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# The Differential Activities of R(+)- and S(−)-Zacopride as 5-$HT_3$ Receptor Antagonists

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R(+)- and S(−)-zacopride Rat Mouse Marmoset Behaviour Ligand binding

5-HYDROXYTRYPTAMINE$_3$ receptor antagonists from different chemical series demonstrate a common profile of action to antagonise chemo- and radiotherapy-induced emesis, moderate mesolimbic dopamine function, reduce aversive behaviour and the consequences of withdrawal from drugs of abuse [see reviews (2, 18, 19, 29)]. The use of 5-$HT_3$ receptor antagonists such as ICS 205-930, GR65630 and zacopride as tritiated ligands has allowed an identification and localisation of 5-$HT_3$ recognition sites in the central nervous system of various species including man (3, 6, 25, 30), and a functional role for these receptors may include a modulation of the release of acetylcholine or dopamine (4,8).

Zacopride and other agents have so far been used as the racemates, but the recent separation of the R(+)- and S(−)isomers of zacopride (Fig. 1) has permitted a more detailed investigation of the stereospecificity of the interaction between an antagonist and the 5-$HT_3$ receptor. In the present study we compare the activities of the R(+) and S(−)isomers of zacopride in behavioural and biochemical tests that have been used to detect 5-$HT_3$ receptor antagonistic effects.

## METHOD

### *Experimental Animals*

Male albino BKW mice (25–30 g) bred at the University of Bradford, and female ICR-DUB albino mice (17–35 g) obtained from Dominion Labs., Dublin, VA, were housed in groups of 10 and 30 respectively in conditions of constant temperature (22 ± 1°C) and controlled lighting (dark period 07.00–19.00 h, BKW mice; and 17.00–05.00 h, ICR-DUB mice) and given free access to water and fed ad lib on a standard laboratory chow.

Male Lister Hooded rats (250–300 g) and male Sprague-Dawley (CD) Bradford strain rats (275–325 g) were housed in groups of 5 and given free access to standard laboratory chow and water. Rats were kept in conditions of constant temperature (21 ± 1°C) on a 12-h light/dark cycle with lights off at 19.00 h.

Common marmosets *(Callithrix jacchus)*, body weights 315 ± 20 g, of either sex were housed as single sex pairs. They were allowed food (Mazuri primate diet, SDS Ltd., Essex) and water ad lib. Additionally, marmosets received an assortment of fruit, brown bread or malt loaf daily and a vitamin supplement (Duphasol B/602; Duphar Veterinary Ltd., Southampton) weekly in fruit juice. Holding rooms were maintained at 25 ± 1°C at a humidity of 55%. Rooms were illuminated for 12 h with a 12-h dark cycle, lights being on between 07.00 and 19.00 h. Simulated dawn and twilight periods were programmed to occur 0.5 h before and after the main lights came on or went off respectively. During the 12-h dark period a single 60-W red bulb was illuminated to avoid complete darkness.

*Stereotaxic Techniques*

Rats were anaesthetised with chloral hydrate (200 mg/kg SC) and placed in a Kopf stereotaxic frame. Chronically indwelling guide cannulae (constructed of stainless steel tubing 0.65 mm diameter held bilaterally in Perspex holders) were implanted using standard stereotaxic techniques to terminate 3.5 mm above the centre of the nucleus accumbens (Ant. 9.4, Vert. 0.0, Lat.1.6) [atlas of De Groot, (20)]. The guides were kept patent during a 14-day recovery period using stainless steel stylets, 0.3 mm diameter, which extended 0.5 mm beyond the guide tips.

*Intracerebral Injection Technique*

Rats were manually restrained and the stylets removed. Intracerebral injection cannulae, 0.3 mm diameter, were inserted and drugs delivered in a volume of 0.5 μl over 5 s (a further 55 s was allowed for deposition) from Hamilton syringes attached via polythene tubing to the injection units. Animals were used on a single occasion only.

*Intracerebral Infusion Technique*

Fourteen days after the implantation of guide cannulae animals were anaesthetised with fluothane for the subcutaneous implantation in the scapula region of two Alzet osmotic minipumps. Each pump was attached via 40–45 mm Bolab V3 polythene tubing to stainless steel injection units (0.3 mm diameter with a 0.65 mm diameter cuff) which were made to fit permanently into the previously implanted guides in place of the stylets, but terminated bilaterally at the centre of the nucleus accumbens. The pumps had been previously filled with a dopamine solution or its solvent (nitrogen bubbled distilled water containing 0.1% sodium metabisulphite) and the entire injection unit primed overnight at 37°C [see Costall and colleagues, (13)]. The pumps and injection units were carefully designed such that their implantation did not cause any obvious disturbance to the free movement of the animals. The pumps delivered dopamine or its solvent at a constant rate of 0.48 μl/h from the time of implantation and, although the pumps were designed to deliver solution for 14 days, removal on day 13 precluded any 'fall-off' effect.

*Histology*

On completion of the experiments rats were anaesthetised and decapitated and the brains removed and fixed in formal saline. Brains were frozen and sectioned on a freezing microtome and the sites of drug or vehicle deposition readily identified from the point of termination of the injection cannulae tracks and from the discrete location of the oxidative products for dopamine. The locations for the injections and infusions were found to be within the defined area and indistinguishable from those previously reported (16).

*Behavioural Tests*

*Light/dark exploration test in mice.* Behaviour of male albino BKW mice and female ICR-DUB albino mice was measured using two different procedures. The measurement of behaviour in the male albino mice was undertaken in an open-topped box, 45 cm long, 27 cm wide and 27 cm high, divided into a small (2/5) area and a large (3/5) area by a partition that extended 20 cm above the walls. There was a $7.5 \times 7.5$ cm opening in the centre of the partition at floor level. The small compartment was painted black and the large compartment white and the floors were marked into 9 cm squares. The white compartment was illuminated by a 60-W tungsten bulb (400 lux) 17 cm above the box and the black compartment by a similarly placed 60-W (0 lux) red bulb. The laboratory was illuminated by red light.

All tests were performed between 13.00 and 18.00 h. Mice were taken in groups of 10 from the holding room during the dark phase of the cycle in a darkened container to the test room illuminated with red light. After a 1-h period of adaptation to the test room, mice received R(+)- or S(−)-zacopride or vehicle (distilled water) treatment IP and, after a 40-min pretreatment period, each mouse was tested by placing it in the centre of the white area of the test box and allowing it to explore the novel environment for 5 min. Its behaviour was recorded on videotape and the behavioural analysis was performed subsequently from the recording. Five parameters were measured: the latency of the initial entry into the dark compartment, the time spent in each area, the number of transitions between compartments, the number of lines crossed in each compartment and the number of rears in each compartment [see Costall and colleagues (17) for detailed methodology]. Since increases or decreases in line crossings and rears occurred concomitant to increases or decreases in the time spent in the two areas, only the % time spent in each area, and the latency of the initial movement from the white to the black compartment are reported. Mice were used once only in treatment groups of five. Results were analysed using single-factor analysis of variance and, where appropriate, followed by Dunnett's procedure for comparing all treatments with control.

The measurement of behaviour in female ICR-DUB albino mice was performed in a sound-attenuated room illuminated with a 25-watt red bulb. Behavioural testing was conducted with three two-compartment automated chambers (Digiscan Model RXYZCM16, Omnitech Electronics, Inc., Columbus, OH). The lit side, illuminated by a high-intensity 75-watt light (260 lux) was separated from the dark side (0 lux, black Plexiglas box) by a $7.5 \times 7.5$ cm passageway.

At approximately 09.00 h mice were taken from the holding room and placed into individual holding cages in the experimental room for a 4-h period. Starting at approximately 13.00 h, mice (7 animals per treatment group) received either an IP or PO dose of either vehicle (0.5% aqueous methylcellulose), R(+)-zacopride, or S(−)-zacopride. Thirty minutes later an animal was placed at the centre of the lit area and behavioural activity was tallied over a 5-min period by the Digiscan Analyser. Eight behavioural measures were recorded: the time spent in the lit and dark areas, locomotor activity counts in each area, locomotor time in each area, number of rearings in each area, rearing time in each area, number of transitions between the lit and dark or dark and lit areas, latency to make the first transition from the lit area to the dark area, and resting time in each area. Previous findings with reference anxiolytic agents have indicated that the time mice spend in the lit area provides the most consistent measure of drug activity (31). Therefore, this data is presented in the present study; statistical analyses were performed using Dunnett's *t*-test.

*Social Interaction Test in Rats*

Tests were conducted between 13.00 and 18.00 h in an illuminated room using a methodology based on the model of File (22). The apparatus used for the detection of changes in social interaction and exploratory behaviour in Lister-Hooded rats consisted of an open-topped box ($51 \times 51$ cm and 20 cm high) with $17 \times 17$ cm areas marked on the floor. Two naïve rats, from separate housing cages, received an intraperitoneal injection of drug or vehicle (40-min pretreatment) and were placed into the box brightly illuminated and their behaviour observed over a 10-min

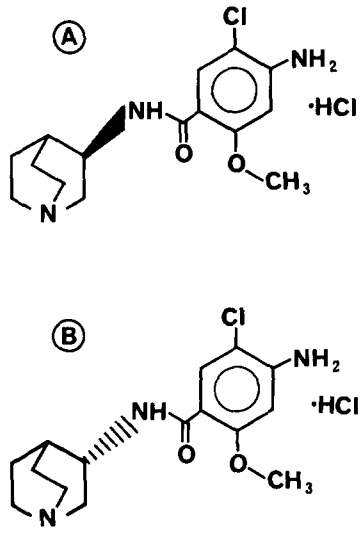


FIG. 1. The structures of (A) R(+)- and (B) S(−)-4-amino-N-(1-azabicyclo[2.2.2]oct-3-yl)-5-chloro-2-methoxybenzamide monohydrochloride (zacopride).

period by remote video recording. Two behaviours were noted, 1) social interaction between the animals was determined by timing (seconds), sniffing of partner, crawling under or climbing over partner, genital investigation of partner, following partner and 2) exploratory locomotion was measured as the number of crossings of the lines marked on the floor of the test box. Rats were used once only in treatment groups of 6 or 12 pairs and data analysed using single factor analysis of variance followed by Dunnett's *t*-test.

*Antagonism of the Hyperactivity Induced by the Injection of d-Amphetamine or Infusion of Dopamine Into the Rat Nucleus Accumbens*

Experiments were conducted between 07.30 and 12.00 h in a quiet room maintained at 22±2°C. Rats were taken from the holding room and allowed 1 h to adapt to the new environment. Locomotor activity was assessed in individual screened Perspex cages (25×15×15 cm high) (banked in groups of 30) each fitted with one photocell unit along the longer axis 3.5 cm from the side; this position has been found to minimise spurious activity counts due, for example, to preening and head movements when the animal is stationary. Interruptions of the light beam were recorded every 5 min. At this time animals were also observed for the presence of any nonspecific change in locomotor activity, e.g., sedation, prostration, stereotyped movements, that could interfere with the recording of locomotor activity. In the dopamine infusion studies the drugs were administered twice daily (at 08.00 and 20.00 h) intraperitoneally and behavioural measurements taken between 10.00 and 12.00 h. Animals were used in treatment groups of 5 and used once only (amphetamine studies) or daily (dopamine infusion studies) and data analysed by single factor analysis of variance followed by Dunnett's *t*-test.

*Influence on Behaviour of the Marmoset Exposed to a Human Threat Situation*

Tests were conducted between 13.30–15.30 h in the normal holding room (to avoid unwanted disruption of behaviour by

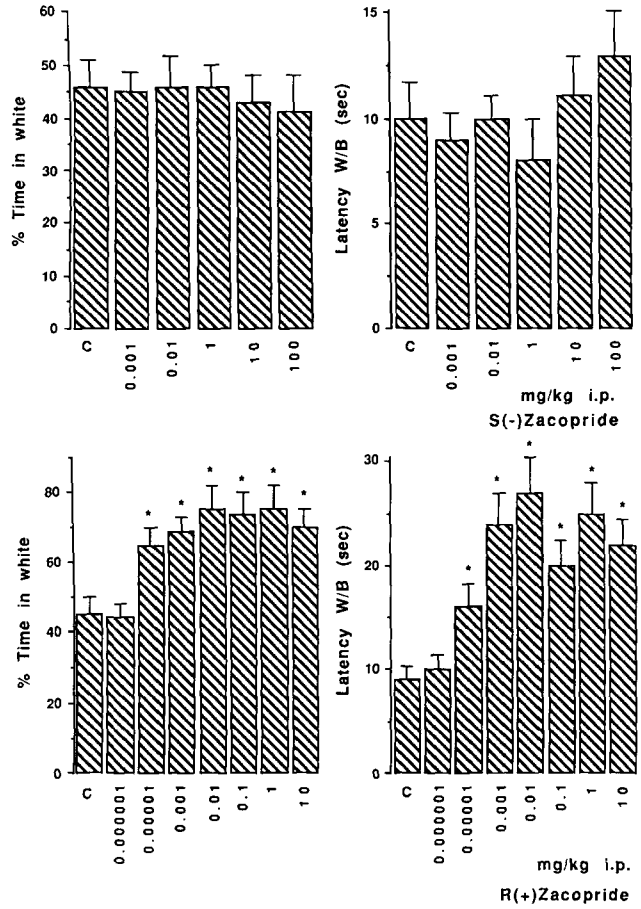


FIG. 2. The effect of R(+)- and S(−)-zacopride on the behaviour of albino BKW mice in the black and white test box. Mice received the injection of drug or vehicle (Control, C) and were placed into the centre of the white area illuminated with white light (60 W), the black area was illuminated with red light (60 W). From behavioural measurements made from remote video recordings taken over a 5-min period, the latency of the first movement from the white (W) to the black (B) section and the % time spent in the black area are presented. Values represent the means±S.E.M.s of 10–15 determinations. Significant increases or decreases in responding compared to controls are indicated as $*p<0.05$–0.001 (one-way ANOVA followed by Dunnett's *t*-test).

movement to a novel room or cage). The holding cages measured 75 cm high, 50 cm wide and 60 cm deep. A behavioural change characterised by retreat from and posturing towards a human threat (a behaviour sensitive to known anxiolytic agents) was initiated by a human observer standing in close proximity in front of the holding cage. Changes in behaviour were recorded over a 2-min period by the observer. The behavioural measures selected for the present study were 1) the % of time spent on the cage front in direct confrontation with the human threat and 2) the number of body postures, primarily shown as raising of the tail to expose the genital region with varying degrees of body piloerection, anal scent marking and slit stare with flattened ear tufts [see Costall and colleagues, (12)].

Twelve marmosets were used at 7-day intervals throughout the study and were subject to a random crossover of acute subcutaneous treatments (2/3 doses of a drug and vehicle control) and assessed after 45 min. Data obtained was analysed using a paired *t*-test.

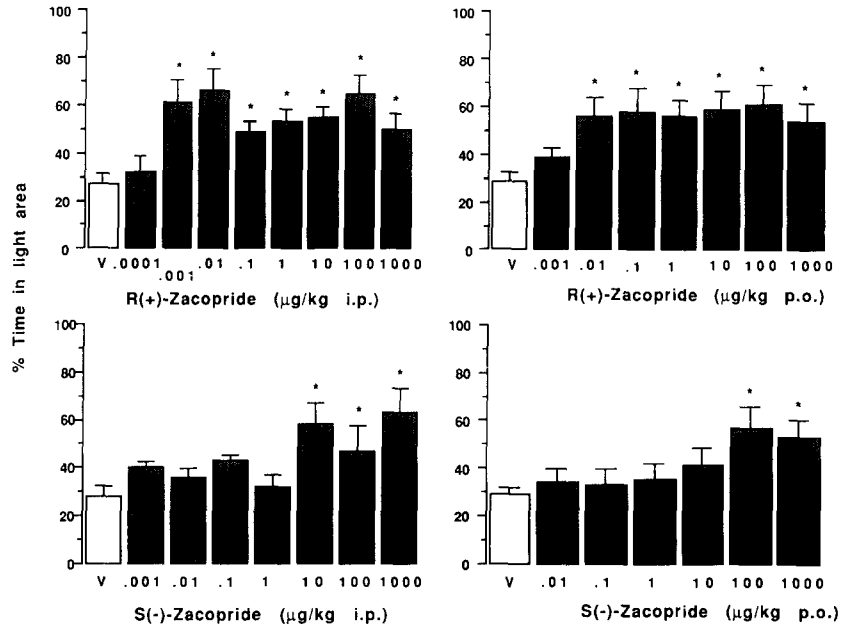


FIG. 3. The effect of R(+)- and S(−)-zacopride on the behaviour of ICR-DUB female albino mice in a two-compartment light and dark automated Digiscan chamber. Mice received either an intraperitoneal (IP) or oral (PO) injection of drug or vehicle (V) and after 30 min were placed into the centre of the brightly illuminated compartment. Behavioural activity was measured by the Digiscan analyser over a 5-min period and the % time spent in the light area presented. Each value is the mean of 7 determinations; a significant increase in response compared to control is indicated $*p<0.05$ (Dunnett's $t$-test).

*Influence on Behaviour of the Mouse in an Habituation Test*

Testing was carried out daily between 08.30 and 12.30 h. Male albino BKW mice were taken from the dark home environment in a dark container to the experimental room maintained in low red lighting and placed into the centre of the white section of the white and black test box (see above). Behaviour was assessed via remote video-recording. A naive mouse moved within 10 to 12 s into the black section of the test box [see Barnes and colleagues, (5)]. On daily testing animals habituated to the test system, moving by the 5th day within 2 to 4 s into the black section. Mice received twice daily IP injections of vehicle or drug treatments (at 08.00 and 20.00 h) and the animals tested between 10.00 and 12.00 h, the habituation profile being recorded over a 10-day period. The ability of the drug treatments to antagonise a scopolamine impairment was also assessed in some animals on the 6th day of testing. Data obtained was analysed using a two-way analysis of variance followed by Dunnett's $t$-test.

At all times the behaviour of mice, rats and marmosets was routinely assessed for the presence of behaviours that would non-specifically interfere with the expression of aversive behaviour and its inhibition, e.g., stereotyped movements, gross excitation, seizures or sedation.

*Biochemical Studies*

*Radioligand binding experiments.* Tissue from the entorhinal cortex of male Hooded-Lister rats (200–250 g) and male albino BKW mice (35–45 g) was dissected out on ice and pooled (approx. 70 mg/rat, 30 mg/mouse) and homogenised (Polytron, setting 7 for 10 s) in 20 volumes of 50 mM Hepes buffer containing all the constituents of Krebs (NaCl 118.0, KCl 4.75, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, glucose 11.0 mM) with a final pH of 7.4. The homogenate was centrifuged at 48,000 × g for 10 min at 4°C and the pellet was then resuspended and again centrifuged. The pellet was finally resuspended in the Hepes/Krebs buffer at a concentration of 0.2–0.3 mg protein/ml. Protein estimation was performed using the Bio-Rad Coomassie blue method using bovine serum albumin as the standard [Bradford (9)]. Assays were always performed on fresh tissue and carried out in replicates of at least three.

Six hundred and fifty μl of displacing drug or buffer (Hepes/Krebs) was added to assay tubes followed by 100 μl [$^3$H]R(+)/S(−)-zacopride (54.9 Ci/mmol, N.E.N.), [$^3$H]R(+)-zacopride (83 Ci/mmol, Amersham) and [$^3$H]S(−)-zacopride (83 Ci/mmol, Amersham) in Hepes/Krebs (final concentration 0.25–0.50 nM for displacement studies or a range of concentrations from 0.1–7.5 nM for saturation studies). Two hundred and fifty μl of the brain tissue homogenate was added to initiate binding. The assay tubes were incubated for 20 min at 37°C, the incubation being terminated by rapid filtration through prewet Whatman GF/B filters which were immediately washed with 9.6 ml of ice-cold Hepes/Krebs buffer (wash time 8 s). Each assay was completed within 60 min of the homogenate preparation.

The filter discs were placed in 10 ml of 'Insta-gel' scintillant, left for dark adaptation for 6 h and radioactivity assayed by liquid scintillation counting. Results are the means ± S.E.M. of at least three separate experiments.

*[$^3$H]Acetylcholine Release in Cortical Tissue*

The method of Barnes and colleagues (4) was used to study

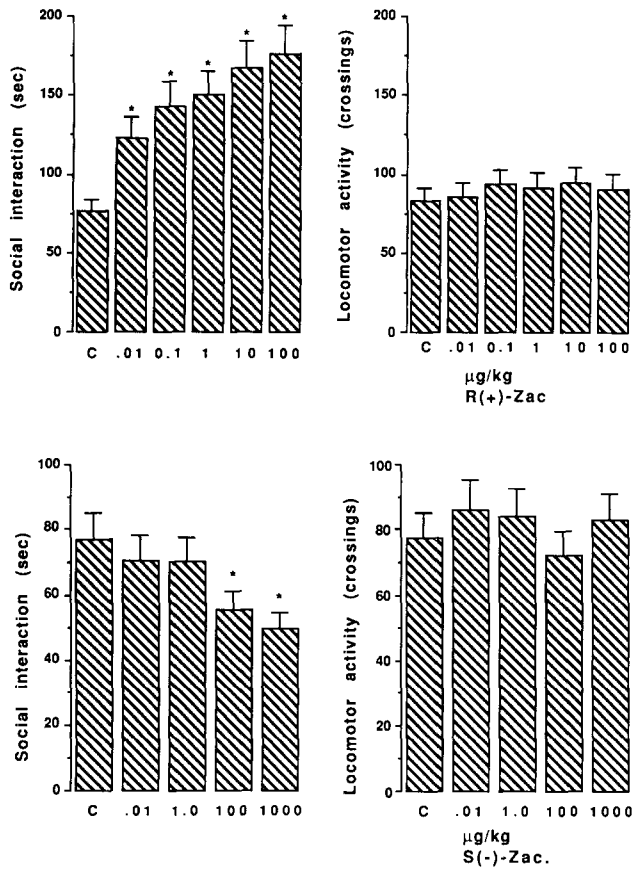


FIG. 4. The effects of R(+)- and S(−)-zacopride administered intraperitoneally in the social interaction test in the rat. The time spent in active social interaction between pairs of rats was recorded during a 10-min period under the experimental conditions of high light and unfamiliarity of rats with each other. Crossings of lines marked on the test box floor were also noted. Data obtained in control (C) and drug-treated rats was analysed by single-factor analysis of variance followed by Dunnett's *t*-test. n = 6–12 pairs. S.E.M.s given. Significant increases/decreases in responding are indicated (*$p<0.05$–$p<0.01$).

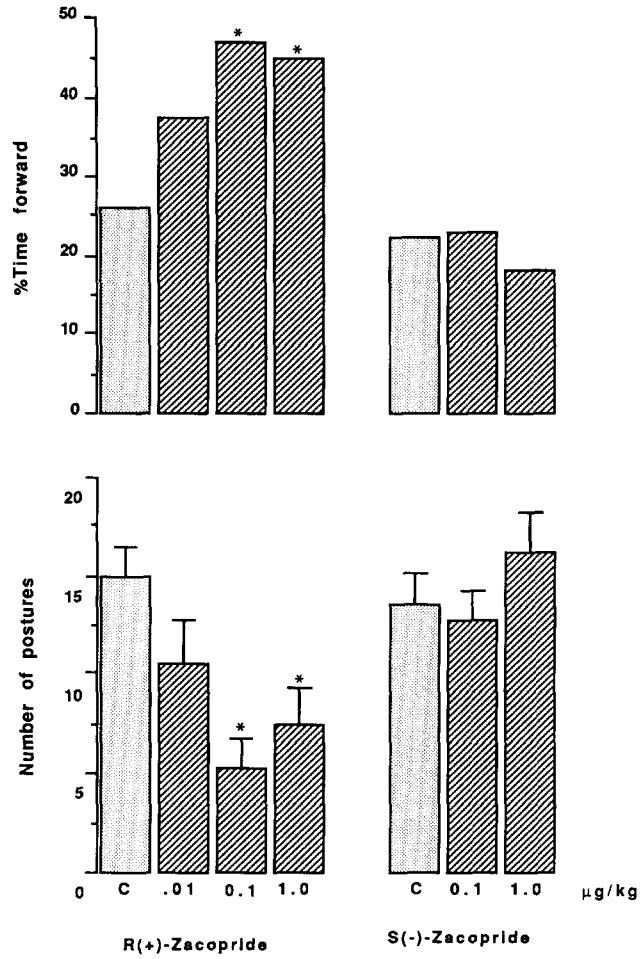


FIG. 5. Effect of R(+)- and S(−)-zacopride on the behavioural changes provoked by a human threat in the common marmoset. Behavioural changes were measured over a 2-min period. The figure shows changes in posturing (S.E.M.s given), and % time spent on the cage front. n = 4. Data obtained for vehicle-treated control (C) and drug-treated animals was analysed by paired *t*-test. Significant increases/decreases in responding are indicated *$p<0.05$.

drug interaction at the 5-$HT_3$ receptor to modify potassium induced release of [$^3$H]acetylcholine. Briefly, the entorhinal cortex from female hooded Lister rats (250–300 g, Bradford bred) was cross-chopped at 90° (McIlwain tissue chopper) to produce slices 0.35 × 0.35 mm × thickness of cortical ribbon. Tissue was obtained from 20 rats for each experiment and each chamber contained tissue from a single rat. The cut tissue was washed three times in gassed (95% $O_2$/5% $CO_2$) Krebs solution (NaCl 118.0, KCl 4.75, $KH_2PO_4$ 1.2, $MgSO_4$ 0.7, $H_2O$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, glucose 11.0 mM). The Krebs solution was then replaced by one containing 39.75 mM KCl (tonicity was maintained by reducing the NaCl concentration) and the tubes shaken at 37°C for 20 min. After being washed the slices were incubated at 37°C for 40 min in normal Krebs buffer containing [$^3$H]choline, 0.1 µM (15 Ci/mmol, Amersham). Two hundred µl of washed settled slices were placed into each of the 20 superfusion baths and superfused with Krebs solution (0.5 ml/min) containing 1.0 µM hemicholinium-3 and ritanserin (1.0 µM) at 37°C. After a 30-min washout period, 4-min samples of perfusate were collected. At 12 min ($S_1$) and 48 min ($S_2$) the slices were depolarised by changing the superfusion fluid for 4 min to a Krebs solution containing 20 mM KCl. Test drugs were added 20 min before, during and after the $S_2$ stimulation, and the $S_2/S_1$ ratio was subsequently calculated. The superfusate samples were collected for a total of 80 min. Tritium content was assayed by liquid scintillation spectroscopy (Tri-Carb 1900CA, efficiency 47%). The evoked release of radioactivity was calculated as the difference between potassium-stimulated and basal release. On completion of the experiment the tritium remaining in the slices was determined. The disintegration per min, per 4-min collection period, was converted to fractional release by dividing by the total amount of radioactivity present in the tissue at the end of each 4-min collection period.

*Drugs*

The R(+)- and S(−)-isomers of zacopride [(R)/(S)-4-amino-N-(1-azabicyclo[2.2.2.]oct-3-yl)-5-chloro-2-methoxybenzamide·HCl] were prepared by the Laboratoires Delalande and by

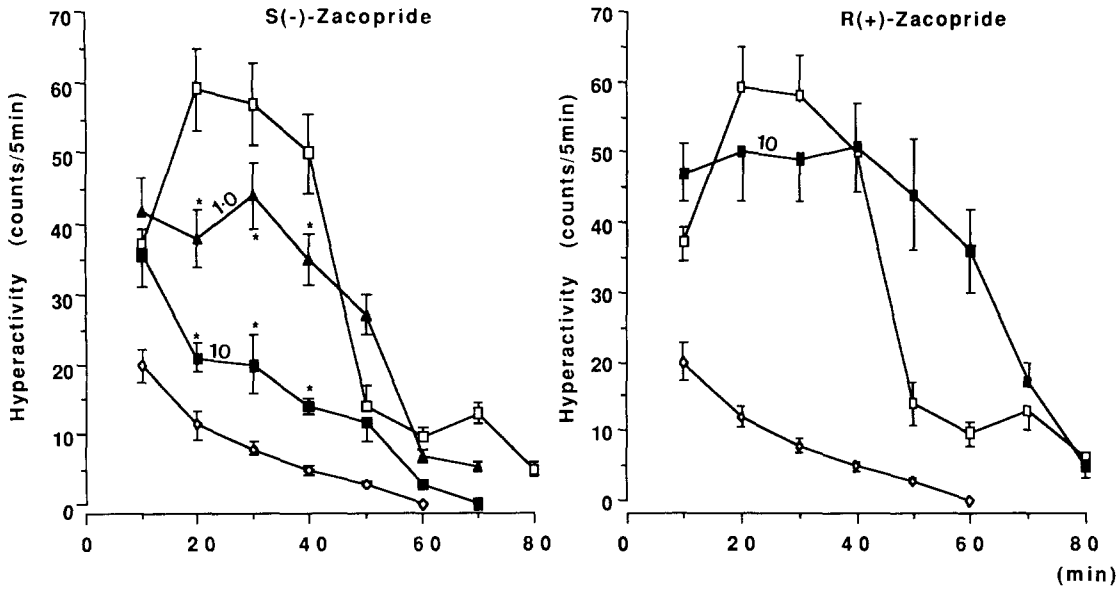


FIG. 6. Abilities of S(−)- and R(+)-zacopride to modify the hyperactivity caused by the bilateral injection of amphetamine (10 μg) into the nucleus accumbens of the rat. The control response of animals receiving amphetamine alone is indicated (□), those receiving vehicle alone (◇). Data are given for R(+)- (▲ 1 ng, ■ 10 ng) and S(−)-zacopride (■ 10 ng) administered as 30-min pretreatments into the nucleus accumbens before amphetamine injection. n = 5. Vertical lines indicate S.E. means. Significant reductions or increases in the amphetamine response are indicated as $*p<0.01$–$0.001$ (one-way analysis of variance followed by Dunnett's $t$-test).

the Chemical Research Department at A.H. Robins using the methodology of Dorme and colleagues (1988; European Patent 208603A). The enantiomeric purity (>99%) of each isomer was established by standard analytical method for chiral compounds and by the HPLC method of Demian and Gripshover (21) for diastereomeric derivatives of chiral 3-aminoquinuclidines. (+)Amphetamine·$SO_4$ and scopolamine hydrobromide from Sigma, and BRL43694 [endo-N-(9-methyl-9-azabicyclo-[3,3,1]- non-3-yl)- 1-methyl-1H-indazol-3-carboxamide·HCl] and 2-methyl-5-hydroxytryptamine·HCl were gifts from Glaxo Group Research. All doses are expressed as the base and were administered in a volume of 1 ml/kg (rat) or 1 ml/100 g (mouse).

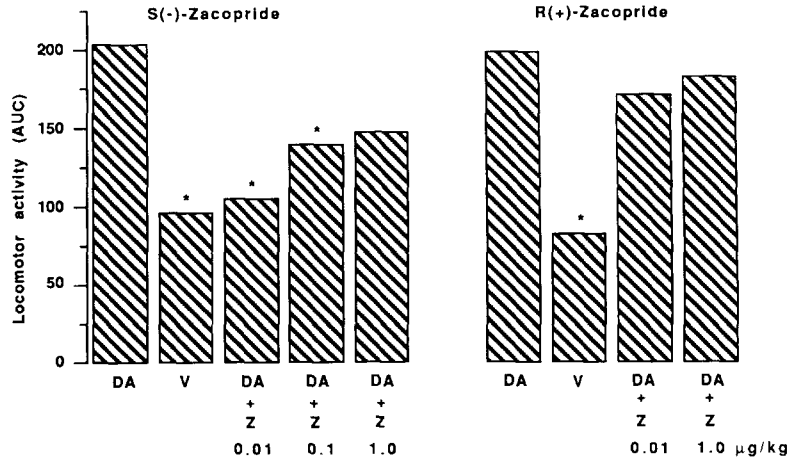


FIG. 7. Abilities of S(−)- and R(+)-zacopride to modify the locomotor hyperactivity of rats during the 13 days of dopamine infusion (25 μg/24 hr) into the nucleus accumbens. The hyperactivity responses of animals were calculated and expressed as areas under the 13-day hyperactivity curves (AUC) of animals receiving dopamine (DA), vehicle (V) or dopamine plus R(+)- or S(−)-zacopride (DA + Z, μg/kg IP b.i.d.). n = 5, significant decreases in locomotor activity to below DA control values are indicated $*p<0.05$ (two-way analysis of variance followed by Dunnetts $t$-test).

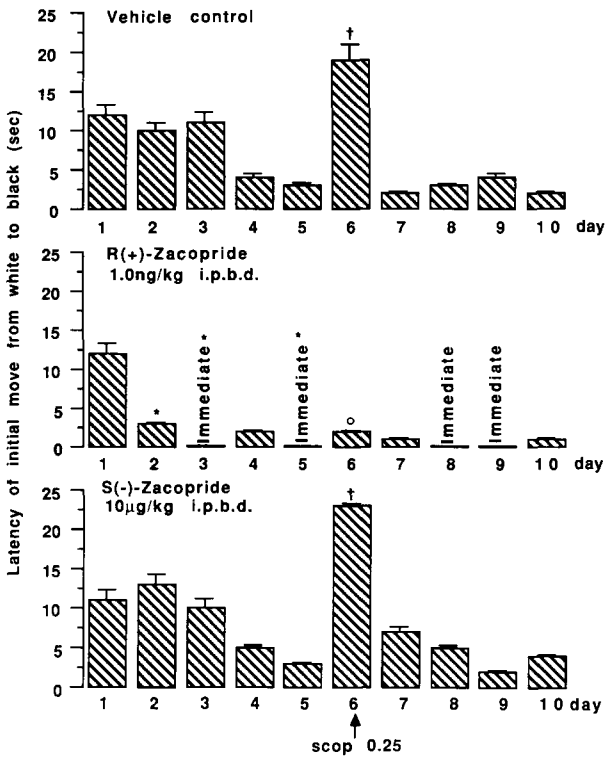


FIG. 8. The influence of R(+)- and S(−)-zacopride to modify mouse habituation responding in a black and white test box, and a scopolamine-induced impairment. Mice received injections of vehicle (control) or R(+)- and S(−)-zacopride (1.0 ng and 10 μg/kg IP b.i.d. respectively). Animals receiving vehicle or drug injections were challenged with a single treatment of scopolamine (Scop. 0.25 mg/kg IP) on the 6th day (↑). Significant differences in the latency of initial movement from the white to the black area between drug treatments with R(+)- and S(−)-zacopride compared to control values are indicated *$p<0.001$ (Dunnett's $t$-test); a significant increase in the latency of movement induced by scopolamine relative to vehicle controls is indicated †$p<0.05$; a significant inhibition of the scopolamine induced impairment is indicated °$p<0.001$ (Dunnett's $t$-test).

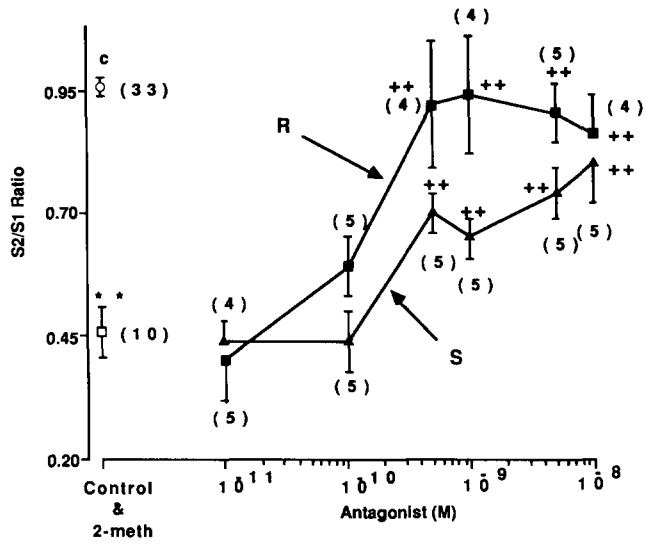


FIG. 9. Inhibition of potassium stimulated [$^3$H]acetylcholine release from the rat entorhinal cortex by 2-methyl-5-hydroxytryptamine and antagonism by R(+)- and S(−)-zacopride. The control potassium- (20 mM) induced [$^3$H]acetylcholine release in the absence of drug treatment is indicated ○ (C) and the inhibition afforded by 2-methyl-5-hydroxytryptamine (2.0 μM) (2-meth) is indicated □ (**$p<0.01$, ANOVA followed by Dunnett's $t$-test). The significance of the antagonism by R(+)-zacopride (■) and S(−)-zacopride (▲) of the inhibitory effect of 2-methyl-5-hydroxytryptamine was assessed using a two-way analysis of variance followed by Dunnett's $t$-test. Each value is the mean ± S.E.M. of (n) determinations. ANOVA $p<0.05$; $^{++}p<0.01$ indicates a significant difference to the value obtained using 2-methyl-5-hydroxytryptamine administered alone.

## RESULTS

### *Behavioural Tests*

*The influence of R(+)- and S(−)-zacopride on mouse behaviour in the light-dark exploration test.* In the black and white test box R(+)-zacopride (0.01 μg–10 mg/kg IP) antagonised the aversive response of male albino BKW mice to the white area, increasing the latency of the initial movement from the white to the black area and increasing the time spent in the white area (Fig. 2). The administration of S(−)-zacopride (0.001 to 100 mg/kg IP) failed to modify mouse behaviour in the black and white test box (Fig. 2).

Using the automated chambers R(+)-zacopride administered orally (0.01–1000 μg/kg) or intraperitoneally (0.001–1000 μg/kg) increased the proportion of time female ICR-DUB mice spent in the white compartment. Mice appeared slightly more sensitive to the effects of R(+)zacopride under these conditions of assessment than using the nonautomated method, responding to doses of 0.001 and 0.01 μg/kg IP respectively. Further, the use of ICR-DUB mice in the automated methodology revealed the ability of S(−)zacopride at ten thousand-fold the dose of R(+)-zacopride to increase the proportion of time that mice spent in the white compartment (Fig. 3).

*The influence of R(+) and S(−)-zacopride on rat social interaction.* R(+)-Zacopride (0.01–100 μg/kg PO) caused a dose-related increase in social interaction with no effect on locomotor activity. In contrast, S(−)-zacopride (0.01 and 1.0 μg/kg PO) failed to increase social interaction and higher doses of 0.1 and 1.0 mg/kg PO caused a small decrease. Similarly to R(+)-zacopride, the S(−)-isomer failed to modify locomotor activity (Fig. 4).

*The influence of R(+)- and S(−)-zacopride on the behaviour of the marmoset exposed to a human threat situation.* In response to a human threat the marmoset retreated from the front of the cage and spent only approximately 20% of its time towards the cage front; during a 2-min observation period animals displayed about 15 postures. Animals treated with R(+)-zacopride (0.01 μg/kg) showed a trend to a reduced number of postures and an increased time spent forward. This achieved significance using treatments with R(+)-zacopride (0.1 and 1 μg/kg) which markedly attenuated the retreat from the cage front and reduced the posturing by some 60%. The administration of S(−)-zacopride (0.1 and 1.0 μg/kg) failed to modify the response of the marmoset to a human threat (Fig. 5).

*The influence of R(+)- and S(−)-zacopride on the hyperactivity induced by the injection of amphetamine into the rat nucleus accumbens.* The bilateral injection of amphetamine (20 μg) into the nucleus accumbens caused a hyperactivity response which peaked within 20 to 30 min of administration. Pretreatment with an injection of S(−)-zacopride (1 and 10 ng) into the nucleus accumbens caused dose-related reductions in the amphetamine response. Pretreatment with R(+)-zacopride 1 ng (results not shown)

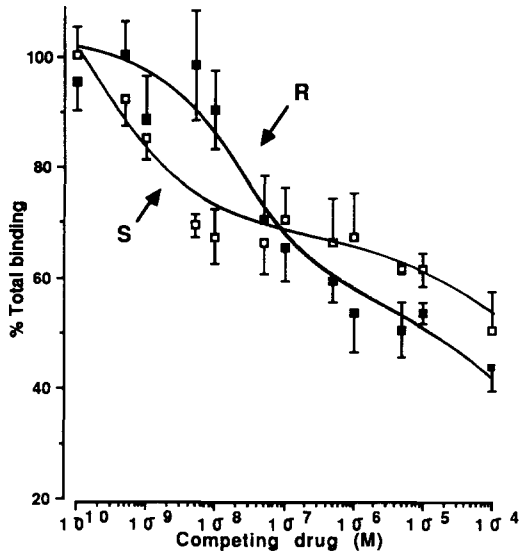


FIG. 10. The ability of R(+)-zacopride (■) and S(−)-zacopride (□) to compete for [$^3$H]R(+)/S(−)-zacopride binding (0.25–0.50 nM) in homogenates of rat entorhinal cortex. Results are the means ± S.E.M. of 3 separate experiments.

and 10 ng failed to reduce the amphetamine induced hyperactivity, response (Fig. 6).

*The influence of R(+)- and S(−)-zacopride on the hyperactivity induced by the infusion of dopamine into the rat nucleus accumbens.* The bilateral infusion of dopamine (25 μg/24 h) into the nucleus accumbens for 12 days caused biphasic peaks of activity on days 4 and 10 [see Costall and colleagues (13)]. The twice daily IP administration of S(−)-zacopride (0.01 μg/kg) during the period of dopamine infusion markedly reduced the hyperactivity response. However, the reduction in hyperactivity caused by the use of higher doses (0.1 and 1.0 μg/kg b.i.d.) was associated with periods of breakthrough hyperactivity, lessening the antagonism which failed to achieve significance at 1.0 μg/kg b.i.d. The administration of R(+)-zacopride (0.01 and 1.0 μg/kg b.i.d.) failed to modify the dopamine-induced hyperactivity response (Fig. 7).

*The influence of R(+)- and S(−)-zacopride on the habituation pattern of mice in the black:white test box.* Naive young adult mice placed into the centre of the white section of the test box moved within 10 to 13 s into the black section. On daily testing animals habituated to the test system, moving by the 4th or 5th day within 2 to 4 s into the black section. Twice daily treatment with R(+)-zacopride (1.0 ng/kg IP) enhanced habituation to the test system, reducing the latency of movement into the black section by the 2nd day. The administration of scopolamine (0.25 mg/kg) on the 6th day impaired habituation to the test box, increasing the latency of movement into the black section to approximately 20 s. Treatment with R(+)-zacopride prevented the effect of scopolamine (Fig. 8).

The twice daily administration of S(−)-zacopride at a dose of

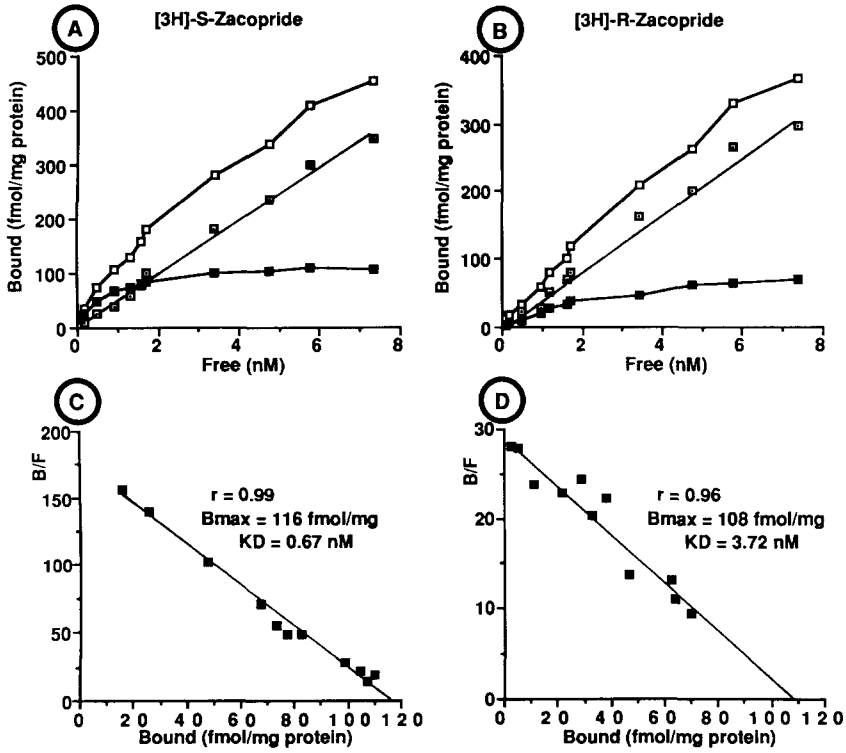


FIG. 11. The binding of [$^3$H]R(+)- and [$^3$H]S(−)-zacopride (0.1–7.5 nM) and its displacement by BRL43694 (10 μM) from homogenates of rat entorhinal cortex. Typical results are presented from a single experiment showing (A) the total (□), nonspecific (⊡) and specific binding (■), (B) the Scatchard transformation. Values represent the mean of a triplicate determination.

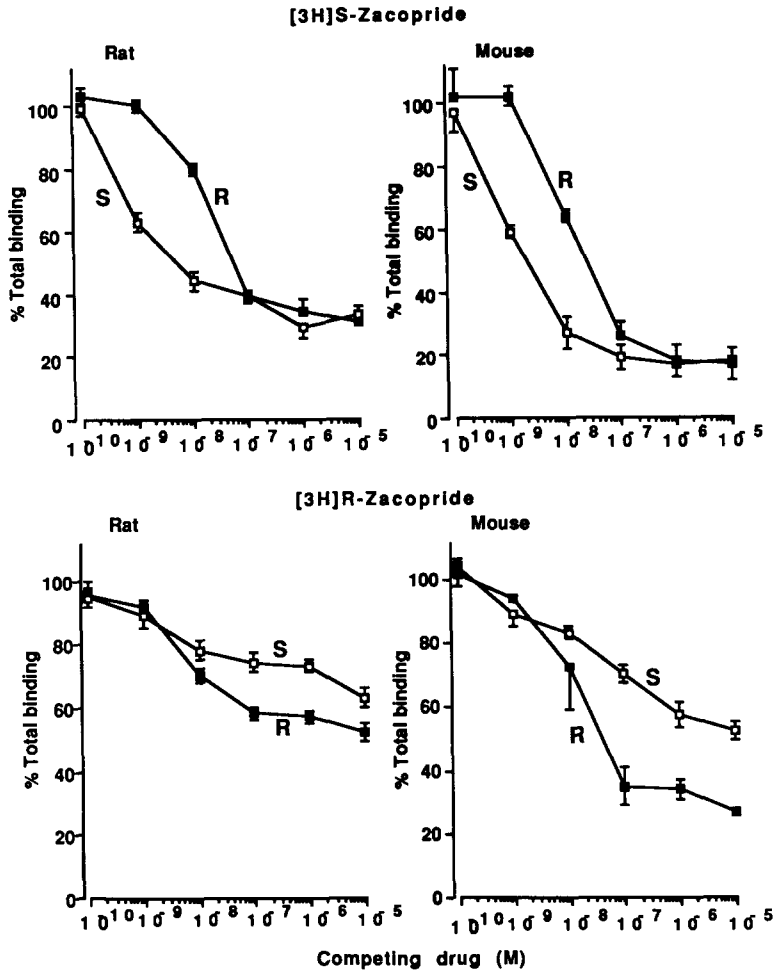


FIG. 12. The ability of R(+)- and S(−)-zacopride (■) and (□) respectively to compete for [$^3$H]R(+)-zacopride (0.25–0.50 nm) and [$^3$H]S(−)-zacopride 0.25–0.50 nM) binding in homogenates of the rat and mouse entorhinal cortex. Typical results are presented from single experiments where each value is the mean of a triplicate determination.

1.0 ng/kg IP (unpublished data) or as high as 10 μg/kg IP failed to modify the habituation response or the scopolamine-induced impairment (Fig. 8).

*Biochemical Tests*

*The ability of R(+)-and S(−)-zacopride to antagonise the effects of 2-methyl-5-hydroxytryptamine (2-Me-5-HT) to inhibit the release of [$^3$H]acetylcholine from rat entorhinal cortex.* The inhibitory effect of 2-Me-5-HT on potassium-stimulated [$^3$H]acetylcholine release was concentration-dependent in the range 0.5–10 μM. A concentration of 2 μM 2-Me-5-HT was selected as causing a maximal reduction of [$^3$H]acetylcholine release [see also Barnes and colleagues (4)], reducing [$^3$H]acetylcholine release by some 60% (Fig. 9). R(+)-Zacopride (0.01–1.0 nM) antagonised the inhibitory effect of 2-Me-5-HT (2.0 μM), the antagonism developing at 0.1 nM and effecting a complete antagonism at 1.0 nM. Higher concentrations of R(+)-zacopride (5.0 and 10.0 nM) had a similar effect. In contrast, the low concentration of S(−)-zacopride (0.1 nM) failed to antagonise the inhibitory effects of 2-Me-5-HT, although a 0.5 nM concentration caused an approximate 50% antagonism of the effect of 2-Me-5-HT. With increasing concentrations of S(−)-zacopride, the effect of a 10 nM concentration was not significantly different from that of R(+)-zacopride (Fig. 9).

*The activity of R(+)- and S(−)-zacopride in radioligand binding assays.* In the initial experiments the binding of [$^3$H]R(+)/S(−)-zacopride to the rat entorhinal cortex [determined using the 5-HT$_3$ receptor antagonist BRL43694 ($10^{-5}$ M) to define no specific binding] indicated a saturable binding of $B_{max}$ 77 fmol/mg protein to a single high-affinity site, $pK_D$ 9.12 [see also Barnes and colleagues (6)]. Using [$^3$H]R(+)/S(−)-zacopride, low nanomolar concentrations of S(−)-zacopride (0.1–5 nM) competed for approximately 35% of binding, increasing the concentration of S(−)-zacopride a 1000-fold failed to cause a further decrease in binding. R(+)-Zacopride, in the contraction range of 5 to 100 nM, competed for approximately 35% of binding. In addition, a further and consistent inhibition of binding (to approximately 55% of control levels) was detected at higher concentrations (0.1–10 μM) that was not achieved using the S(−)-isomer. At the very high concentration of 0.1 mM, both isomers caused a further decrease in binding (Fig. 10). $pIC_{50}$ values for S(−)- and R(+)-zacopride were 8.70 ± 0.14 and 7.57 ± 0.19 respectively (mean ± S.E.M., n = 5).

In subsequent experiments, the binding of [$^3$H]S(−)-zacopride (0.1–7.5 nM) to homogenates of the rat entorhinal cortex indicated a saturable high affinity binding site ($B_{max}$ 120 ± 5 fmol/mg protein, $pK_D$ 9.34 ± 0.08, mean ± S.E.M., n = 4). A Scatchard analysis of the binding of [$^3$H]R(+)-zacopride indicated a $B_{max}$ of 124 ± 8 fmol/mg protein and $pK_D$ 8.34 ± 0.04 (mean ± S.E.M., n = 4; Fig. 11).

Using [$^3$H]S(−)-zacopride, S(−)- and R(+)-zacopride displaced from the rat tissue approximately 65% of binding (Fig. 12). $pIC_{50}$ values for the S(−)- and R(+)-zacopride being 9.02 ± 0.10 and 7.58 ± 0.06 respectively (mean ± S.E.M., n = 3). Similarly, experiments were performed using homogenate preparations from the mouse entorhinal cortex and a similar profile was observed, the $pIC_{50}$ values for the S(−)- and R(+)-isomers to displace [$^3$H]S(−)-zacopride being respectively 8.97 ± 0.08 and 7.78 ± 0.04 (mean ± S.E.M., n = 3; Fig. 12).

Using [$^3$H]R(+)-zacopride, a competition profile was observed for the R(+)- and S(−)-isomers in the rat homogenate that was broadly comparable to their displacement of [$^3$H]R(+)/S(−)-zacopride. Thus, S(−)- and R(+)-zacopride at $10^{-8}$ M caused the same displacement as S(−)-zacopride (approximately 20–30%), but at higher concentrations ($10^{-7}$–$10^{-5}$ M) the R(+)-isomer caused 10–20% more inhibition than recorded using the S(−)-isomer. Such differences were more marked using homogenate preparations from the mouse entorhinal cortex, the R(+)-isomer ($10^{-9}$–$10^{-7}$ M), causing a concentration related inhibition and a maximum reduction of 65% of total [$^3$H]R(+)-zacopride binding. In contrast, S(−)-zacopride ($10^{-10}$–$10^{-5}$ M) caused a concentration-dependent inhibition of [$^3$H]R(+)-zacopride which competed for only 40% of total binding (Fig. 12).

## DISCUSSION

A stereoselectivity of drug-receptor interaction is a common observation at numerous receptor sites, and the finding that the R(+)isomer of zacopride was orders of magnitude more potent than the S(−)isomer to release suppressed behaviour is indicative of a stereoselective interaction at the 5-$HT_3$ receptor. In the mouse black and white test box, rat social interaction and marmoset human threat test, anxiolytic agents and the 5-$HT_3$ receptor antagonists reduce aversive responding (12,24). R(+)-Zacopride attenuated the aversive response of male and female mice to the brightly illuminated white area of the test box using automated and nonautomated methodologies, enhanced rat social interaction and reduced aversive responding in the marmoset to a human threat. The effect of R(+)-zacopride was recorded over an extensive dose range and contrasted with the general failure of S(−)-zacopride to reduce aversive responding in the three species. An exception was the ability of large doses of S(−)-zacopride to release suppressed behaviour in the female mouse using the automated light/dark test box methodology. This may reflect a sex or strain difference, but the higher basal level of responding in the automated methodology may more readily reveal an action to release suppressed behaviour. In any event, it is clear that in all test procedures, R(+)-zacopride was indisputably the more potent agent to reduce aversive responding.

In addition to releasing a suppressed behaviour, R(+)-zacopride was also at least 10 times more potent than S(−)-zacopride to improve cognitive performance in a mouse habituation test and to prevent a scopolamine-induced impairment. These properties are characteristically shown by the 5-$HT_3$ receptor antagonists in tests of cognition (5,11) and drug action to facilitate cholinergic transmission is considered to increase performance in tests of cognitive function (7). It is therefore relevant that serotonergic action on 5-$HT_3$ receptors has been shown to reduce acetylcholine release and that 5-$HT_3$ receptor antagonists can antagonise this effect in the rat entorhinal cortex and hippocampus [(3); Barnes, unpublished data]. The present finding that R(+)-zacopride antagonised the inhibitory effect of 2-methyl-5-HT to reduce [$^3$H]acetylcholine release from the rat entorhinal cortex may be relevant to its effects to improve cognitive performance, particularly when S(−)-zacopride was less potent to antagonise the inhibitory response to 2-methyl-5-HT. Thus, in both behavioural and biochemical tests, the R(+)-isomer of zacopride was revealed to interact more effectively at the 5-$HT_3$ receptor.

Therefore, it was an unexpected finding that in a quite different behavioural paradigm, the S(−)-isomer of zacopride was found to antagonise a hyperactivity response induced by an increased mesolimbic dopamine function. Thus, the peripheral administration of S(−)-zacopride effectively antagonised the increased locomotor activity induced by dopamine infusion into the nuleus accumbens; the hyperactivity induced by an injection of amphetamine into the nuleus accumbens was also antagonised by the S(−)-zacopride administered into the same site. In these behavioural tests a precise measurement of the relative inhibitory potency of the S(−)- and R(+)-isomer is frequently made difficult by the presence of a bell-shaped dose-response curve [see also Costall and colleagues (16,19)]. But over the 10- to 100-fold dose ranges used, it may be concluded that the S(−)-isomser and not the R(+)-isomer is effective to antagonise a limbic dopamine hyperactivity.

A stereoselectivity of action of the S(−)-isomer has also been shown in an antiemetic study. Zacopride racemate is highly effective to antagonise emesis induced by many types of chemotherapeutic agents and radiotherapy (1, 10, 14, 28). Observations that the S(−)-isomer is some fifty times more potent than the R(+)-isomer indicates that a significant component of the antiemetic action of the racemate resides in the S(−)-isomer (Smith, personal communication).

In the present study a stereoselective action of the S(−)-isomer was also confirmed in a ligand binding assay using [$^3$H]zacopride racemate, where the S(−)-isomer was ten times more potent as a displacing agent than the R(+)-isomer. A similar potency difference has previously been recorded for the (−)isomer of zacopride (27) and for the (−)isomer of the indole derivative SDZ210-204 [(−)(1R,2R,4S)-1H-indole-3-carboxylic acid-7-methyl-7-azabicyclo-[2.2.1.]hept-2-yl-ester] to inhibit [$^3$H]ICS 205-930 binding (26).

It becomes clear that both the R(+)- and S(−)-isomers of zacopride may contribute individually to the activity of the racemate. R(+)-Zacopride may retain potential activity in all tests, with the possible exception of an ability to modify dopamine function; the S(−)-isomer appears to be of major importance for the antiemetic effect and to influence limbic dopamine function. Evidence that R(+)-zacopride may be influencing a receptor site not influenced by S(−)-zacopride is also obtained from ligand binding studies. Thus, although the S(−)-isomer is 10 times more potent than R(+)-zacopride to displace [$^3$H]racemic zacopride from the rat entorhinal cortex, the R(+)-isomer at high concentrations displaced a further 10–20% of the total binding in excess of that achieved using S(−)-zacopride. Further evidence was obtained using [$^3$H]R(+)-zacopride as ligand, where R(+)-zacopride was approximately equal in affinity with the S(−)-isomers as a displacing agent but, more importantly, displaced in the rat and particularly in the mouse entorhinal cortex 20 percent more of the total binding. Thus, R(+)-zacopride appears to label a recognition site to which the S(−)-isomer has little or no affinity. This may contribute to the differences observed between the isomers in the above functional test systems and studies are in progress to identify the additional recognition site. However, the possibility that the different behavioural profiles result from partial agonistic properties are unlikely since neither isomer of zacopride de-

polarised the rabbit vagus nerve (Owera-Ataepo, unpublished observation), a functional preparation responsive to 5-$HT_3$ receptor agonists and partial agonists (23).

In summary, the pharmacological activities of racemate zacopride as a 5-$HT_3$ receptor antagonist to antagonise emesis, inhibit mesolimbic dopamine function, antagonise aversive responding, enhance performance in tests for cognition and antagonise the inhibitory effects of 2-methyl-5-HT to reduce acetylcholine release can be differentiated as properties of the R(+)- or S(−)-isomers. A stereoselectivity of action can be demonstrated in all tests, and the differential activity of the isomers indicates the possibility of drug action at different recognition sites, with the possibility of further refinements in drug design.

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