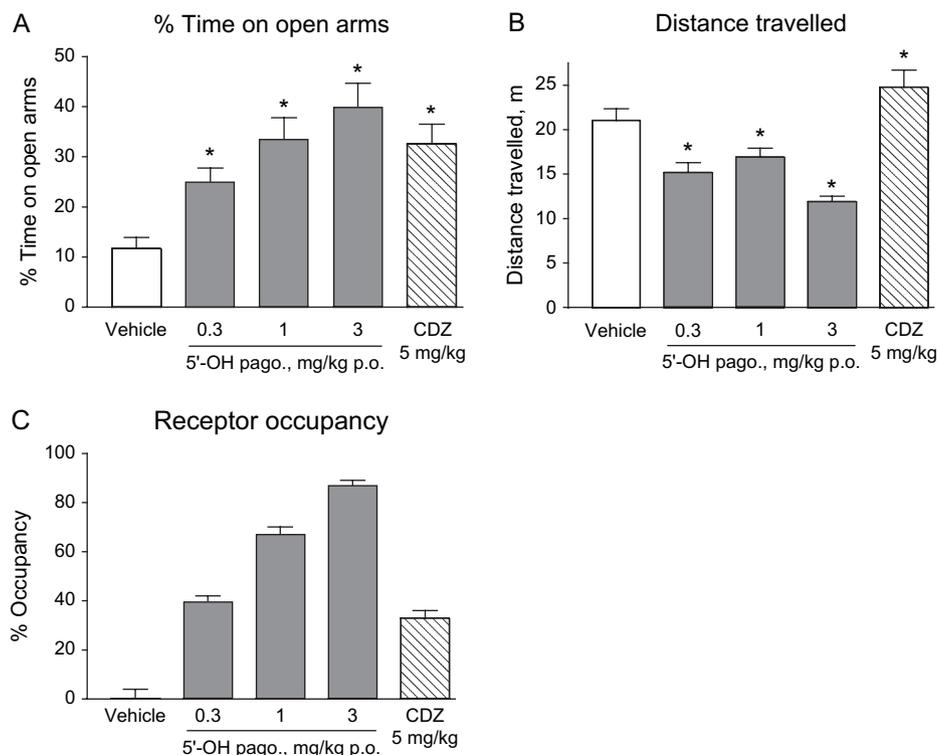


Fig. 7. Performance on the elevated plus maze and benzodiazepine binding site occupancy in rats dosed p.o. with 5'-hydroxy pagoclone (5'-OH pago.). (A) Effects of 5'-hydroxy pagoclone on the % time spent on the open arms of the elevated plus maze expressed as a percentage of the total time (5 min). 5'-Hydroxy pagoclone produced a dose-dependent increase in time spent on the open arms, which was significantly different from vehicle (0.5% methyl cellulose) even at the lowest dose tested (0.3 mg/kg). The positive control chlordiazepoxide (CDZ, 5 mg/kg i.p.) produced a robust increase in time spent on the open arms. Values shown are mean ± S.E.M. (*n* = 18/group). *Significantly different from vehicle using an analysis of variance followed by Dunnett's post hoc *t*-tests. (B) Total distance travelled during the 5 min plus maze trial. Whereas chlordiazepoxide (CDZ) produced a significant increase in the total distance travelled, all three doses of 5'-hydroxy pagoclone showed a significant decrease. Values shown are mean ± S.E.M. (*n* = 18/group). *Significantly different from vehicle using an analysis of variance followed by Dunnett's post hoc *t*-tests. (C) Receptor occupancy of 5'-hydroxy pagoclone in a subgroup of rats taken after completion of their plus maze trial. Values shown are mean ± S.E.M. (*n* = 9/group).

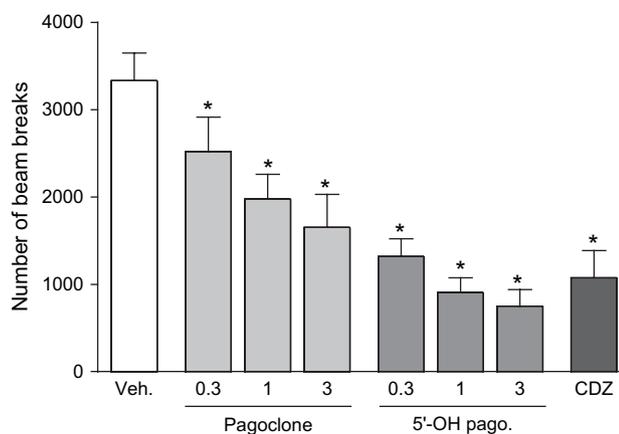


Fig. 8. The effects of pagoclone and 5'-hydroxy pagoclone (5'-OH pagoclone) on spontaneous locomotor activity in rats. Animals received vehicle (0.5% methyl cellulose), various doses (0.3, 1 or 3 mg/kg) of pagoclone or 5'-hydroxy pagoclone or chlordiazepoxide (CDZ; 10 mg/kg i.p.) via the oral (vehicle, pagoclone and 5'-hydroxy pagoclone) or i.p. (chlordiazepoxide) routes. Animals were then placed in a novel environment and activity was measured in terms of the number of infra-red beam breaks over a 20 min period. Values shown are mean \pm S.E.M. ($n = 6-8$ /group). *Significantly different from vehicle using an analysis of variance followed by Dunnett's post hoc t -tests.

4.1. *In vitro* properties of pagoclone

RP 59037 is a cyclopyrrolone racemate which is structurally related to suriclone (Blanchard and Julou, 1983) and zopiclone (Noble et al., 1998). Pagoclone is the (+)-enantiomer of this racemate (Kinsora et al., 2000) with the implicit assumption being that the (+)-enantiomer is pharmacologically more active than the (–)-enantiomer. Accordingly, we observed that pagoclone does indeed possess higher affinity for the benzodiazepine binding site compared with the (–)-enantiomer (Table 1). In addition, RP 59037 appears selective for the benzodiazepine site of GABA_A receptors since it does not

have appreciable affinity for a variety of receptors or uptake sites, including adrenergic, muscarinic, dopamine D2, histamine H1, serotonergic 5-HT₂ or neuropeptide (somatostatin, neurokinin1, neurotensin) receptors or 5-HT reuptake and calcium and sodium channel binding sites (Doble et al., 1993).

With respect to efficacy, previously published data using a variety of *in vitro* binding assays (i.e., [³H]Ro 15-1788, [³H]flunitrazepam or [³⁵S]TBPS) have variously suggested that pagoclone behaves either similar to the full agonist diazepam (Doble et al., 1993), comparable to a partial agonist (Piot et al., 1990, 1992; Doble et al., 1993) or consistent with an inverse agonist (Cordon et al., 2001). Whilst these binding assays can provide useful indices of intrinsic efficacy of GABA_A receptors (Möhler and Richards, 1981; Supavilai and Karobath, 1983; Braestrup et al., 1984), they lack the resolution of electrophysiological methods (Smith and Simpson, 2003) and this may, in part, explain the variability in the published efficacy profile of pagoclone. Consequently, in the present study we characterized the *in vitro* efficacy of pagoclone, as well as its hydroxylated metabolite (see below), using high-resolution patch clamp electrophysiology in cells expressing individual GABA_A receptor subtypes. These data show that although pagoclone does not possess appreciable binding selectivity for the different human recombinant GABA_A receptor subtypes, it does have a degree of efficacy selectivity. Hence, whilst pagoclone is a partial agonist at $\alpha 1$, $\alpha 2$ and $\alpha 5$ subtypes, potentiating GABA EC₂₀-induced currents by 66%, 42% and 45%, respectively, its profile is more like a full agonist at the $\alpha 3$ subtype where it potentiated by 118%. It should be emphasized that this efficacy-selectivity differs from that of efficacy selective compounds such as L-838417 and TPA023 (McKernan et al., 2000; Atack et al., 2006), in that whereas pagoclone (and its metabolite) possess varying degrees of agonism at all four subtype, L-838417 and TPA023 are antagonists at some subtypes and partial agonists at others.

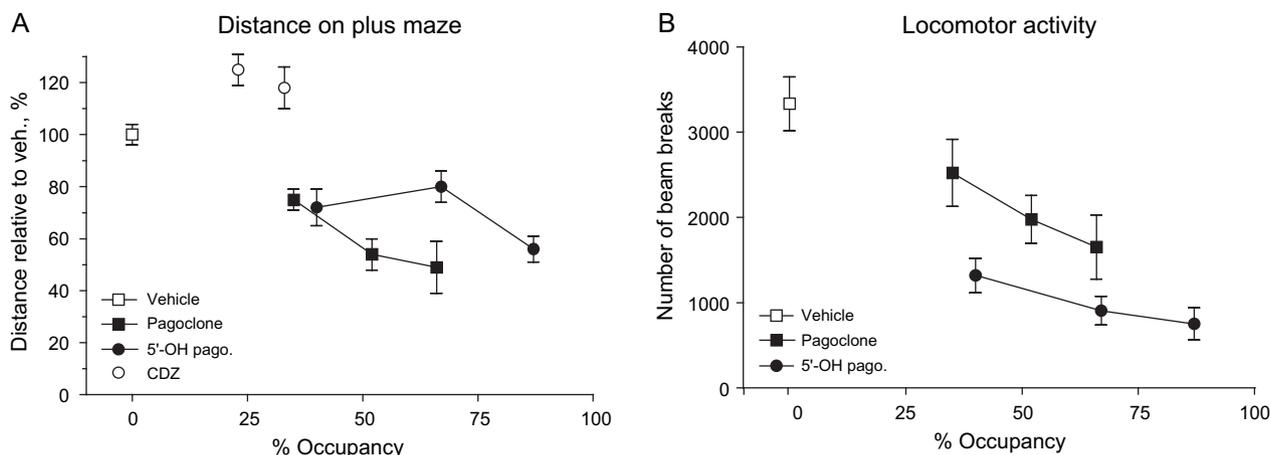


Fig. 9. Impairment of locomotor activity on the elevated plus maze or spontaneous locomotor activity plotted as a function of receptor occupancy. (A) Distance travelled on the elevated plus maze in pagoclone- and 5'-hydroxy pagoclone-treated rats (data derived from Figs. 3 and 7, respectively) plotted versus occupancy measured in the same animals. Since these data are derived from two separate experiments, they have been normalized relative to the distance travelled by vehicle-treated rats (19 ± 1 and 21 ± 1 m in the pagoclone and 5'-hydroxy pagoclone experiments, respectively). Values shown are mean \pm S.E.M. ($n = 18$ /group). (B) The same activity data shown in Fig. 8 plotted as a function of receptor occupancy. Occupancy data was derived from the corresponding elevated plus maze experiments (Figs. 3 and 7). Values shown are mean \pm S.E.M. ($n = 6-8$ /group).

4.2. Effects of pagoclone in vivo are consistent with a partial agonist efficacy

The key characteristic of a partial agonist in vivo is that it requires greater occupancy to produce its effects compared with a full agonist (Haefely et al., 1990; Facklam et al., 1992). Thus, in order to establish the in vivo efficacy profile of pagoclone, it is important to consider the behavioural outcomes in terms of receptor occupancy compared with full agonist comparators, such as diazepam or chlordiazepoxide.

In the present study, whilst there was a clear trend towards anxiolytic-like activity at the lower doses (0.3 and 1 mg/kg, 35% and 52% occupancy, respectively), the minimum significantly effective anxiolytic dose for pagoclone in the elevated plus maze was 3 mg/kg (p.o.), which corresponded to a benzodiazepine site occupancy of 66%. On the other hand, RP 59037 has previously been reported to have a minimum effective dose (MED) on the elevated plus maze after oral dosing of 0.63 mg/kg (Piot et al., 1990, 1992; Doble et al., 1993), which given the occupancy ID₅₀ of 2.1–6.8 mg/kg (Piot et al., 1990, 1992; Doble et al., 1993) would correspond to a predicted occupancy in the region of 10–25%. Due to differences in methodology, it is difficult to compare the discrepancies in anxiolytic behaviour on the plus maze between the present study, which required 62% occupancy and the previous studies requiring only 10–25%; within study comparisons with a positive control are more meaningful. Thus, in the present study, the positive control chlordiazepoxide (which, like diazepam, is a non-selective full agonist) required only 23% occupancy to produce a robust anxiolytic effect (suggesting that at a MED the occupancy for chlordiazepoxide would be appreciably lower than 23%), thus clearly defining pagoclone, which required 62% occupancy, as a partial agonist. In contrast, in the same study in which RP 59037 required a predicted occupancy of around 10–25%, the positive control diazepam (occupancy ID₅₀ = 6.5 mg/kg p.o.) had a MED of 5 mg/kg, which would be equivalent to an occupancy of around 43%; in other words in the elevated plus maze, pagoclone appeared to have greater efficacy than diazepam (Doble et al., 1993). Nevertheless, in a number of other respects, pagoclone behaved like a partial agonist, including anticonvulsant activity and ethanol interaction (Doble et al., 1993). Additional evidence that pagoclone behaves as a partial agonist in vivo comes from observations that even at doses claimed to give near maximal receptor occupancy, RP 59037 produced a much reduced hypothermic response compared with the full agonist suriclone (Jackson et al., 1992).

Finally, pagoclone also appears to have partial agonist activity in man. Hence, in a positron emission tomography study, 0.4 mg pagoclone produced receptor occupancy of 15% whereas the non-selective full agonist lorazepam (1 mg) was associated with 6% occupancy (Lingford-Hughes et al., 2005). Despite pagoclone producing greater occupancy than lorazepam, both drugs caused an equivalent impairment of saccadic eye movements, consistent with pagoclone behaving as a partial agonist in man (Lingford-Hughes et al., 2000). In summary, therefore, the in vivo data would suggest that

following administration of pagoclone, the behavioural effects are consistent with a partial agonist in vivo efficacy.

4.3. Pagoclone causes sedation in rats

The most obvious difference between the present study and previously published data is that whereas previously pagoclone has been reported to give a good separation between anxiolytic and sedative doses (Piot et al., 1990, 1992; Doble et al., 1993; Kinsora et al., 2000), in the present study, no such separation was observed. Thus, at the minimally significantly effective anxiolytic dose on the elevated plus maze (3 mg/kg), as well as at lower doses of 0.3 and 1 mg/kg, there was a significant decrease in the total distance travelled, indicative of a sedative-like effect (Fig. 3). This sedative action of pagoclone was also detected in the rat chain-pulling and spontaneous locomotor assays, in which significant impairments were observed at 1 and 0.3 mg/kg, respectively. In other words, the ratio of the MEDs for pagoclone (the therapeutic index) in the assays of sedation (0.3 mg/kg as judged by distance travelled on the plus maze and spontaneous locomotor activity or 1 mg/kg in the chain-pulling assay) and anxiolysis (3 mg/kg in the elevated plus maze) was 0.1–0.3. Strictly speaking, the doses used in the sedation assays do not define a MED since the no-effect dose was not established. However, evaluating lower doses in the sedation assay would not increase the therapeutic window, they would merely potentially decrease it further.

4.4. The major metabolite of pagoclone, 5'-hydroxy pagoclone, possesses pharmacological activity

The recognition that the metabolite 5'-hydroxy pagoclone occurs in plasma and brain at 10–20-fold higher concentrations than pagoclone (Fig. 5) raises the possibility that it may play a role in the pharmacological actions that occur following pagoclone administration. This metabolite has equivalent or slightly higher affinity than pagoclone not only for recombinant human (Table 3), but also native rat brain GABA_A receptors (affinity of 0.5 to 1.0 nM; Barreau et al., 1996). Moreover, it has intrinsic efficacy comparable to that of pagoclone (α 2 and α 5 partial agonism, α 3 full agonism) with the exception that it has full agonism at the α 1 subtype (150% modulation of a GABA EC₂₀) compared with the partial agonism of pagoclone (66% modulation). Furthermore, 5'-hydroxy pagoclone has good CNS penetration as judged by the fact that it inhibits the in vivo binding of [³H]Ro 15-1788 with an ID₅₀ of 0.5 mg/kg which is comparable to the ID₅₀ seen following pagoclone administration (0.9 mg/kg). Finally, the metabolite was able to produce pharmacological effects in rat comparable to those of pagoclone itself, namely an anxiolytic-like activity on the elevated plus maze which occurred at doses (0.3–3 mg/kg p.o.) similar to those which produced sedation as measured by distance travelled on the elevated plus maze or in a spontaneous locomotor assay (0.3–3 mg/kg). The in vivo benzodiazepine site-mediated GABA_A receptor agonism of 5'-hydroxy pagoclone is consistent with its reported

anticonvulsant activity in the mouse pentylenetetrazole assay (Bareau et al., 1996). Hence, the *in vivo* effects of 5'-hydroxy pagoclone are consistent with this metabolite mediating the pharmacological effects observed in rats following administration of pagoclone.

4.5. Does the intrinsic efficacy of pagoclone and 5'-hydroxy pagoclone explain the *in vivo* effects?

The fact that neither pagoclone nor 5'-hydroxy pagoclone is a full agonist at all four benzodiazepine-sensitive GABA_A receptor subtypes is consistent with the observations that the *in vivo* effects observed after pagoclone administration are generally consistent with partial agonist efficacy (see above). Thus, pagoclone is a full agonist at the $\alpha 3$ and partial agonist at the $\alpha 1$, $\alpha 2$ and $\alpha 5$ subtypes whereas 5'-hydroxy pagoclone is a full agonist at the $\alpha 1$ and $\alpha 3$ subtypes and partial agonist at the $\alpha 2$ and $\alpha 5$ subtypes (Fig. 6). However, most striking is the fact that both pagoclone and its metabolite possess appreciable efficacy at the $\alpha 1$ subtype; indeed, 5'-hydroxy pagoclone appears to have greater $\alpha 1$ efficacy than the full agonist standard chlordiazepoxide. Given that the $\alpha 1$ subtype of GABA_A receptors is clearly associated with sedation (Rudolph et al., 1999; Crestani et al., 2000; McKernan et al., 2000; Johnstone et al., 2004; Rudolph and Möhler, 2004), it is therefore not surprising that, at least in our hands, pagoclone produced sedation in rats with little separation between anxiolytic-like and sedative doses.

4.6. Does 5'-hydroxy pagoclone occur in man?

In man, the only published pharmacokinetic data for pagoclone states that based on an indirect bioassay it has an estimated half-life of around 4 h (Sandford et al., 2001) but no mention is made of its metabolism. Consequently, it is not clear to what extent 5'-hydroxy pagoclone occurs in man although in squirrel monkeys plasma metabolite concentrations were observed to be much greater than those of the parent (unpublished observations), indicating that the production of metabolite is not a rat-specific phenomenon. In addition, the fact that metabolite levels were measured (but not described) in a single (0.4 mg) dose PET study (Lingford-Hughes et al., 2005) suggests that they do occur in man. Furthermore, it was noted that whereas occupancy was related to lorazepam plasma concentrations, this was not the case with pagoclone (Lingford-Hughes et al., 2000, 2005), suggesting that in pagoclone-treated subjects, occupancy is not a function of the concentration of the parent molecule but might be related to the concentration of a metabolite.

In conclusion, following dosing with pagoclone, the 5'-hydroxy metabolite of pagoclone occurs at much higher plasma and brain concentrations than the parent. This metabolite has affinity and brain penetration (occupancy) comparable to pagoclone and its intrinsic efficacy is similar to pagoclone, except that it has greater $\alpha 1$ efficacy. Moreover, when dosed by itself, 5'-hydroxy pagoclone produces anxiolytic and sedative effects similar to pagoclone. Taken together, these data

suggest that the pharmacological effects of pagoclone in rats are due primarily to the metabolite. Whether this same metabolite occurs in man at levels that might explain pagoclone's effects (Sandford et al., 2001) remains to be established.

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