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The potential anxiolytic activity of GR38032F, a 5-HT_3-receptor antagonist

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1 The highly selective 5-HT_3-receptor antagonist, GR38032F, has been tested in five animal models predictive for anxiolytic activity.

2 In the social interaction test in the rat and in a light/dark exploration test in the mouse, GR38032F dose-dependently released suppressed behaviour without modifying locomotor activity.

3 In the cynomolgus monkey and the marmoset, GR38032F reduced anxiety-related symptoms without causing sedation. In the marmoset, the effects were clearly dose-related.

4 GR38032F did not have any detectable activity in the water-lick conflict test in the rat.

5 We conclude that GR38032F is potentially a very potent anxiolytic agent without sedative, anticonvulsant or hypnotic activity.

Introduction

For many years, 5-hydroxytryptamine (5-HT) has been implicated in the processes underlying anxiety and a great deal of research has been undertaken on this topic (see Iversen, 1984). Until recently, there have not been any indications that new anxiolytic drugs might develop from this research. One of the major problems has been the absence of selective agonists and antagonists for the 5-HT receptor subtypes that have now been identified (Bradley *et al.*, 1986). In recent years, however, new selective compounds have been discovered and there has been a resurgence of interest in the involvement of 5-HT in anxiety. Several compounds with potential anxiolytic activity have emerged from these investigations. For instance, the 5-HT_{1A}-receptor selective compounds, buspirone, gepirone and ipsapirone (Peroutka, 1985; Glaser & Traber, 1985; Eison *et al.*, 1986) and the 5-HT_2-receptor antagonist ritanserin (Ceulemans *et al.*, 1985) are believed to have anxiolytic activity.

We now describe evidence from experiments using the selective 5-HT_3-receptor antagonist GR38032F (Brittain *et al.*, 1987) that the 5-HT_3-receptor, (which has been identified in peripheral neurones but not yet unequivocally in the CNS; Richardson & Engel, 1986), may also be involved in anxiety.

The results described here were presented to the British Pharmacological Society in preliminary form (Jones *et al.*, 1987; Costall *et al.*, 1987).

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Methods

Social interaction test in rats

Male Hooded Lister rats (Glaxo bred, 200–250 g), were housed 5 to a cage and kept in the laboratory environment for at least a week before testing. Rats paired in the test were taken from separate cages.

Procedure The method was based on that described by File (1980). The test arena consisted of an open-topped box, 62 × 62 × 33 cm with a 7 × 7 matrix of infra-red photocell beams in the walls, 2.5 cm from the floor. The light intensity at the floor of the arena was 380 lux under high light conditions and 3.5 lux under low light conditions.

Drugs were tested by treating both members of a pair of rats with the same treatment at the predetermined time before testing. Where a 45 min pretreatment time was used, the rats were placed singly in small cages immediately after dosing until they were tested. Where longer pretreatment times were used, the rats were returned to their home cages after dosing and placed in the single cages 45 min before testing.

Testing involved placing each member of a pair of rats in opposite corners of the arena and then leaving them undisturbed for 10 min while recording their behaviour remotely on videotape. The behavioural assessments were made subsequently from the recordings. The time spent in social interaction was measured and expressed as a cumulative total for the 10 min session. The behaviours that comprised social interaction were: following with contact, sniffing (but not sniffing of the hindquarters), crawling over and under, tumbling, boxing and grooming. Sniffing of the hindquarters was excluded because it was markedly influenced by the degree of urination and defaecation. Furthermore, it has been suggested that drugs can create olfactory stimuli by altering the odour of the urine (Dixon, 1982), but this may not be a significant problem in the rat (File & Hyde, 1978).

When the familiar condition was used, rats were exposed to the arena in pairs for 10 min on the day before the experiment. Rats were paired with different partners on the test day.

GR38032F, administered orally, was tested under high light, unfamiliar conditions over the dose ranges 0.0025–0.1 mg kg^{-1} and 0.1–10 mg kg^{-1} in two separate experiments. A single dose of 10 μg kg^{-1} GR38032F was also tested under low light, unfamiliar and low light, familiar conditions. The time course of the effect produced by 10 μg kg^{-1} GR38032F was determined by testing 5 separate groups of rats at pretreatment times of 0.75, 1.5, 3, 6 and 12 h.

Water-lick conflict test

Male Hooded Lister rats were used as in the social interaction test.

Procedure The technique was based on the one described by Vogel *et al.* (1971). The test chamber was a 30 × 30 × 30 cm acrylic box with a stainless steel grid floor. A drinking spout projected 2 cm through a hole in one wall. A small bulb (2 W) in the roof provided illumination. The apparatus was enclosed in a sound-attenuated chamber.

Rats were deprived of water for 24 h before the test. Testing involved placing each rat in the test chamber and allowing it to drink at the water spout for 3 min. Licking was automatically punished with 0.75 mA, 20 ms electrical stimuli across the grid floor and the spout, either every 5 licks or every 0.75 s of contact with the spout. The number of shocks received in the test period was recorded automatically.

Drugs were administered intraperitoneally suspended or dissolved in 0.5% Tween 80 in saline, 30 min before testing. Diazepam, 5 mg kg^{-1}, was used as a positive control and the doses of GR38032F were 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 mg kg^{-1}.

Light/dark exploration test in mice

Male albino BKW mice, 25–30 g, were housed 10 to a cage and allowed free access to food and water. They were kept on a reversed light cycle with the lights on between 22 h 00 min and 10 h 00 min.

Procedure The apparatus was 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 × 7.5 cm opening in the partition at floor level. The small compartment was painted black and the large compartment white. The floor of each compartment was marked into 9 cm squares. The white compartment was illuminated by a 100 W tungsten bulb 17 cm above the box and the black compartment by a similarly placed 60 W red bulb. The laboratory was illuminated with red light.

All tests were performed between 13 h 00 min and 18 h 00 min. Each mouse was tested by placing it in the centre of the white area 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 to 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.

Drugs were administered intraperitoneally 45 min before testing. GR38032F, dissolved in distilled water, was administered to 5 groups of mice, covering the dose range 0.05–10 μg kg^{-1}. Diazepam (0.063–10 mg kg^{-1}) was dissolved in the minimum quantity of polyethylene glycol diluted to volume with distilled water and given to 7 groups of mice.

Behavioural observation of marmosets

Common marmosets (*Callithrix jacchus*), body weights 315 ± 20 g, of either sex were housed as single sex pairs. They were tested in their home cages. Only marmosets that responded consistently and reliably to confrontation by an observer (see below) were used in the experiments. It was essential to allow 1 or 2 days between test days and no marmoset was tested more than 3 times in a week. Marmosets were subjected to one test only on each test day.

Drugs were injected subcutaneously (dissolved or suspended in saline), each member of the pair receiving the same treatment. Forty-five minutes after

drug treatment, the marmosets were confronted by an observer standing 0.6 m in front of the cage. Over a 2 min period, the number of 'aggressive' postures shown by one member of the pair was recorded: tail erect with exposure of genitals, slit stare facial expression, scenting and arch piloerect locomotion (marmoset moves to and fro along perch with back arched and full body piloerection) (Stevenson & Poole, 1976). In the following 2 min period, the amount of time spent on the wire cage front was recorded. Any overt behavioural changes were also noted. The responses of one member of each of the other treated pairs of marmosets were assessed before returning immediately to the second member of the previous pair. At least 2 pairs of marmosets were tested on any one occasion. The intensity of responses evoked in the marmosets varied between observers. Consequently, the experiments described here were performed by the same observer.

Behavioural observation of cynomolgus monkeys

Four cynomolgus monkeys (*Macaca mulatta*), 2 male and 2 female, weighing 3.1–5.4 kg, were selected on the basis that they showed a moderately high level of emotionality when tested by the procedure outlined below. They were housed individually and allowed free access to food and water. On test days, they were deprived of food throughout the test session except when they were offered a small amount of apple to test appetite.

The experimental design was a 4 × 4 crossover so that each monkey received the 4 treatments over 4 weekly test sessions. The treatments, administered orally, were: vehicle (1% tragacanth); diazepam, 2.5 mg kg^{-1}; GR38032F, 10 μg kg^{-1} and 100 μg kg^{-1}. The behavioural analysis consisted of the systematic rating of 33 parameters before dosing and then every 30 min after dosing for 5 h. The parameters included a range of autonomic symptoms, motor behaviours, provoked behaviours and others such as vocalisation, facial expression and alertness. The response to offering a small piece of apple 1.5 h after dosing was also rated. The parameters that appear in the Results section are described below: Locomotor activity: the amount of spontaneous spatial movement. Restlessness: a measure of the inability of the monkey to remain still. Agitation: the degree by which the monkey is disturbed by spontaneous environmental stimuli such as vocalisation and movements of other monkeys. Startle: the response to a sudden noise, i.e. a hand clap. Alertness: a measure of general awareness. Incoordination: a measure of the inability of the monkey to perform co-ordinated movements such as climbing. Observer approach: response to the observer walking deliberately towards the cage. Cage bar rattle: response to the observer rattling the bars of the cage. Pole-prod response: response to the introduction of a wooden pole through the bars of the cage. Aggression: the degree of aggressive behaviour directed toward the observer with and without provocation.

Pentylenetetrazole-induced convulsions

Groups of 10 male, Glaxo bred CR/H mice (20–26 g) were treated orally with vehicle (5% acacia solution), diazepam (2.5 mg kg^{-1}) or GR38032F (0.001, 0.01, 0.1 or 1 mg kg^{-1}). One hour later, pentylenetetrazole, 60 mg kg^{-1}, was injected intravenously and the numbers of mice displaying tonic extensor convulsions were recorded.

Pentobarbitone sleeping time

Groups of 10 mice as above were treated orally with vehicle, diazepam (2.5 mg kg^{-1}) or GR38032F (0.01 or 1 mg kg^{-1}). One hour later, pentobarbitone, 40 mg kg^{-1}, was injected intravenously. The time between loss and return of the righting reflex was determined.

Radioligand binding assays

The affinity of GR38032F for benzodiazepine and 5-HT$_{1A}$-receptor binding sites *in vitro* was determined by displacement of [^{3}H]-flunitrazepam or [^{3}H]-8-hydroxy-dipropylaminotetralin (8-OH-DPAT), respectively. Crude membrane preparations from rat hippocampus were incubated with GR38032F, 10^{-5} M and either [^{3}H]-flunitrazepam, 0.5×10^{-9} M at 0°C for 20 min or [^{3}H]-8-OH-DPAT, 0.5×10^{-9} M at 37°C for 15 min. Non-specific binding was defined with diazepam, 5×10^{-6} M and 5-HT, 10^{-6} M respectively. Membrane bound radioactivity was determined by filtration through Whatman GF/B filters followed by liquid scintillation spectroscopy.

Drugs

The following drugs (source in parentheses) were used: GR38032F (1,2,3,9-tetrahydro-9-methyl-3-[(2-methy-1H-imidazol-1-yl)methyl]-4H-carbazol-4-one hydrochloride dihydrate: synthesised in our Chemical Research Laboratories at Ware); diazepam (Evans Medical); pentylenetetrazole (Sigma); pentobarbitone (May & Baker); [^{3}H]-flunitrazepam and [^{3}H]-8-OH-DPAT (Amersham).

Analysis of data

The results from the social interaction, light/dark exploration, marmoset behaviour and pentobarbitone sleeping time tests were analysed by analysis of variance followed by Dunnett's test for multiple comparisons. In the water-lick conflict test, the non-parametric multiple comparison test described by Levy (1980) was used. The results from the cynomolgus monkey behaviour experiment were not analysed statistically.

Results

Social interaction test in rats

GR38032F, 0.5–1000 $\mu g\,kg^{-1}$, administered orally, significantly ($P < 0.05$) increased social interaction under high light, unfamiliar conditions. At higher doses, the effect on social interaction declined, but there were no significant changes in locomotor activity throughout the dose range (Figure 1). Although

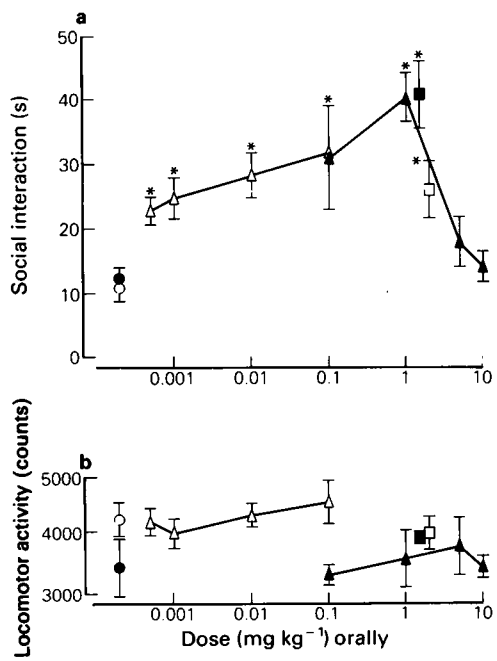

Figure 1 The effect of GR38032F on social interaction in rats (a) and lack of effect on locomotor activity (b). Pairs of rats were treated with GR38032F (△), diazepam (□) or vehicle (○) orally 45 min before testing under high light, unfamiliar conditions. $n = 8$ pairs per group. Open symbols: experiment 1; closed symbols: experiment 2. $^*P < 0.05$ compared to controls.

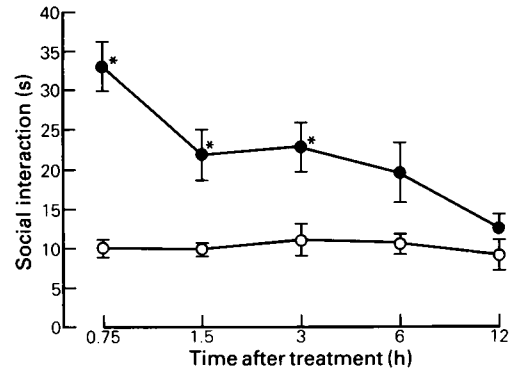

Figure 2 Time course of effect of GR38032F on social interaction of rats under high light, unfamiliar conditions. Pairs of rats were treated orally at the specified times with either GR38032F, 10 $\mu g\,kg^{-1}$ (●) or vehicle (○) before testing. $n = 8$ pairs per group. $^*P < 0.05$ compared to controls.

in these experiments only a single dose of diazepam was used as a positive control, previous experiments have indicated that GR38032F is at least 500 times more potent than diazepam in this test.

The effect of a dose of 10 $\mu g\,kg^{-1}$ GR38032F reached a peak 45 min after dosing and declined to control values over 6–8 h (Figure 2). The same dose did not significantly modify social interaction under the minimally adversive low light, familiar condition but was still effective under the intermediate low light, unfamiliar condition (Figure 3).

Water-lick conflict test in rats

In the water-lick conflict test in rats GR38032F, 0.05–1.6 $mg\,kg^{-1}$ i.p., failed to alter significantly the number of shocks received by the rats. In contrast, diazepam, 5 $mg\,kg^{-1}$, caused a marked increase. In most of the groups of rats treated with GR38032F, there did appear to be an increase in variation (Figure 4).

Light/dark exploration test in mice

GR38032F, like diazepam, dose-dependently increased the proportion of time the mice spent in the larger, light area of the test chamber. The numbers of line crossings and rears in the light compartment correspondingly increased at the expense of those in the dark compartment. The total number of rears and crossings did not change significantly, indicating that GR38032F did not have any sedative activity. In contrast, the highest dose of diazepam (10 $mg\,kg^{-1}$) was markedly sedative (Figure 5). The latency to entering the dark compartment was

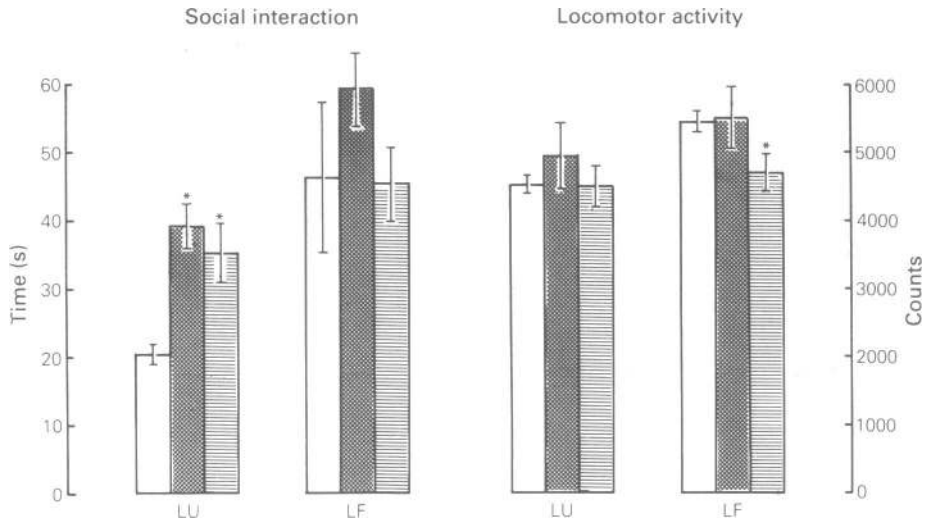

Figure 3 The effect of GR38032F on social interaction and locomotor activity in rats under low light familiar and unfamiliar conditions. Pairs of rats were treated orally with GR38032F, 10 $\mu g\,kg^{-1}$ (stippled columns), diazepam, 2 $mg\,kg^{-1}$ (hatched columns) or vehicle (open columns) 45 min before testing. LU = low light, unfamiliar conditions; LF = low light, familiar conditions. $n = 8$ pairs per group. $^*P < 0.05$ compared to controls.

increased dose-dependently by both drugs, the peak effects being obtained with 0.25 $mg\,kg^{-1}$ diazepam (latency = 20.6 ± 2.3 s) and 0.1 $\mu g\,kg^{-1}$ GR38032F (latency = 23.7 ± 2.5 s; vehicle latency = 11.6 ± 1.3 s). Neither GR38032F nor diazepam altered the number of transitions.

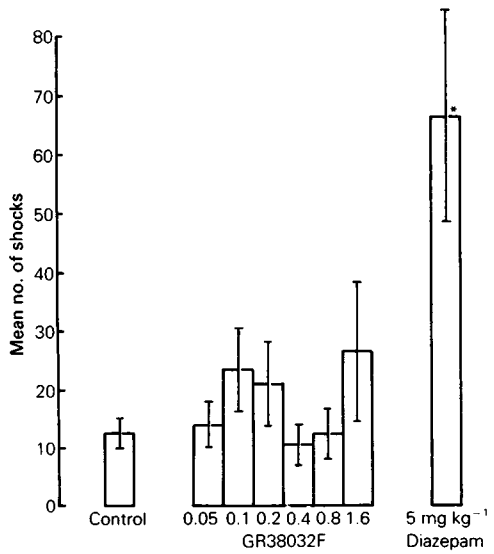

Figure 4 The effect of GR38032F in the water-lick conflict test in rats. Treatments were administered intraperitoneally at the doses specified 30 min before testing. $n = 10$ per group. $^*P < 0.05$ compared to controls.

Under conditions where both compartments were illuminated with red light, neither GR38032F 1 $\mu g\,kg^{-1}$ nor diazepam 1.25 $mg\,kg^{-1}$ affected the patterns of behaviour of the mice in the apparatus. Thus the mean proportions of time spent in the larger compartment were 68 ± 7.3% for control, 66 ± 7.3% for GR38032F and 73 ± 7.9% for diazepam.

Behavioural observations of marmosets

The amount of time the marmosets spent at the front of their cages increased dose-dependently after GR38032F or diazepam treatment. The number of aggressive postures correspondingly decreased. The minimum effective doses of GR38032F and diazepam were 0.1 $\mu g\,kg^{-1}$ and 10 $\mu g\,kg^{-1}$, respectively (Figure 6). No other behavioural changes were observed with either compound at the doses tested.

Behavioural observation of the cynomolgus monkey

Ten of the behavioural parameters assessed were altered by GR38032F, 10 and 100 $\mu g\,kg^{-1}$, or diazepam, 2.5 $mg\,kg^{-1}$, and these results are shown in Figure 7. Most of the effects reached a maximum 2 h after dosing with GR38032F and 2–3 h after dosing with diazepam. Only slight effects remained 5 h after

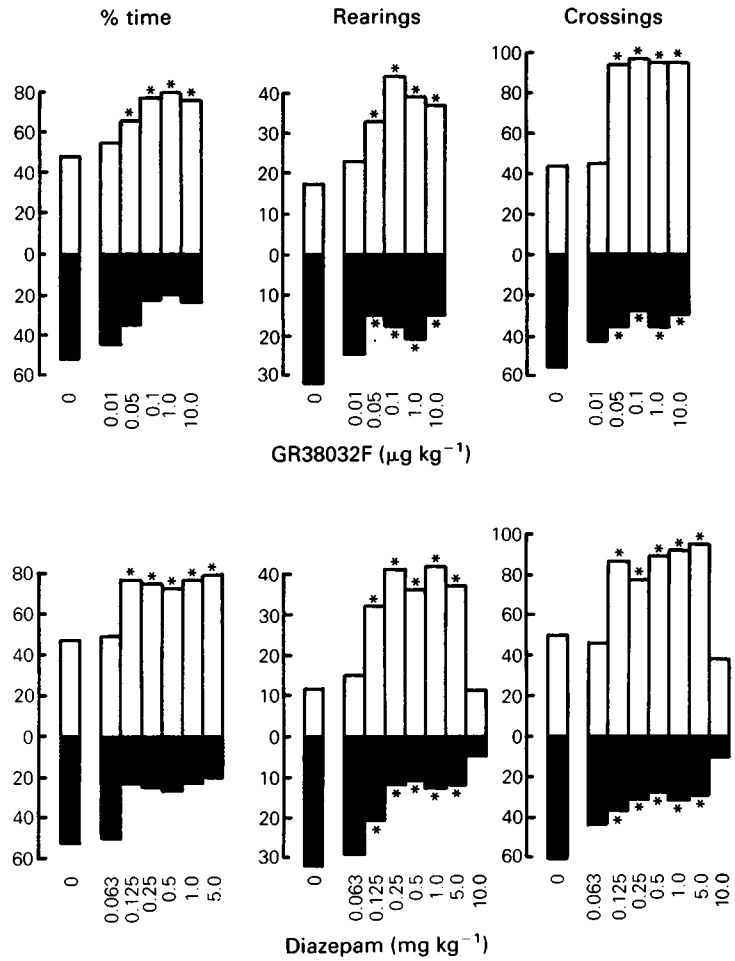

Figure 5 The effects of GR38032F and diazepam in the light/dark exploration test in the mouse. Treatments were given intraperitoneally at the doses specified 45 min before testing. Open columns = light area; solid columns = dark area. $n = 6$–8 per group. $^*P < 0.05$ compared to controls. Standard errors of the means were 6.3 – 11.3% of the means.

dosing. The main differences between the effects of the two compounds were that diazepam had stronger effects in all cases and that GR38032F, unlike diazepam, had little or no effect on the startle response, motor co-ordination or alertness. The effects of GR38032F were not consistently dose-related.

Pentylenetetrazole-induced convulsions in mice

GR38032F, 0.001–1 mg kg^{-1}, did not inhibit the convulsions whereas diazepam, 2.5 mg kg^{-1}, had a marked anticonvulsant effect (Table 1).

Table 1 The effect of GR38032F on pentylenetetrazole-induced convulsions in mice

Treatment (mg kg^{-1})	*Number convulsing*	n
Vehicle	9	10
Diazepam, 2.5	2*	10
GR38032F, 0.001	9	9
GR38032F, 0.01	10	10
GR38032F, 0.1	8	10
GR38032F, 1.0	9	10

Treatments were administered orally 1 h before the mice were injected with pentylenetetrazole, 60 mg kg^{-1} intravenously. $^*P < 0.01$ compared to control (Chi-squared test).

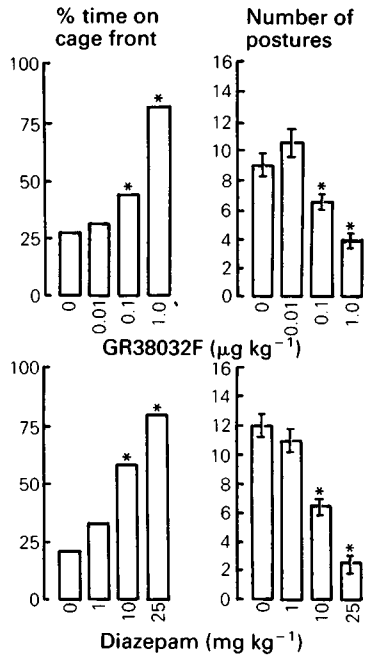

Figure 6 The effect of GR38032F and diazepam on anxiety-related behaviours in marmosets. Treatments were administered subcutaneously 45 min before the behavioural observations were made. $n = 4$–6 per group. $^*P < 0.05$ compared to controls.

Pentobarbitone sleeping time in mice

GR38032F, 0.01 and 1 mg kg^{-1}, did not alter the duration of pentobarbitone sleeping time whereas diazepam, 2.5 mg kg^{-1}, approximately doubled the duration (Table 2).

Radioligand binding assays

GR38032F 1×10^{-5} M, did not significantly displace either [^{3}H]-flunitrazepam or [^{3}H]-8-OH-DPAT from rat brain tissue.

Discussion

The present results provide strong indications that GR38032F will have anxiolytic activity in man. In the five predictive tests that we used, GR38032F was active in four. Of these, the social interaction test in the rat is the one that has been most extensively validated (File, 1981). GR38032F enhanced social interaction dose-dependently under the high light, unfamiliar and low light, unfamiliar conditions, but not under the minimally aversive low light, familiar condition. Thus, GR38032F selectively released social interaction suppressed by aversive conditions, the property that is believed to predict potential anxiolytic activity.

Table 2 The effect of GR38032F on pentobarbitone sleeping times in mice

Treatment (mg kg^{-1})	*Sleeping times* (min)
Vehicle	35.0 ± 4.4
Diazepam, 2.5	67.3 ± 7.2*
GR38032F, 0.01	40.1 ± 4.7
GR38032F, 1.0	37.6 ± 4.1

Treatments were administered orally 1 h before the mice were injected intravenously with pentobarbitone, 40 mg kg^{-1}. $n = 8$–10. Mean values ± s.e. mean are shown. $^*P < 0.05$ compared to control (ANOVA and Dunnett's test).

In the light/dark exploration test in the mouse, GR38032F similarly reinstated the behaviours suppressed by mildly aversive environmental conditions (high light). This was clearly a release of suppressed behaviour because under conditions where the aversive element was absent, GR38032F did not change the pattern of behaviour. In this test and in the social interaction test, GR38032F did not cause any sedation. Even at very high doses, when the effect on social interaction was declining, there was no evidence of sedation.

The value of the tests in primates that we used is based on the premise that we can more readily classify behaviours as anxiety symptoms because of their similarities to human behaviour. The difficulties are that the observer must be skilled in the observation of the behaviour of the individual animals and quantitative data are difficult to obtain. We adopted a dual approach: we obtained semi-quantitative data from cynomolgus monkeys using the subjects as their own controls and also developed a quantitative test in marmosets after selecting two behaviours that appeared to be very sensitive to anxiolytic drug action. In both tests, GR38032F caused behavioural changes that might be expected from an anxiolytic agent. The test in the marmoset proved to be very sensitive to both GR38032F and diazepam and both drugs caused dose-related changes in the behaviours observed. The test in the cynomolgus monkey appeared to be less sensitive to both drugs and the changes caused by GR38032F were not consistently dose-related. However, the doses selected may not have been the most appropriate. Diazepam caused mild sedation and this undoubtedly enhanced the scores. Consequently, the anxiolytic effects could not be disentangled from the sedative effects. GR38032F was not sedative and accordingly produced smaller changes in behaviour.

The observation that GR38032F did not have any

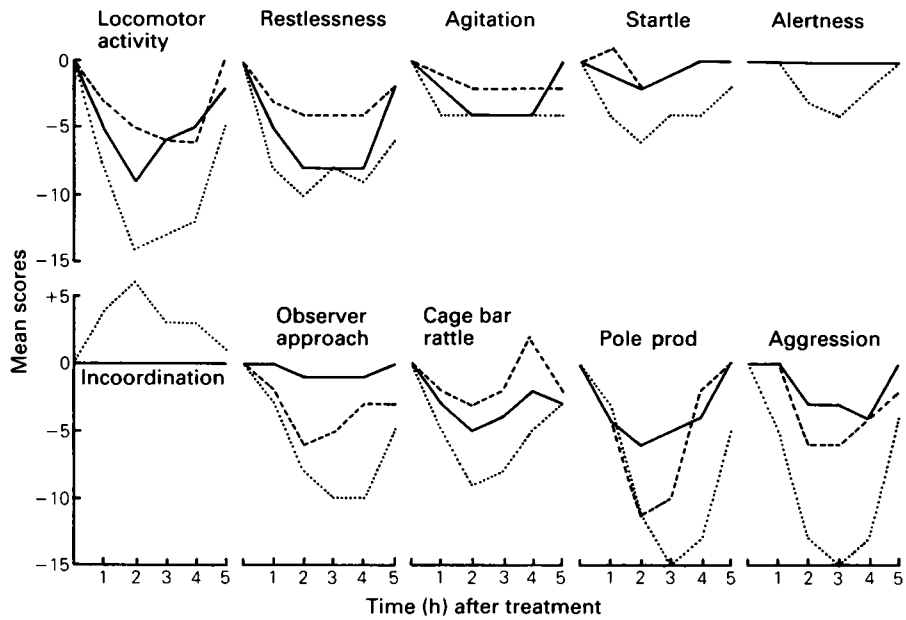

Figure 7 The effect of GR38032F on the behaviour of cynomolgus monkeys. The monkeys were treated orally with GR38032F, 10 $\mu g\,kg^{-1}$ (continuous line); GR38032F, 100 $\mu g\,kg^{-1}$ (dashed line); diazepam, 2.5 $mg\,kg^{-1}$ (dotted line) or vehicle. The points show the mean changes in scores from the control conditions at hourly intervals from the time of treatment. Only the parameters that were changed by the drugs are shown. $n = 4$.

activity in the water-lick conflict test in the rat may be interpreted in several different ways. As an animal model of anxiety, it has only been validated by demonstrating that known anxiolytic agents are effective in the test. Thus, it could be argued that an anxiolytic with a novel mode of action would not necessarily be active in this test.

The observation that GR38032F seemed to increase the variability of the results might be an indication that the compound does exert some action, but that the conditions of the test are inappropriate for its detection. This possibility is being investigated. Alternative interpretations are that (i) the water-lick conflict test may be predictive for a particular type of anxiety in which benzodiazepines, but not GR38032F, are effective; (ii) the test has no predictive value for non-benzodiazepine anxiolytic agents or, (iii) it is the only test with predictive value and GR38032F therefore may not have clinically useful anxiolytic activity. The correct interpretation will only become clear when GR38032F has been evaluated in man.

In addition to its lack of effect in the above test and its lack of sedative effect, GR38032F differs from the benzodiazepines in having no anticonvulsant or hypnotic activity. Thus, we observed that GR38032F did not modify pentylenetetrazole-induced convulsions or pentobarbitone-induced sleeping time in mice. Furthermore, GR38032F did not have any affinity for benzodiazepine receptors *in vitro*.

An important question posed by this work is whether the potential anxiolytic activity of GR38032F is directly related to its 5-HT_3-receptor antagonist activity. In the rat, the doses of GR38032F that are known to block peripheral 5-HT_3-receptors *in vivo* are similar to those that are effective in the social interaction test. Thus, the oral dose required to inhibit the 5-HT-induced von Bezold-Jarisch reflex by 50% is about 8 $\mu g\,kg^{-1}$ (Butler *et al.*, 1988).

From initial evidence we have obtained with ICS 205-930 and MDL 72222, it appears that other 5-HT_3-receptor antagonists produce similar behavioural effects to those observed after GR38032F (Tyers *et al.*, 1987). Furthermore, GR38032F does not have any affinity for 5-HT_{1A}-receptors (this paper) or 5-HT_2-receptors (Brittain *et al.*, 1987).

We conclude, therefore, that GR38032F is potentially a very potent non-sedative anxiolytic agent, without anticonvulsant or hypnotic properties. Furthermore, its anxiolytic activity is probably conferred by its ability to block 5-HT_3-receptors. If GR38032F proves to have anxiolytic activity in man, it will represent not only a major advance in treatment but we shall have gained a better understanding of the mechanisms underlying anxiety.

Note added in proof

Direct evidence for the existence of 5-HT_3-receptors in rat brain was reported recently (Kilpatrick, Jones & Tyers (1987), *Nature*, **330**, 746–748).

We are grateful to David Beard for performing the cynomolgus monkey experiments.

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