The pharmacology of fluparoxan: a selective α_2 -adrenoceptor antagonist

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1 This paper describes the pharmacology of the novel α_2 -adrenoceptor antagonist fluparoxan (GR 50360) which is currently being studied clinically as a potential anti-depressant. Idazoxan and yohimbine were included in many studies for comparison.

2 In the rat isolated, field-stimulated vas deferens and the guinea-pig isolated, field-stimulated ileum preparations, fluparoxan was a reversible competitive antagonist of the inhibitory responses to the α_2 -adrenoceptor agonist UK-14304 with pK_B values of 7.87 and 7.89 respectively. In the rat isolated anococcygeus muscle, fluparoxan was a much weaker competitive antagonist of the contractile response to the α_1 -adrenoceptor agonist phenylephrine with a pK_B of 4.45 giving an $\alpha_2:\alpha_1$ -adrenoceptor selectivity ratio of greater than 2500.

3 In the conscious mouse, fluparoxan ($0.2\text{--}3.0\text{ mg kg}^{-1}$) was effective by the oral route and of similar potency to idazoxan in preventing clonidine-induced hypothermia and antinociception. In the rat, UK-14304-induced hypothermia ($ED_{50} = 1.4\text{ mg kg}^{-1}$, p.o. or 0.5 mg kg^{-1} , i.v.) and rotarod impairment ($ED_{50} = 1.1\text{ mg kg}^{-1}$ p.o. or 1.3 mg kg^{-1} , i.v.) were antagonized by fluparoxan. Fluparoxan, $0.67\text{--}6\text{ mg kg}^{-1}$, p.o., also prevented UK-14304-induced sedation and bradycardia in the dog.

4 In specificity studies fluparoxan had low or no affinity for a wide range of neurotransmitter receptor sites at concentrations up to at least $1 \times 10^{-5}\text{ M}$. It displayed weak affinity for 5-HT_{1A} ($pIC_{50} = 5.9$) and 5-HT_{1B} ($pK_i = 5.5$) binding sites in rat brain.

5 We conclude that fluparoxan is a highly selective and potent α_2 -adrenoceptor antagonist. The density of rat brain [³H]-dihydroalprenolol binding sites was reduced by 26% when fluparoxan was administered chronically for 6 days at a dose of 12 mg kg^{-1} orally twice daily. The down-regulation of β -adrenoceptors by fluparoxan is consistent with its antidepressant potential.

Keywords: α_2 -Adrenoceptor antagonist; fluparoxan; GR 50360; idazoxan

Introduction

The classification of α -adrenoceptors into α_1 and α_2 subtypes is well recognised (see McGrath *et al.*, 1989). Both agonists and antagonists for these subtypes are known and have proved to be useful therapeutically. However, highly selective antagonists at α_2 -adrenoceptors are relatively novel, e.g. Merck 912 (Pettibone *et al.*, 1987), CH38083 (Vizi *et al.*, 1988) and RS-15385-197 (Clark *et al.*, 1989), and await therapeutic evaluation. The autoreceptor on noradrenergic neurones mediating the feedback inhibitory effects of noradrenaline is widely recognised as belonging to the α_2 -subtype (Langer, 1980). Antagonists at this receptor facilitate noradrenergic neurotransmission as evidenced by a rapid increase in noradrenaline turnover (Anden *et al.*, 1982; Walter *et al.*, 1984). An α_2 -adrenoceptor antagonist could therefore be beneficial in conditions such as depression which have been associated with a deficiency of noradrenaline in the CNS (Schildkraut, 1965).

Several novel α_2 -adrenoceptor antagonists with varying degrees of α_2 - to α_1 -adrenoceptor selectivity have been described since the appearance of imiloxan in 1981 (Michel & Whiting, 1981) and idazoxan in 1983 (Doxey *et al.*, 1983). We describe here the *in vitro* and *in vivo* pharmacology of the novel benzodioxinopyrrole, fluparoxan (formerly known as GR50360), (Figure 1), a potent and selective α_2 -adrenoceptor antagonist that is currently undergoing clinical evaluation. Preliminary accounts of some of the data reported here were presented to the British Pharmacological Society (Halliday *et al.*, 1988a,b).

Methods

Rat vas deferens

The prostatic halves of vasa deferentia were carefully removed from adult Sprague-Dawley rats (300–400 g; Charles River) killed by stunning and cervical dislocation.

The tissues were mounted under 0.5 g tension at 31°C in oxygenated Krebs-Henseleit solution. Electrical stimulation was provided through two stainless steel ring electrodes, 20 mm apart, placed around the tissue and consisted of supra-maximal rectangular monophasic pulses of 2 ms duration and approximately 20 V amplitude delivered every 10 s. Twitch responses were recorded isometrically by use of Ormed Dynamometer UF1 strain-gauge transducers. UK-14304 or clonidine was added to the bath in incremental doses to produce cumulative inhibitory concentration curves. For antagonist studies the antagonist drugs were added 25–30 min before addition of the agonist.

Guinea-pig ileum

Guinea-pig terminal ileum (3 cm lengths) was obtained from male Dunkin Hartley guinea-pigs (450–550 g; Porcellus) killed by cervical dislocation. The tissues were set up under 0.5 g

Figure 1 Structural formula of (±)-fluparoxan.

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tension in 12.5 ml jacketed organ baths in oxygenated Krebs-Henseleit solution at 36°C and subjected to electrical field stimulation as described by Drew (1978).

Twitch responses were recorded isometrically as for rat vas deferens. After equilibration, the tissues were exposed to either fresh Krebs-Henseleit solution or Krebs-Henseleit solution containing antagonist for 15–30 min. Successive doses of UK-14304 were then added to the bath to produce a cumulative inhibition of the twitch response, each dose being added when the previous dose had produced its maximal inhibitory effect.

Rat anococcygeus muscle

Anococcygeus muscles were dissected from male Sprague-Dawley rats (300–400 g; Charles River) killed by stunning and cervical dislocation. Tissues were soaked overnight for 17 h at 4°C in Krebs-Henseleit solution containing 6-hydroxydopamine, 1×10^{-3} M, to destroy presynaptic sites of uptake and noradrenaline release as described by Doggrell (1980). The anococcygeus muscles were then mounted under 1 g tension in 12.5 ml organ baths and washed repeatedly with oxygenated Krebs-Henseleit solution at 36°C for 1 h. Thereafter the tissues were attached to isometric strain gauge transducers and the tension reduced to 0.5 g. The tissues were primed with high doses of phenylephrine (final bath concentration of 6×10^{-6} M) and a 30 s contact time until reproducible contractions were obtained. The maximum contractile response of each tissue to either phenylephrine or methoxamine was measured and tissues were then washed with fresh Krebs-Henseleit solution and incubated with either this solution or Krebs-Henseleit solution containing antagonist for a period of 15–30 min. Concentration-response curves were obtained by cumulative addition of either phenylephrine or methoxamine, each dose being added after the previous dose had produced its maximum effect.

Rabbit aorta

Lengths of aorta (8 cm approx.) were removed from adult rabbits (Frodox NZW) killed by cervical dislocation. Excess fatty tissue was removed from the aorta which was then threaded on to a 20 mm diameter glass rod and cut helically into strips approximately 4 mm wide and 4 cm long. The strips were mounted under 0.5 g tension in oxygenated Krebs-Henseleit solution at 37°C in 15 ml organ baths. Agonist-induced contractures were recorded isometrically. Four cumulative dose-response curves to phenylephrine were obtained for each tissue strip. The antagonist was introduced at least 15 min before the fourth curve. The shift in the agonist potency between the third and fourth curves was then calculated after correcting for any sensitivity change calculated from tissues not exposed to antagonist. The largest sensitivity change was usually between the first and second curves, hence the design employed here.

Estimation of potency in vitro

Lateral displacements of agonist concentration-response curves were measured at the control half-maximal response level. The negative logarithm of the dissociation constant of each antagonist (pK_B) was calculated using the Schild/Gaddum equation (Gaddum, 1957) from the relationship: $pK_B = \log (\text{dose-ratio} - 1) - \log [\text{antagonist}]$ where dose-ratio (DR) is the ratio of the agonist concentration producing half maximal responses (IC_{50}) in the presence and absence of the antagonist at the molar concentration [antagonist]. Individual pK_B determinations were made using 3 or 4 concentrations for each antagonist such that a mean ($\pm$ s.e.) pK_B value could be calculated. The results were also plotted according to the method of Arunlakshana & Schild (1959) to yield Schild plot gradients. The effect of each concentration of antagonist was measured on 1–6 preparations of a given tissue, each from a separate animal.

Antagonism of clonidine-induced hypothermia in the mouse

Male mice (Glaxo CRH 20–26 g), housed in groups of 5 at room temperature (22°C), were given gum acacia 5% w/v (vehicle), fluparoxan or idazoxan orally in a dose volume of 10 ml kg⁻¹. After 60 min the oesophageal temperature of each mouse was recorded with an electronic thermistor probe (Comark) and clonidine, 0.15 mg kg⁻¹, was given intraperitoneally. Temperatures were remeasured 30 min later and the changes in temperature were calculated.

Antagonism of clonidine-induced antinociception in the mouse

Male mice (Glaxo CRH 20–26 g) were given vehicle (5% w/v gum acacia 10 ml kg⁻¹), fluparoxan, idazoxan or yohimbine; 60 min later either saline or clonidine, 0.05 mg kg⁻¹, was given subcutaneously. After another 30 min each mouse received an intraperitoneal injection of 0.25 ml phenylbenzoquinone, 0.02% w/v, and was observed for 10 min for the appearance of abdominal constriction accompanied by stretching.

Antagonism of UK-14304-induced hypothermia and rotarod impairment in the rat

Male hooded rats (70–120 g; Glaxo) were given vehicle (5% w/v gum acacia-oral route; 0.9% w/v sodium chloride solution-intravenous route), fluparoxan or idazoxan by either the oral or intravenous (tail vein) routes (10 ml kg⁻¹ and 5 ml kg⁻¹ dose volume respectively). After 30 min the oesophageal temperature of each rat was recorded with an electronic thermistor probe (Comark) and either 0.9% w/v sodium chloride solution or UK-14304 (2 mg kg⁻¹) was given subcutaneously (5 ml kg⁻¹ dose volume). Oesophageal temperature was recorded again after an additional 30 min; 10 min later each rat was placed on an accelerating rotarod (Jones & Roberts, 1968) and the time it remained on the revolving drum was measured.

Antagonism of UK-14304-induced sedation and bradycardia in the dog

Studies were performed in beagles (6 males, 6 females; Glaxo; 6.8–10 kg weight range). Fluparoxan was given orally (0.22–6 mg kg⁻¹ in gelatin capsules) 30 min before a subcutaneous injection of UK-14304 (0.05 mg kg⁻¹). Sedation and heart rate were measured before giving fluparoxan and at various times after injection of UK-14304. For sedation, both spontaneous activity and reactivity to an audible stimulus (hand clap) were assessed on pairs of dogs housed in their home kennel and again when placed alone in an unfamiliar environment. Behaviour each time was scored from 0 (inactive) to 6 (maximal activity) such that by combination of the 4 behavioural scores a maximum activity score of 24 was possible. Heart rate was measured by counting the number of pulses in the femoral artery in 30 s.

Estimation of potency in vivo

The potencies of test compounds in antagonizing α_2 -adrenoceptor agonist-induced behavioural effects were calculated in the form of ED_{50} values. For antagonism of antinociceptive effects the data were quantal and ED_{50} values were used to represent the dose of antagonist reducing the incidence of the response to half that of the control animals. This was obtained from the best line fit to a log dose-logit plot of the quantal data. Confidence limits (95%) were obtained by use of Fieller's theorem. For antagonism of the hypothermia and sedation, the ED_{50} value represents the dose of antagonist reducing the magnitude of the response to 50% of that in animals given agonist alone. Half maximal response values

and standard errors were obtained by fitting dose-response curves to the data by the computer programme ALLFIT (De Lean *et al.*, 1978).

Evaluation of activity or affinity at non α -adrenoceptor sites

β -Adrenoceptors Affinity for β_1 -adrenoceptors was assessed in the rat (male 200 g; Glaxo AHA strain) isolated, electrically stimulated left atrium by a method based on that of Blinks (1965). The isolated atrium was impaled on a two pin electrode and placed in a 2 ml organ bath in oxygenated Krebs-Henseleit solution and stimulated at 1 Hz and 2 ms using just suprathreshold voltage.

Activity at β_2 -adrenoceptors was measured in guinea-pig (Dunkin Hartley male 300–400 g; Porcellus) isolated trachea as described by Coleman & Nials (1989).

Dopamine receptors The effect of fluparoxan at functional peripheral dopamine receptors was assessed by use of dopamine-induced increases in mesenteric blood flow in the anaesthetized dog for D_1 -receptor-mediated effects as described by Hilditch & Drew (1984) and dopamine-induced inhibition of electrically stimulated increases in heart rate of the anaesthetized cat for D_2 -receptor-mediated effects (Dallas *et al.*, 1986).

Radioligand binding assays for the D_2 site were performed on rat striatal membranes. Corpora striata were obtained from male hooded rats (200–250 g; Glaxo) killed by stunning and decapitation. The tissues were homogenized in 30 volumes of 50 mM Tris-HCl buffer at 4°C and pH 7.4 in an Ultra-Turrax homogeniser (lowest setting for two periods of 10 s with 30 s on ice in between). The homogenate was centrifuged (40 000 *g* for 20 min at 4°C) and the resulting pellet resuspended in 30 vol of fresh Tris-HCl buffer. The centrifugation was repeated and the final pellet resuspended in 30 vol of Tris-Krebs buffer containing (mM): Tris-HCl 50, NaCl 120, KCl 5, MgCl₂ 1, CaCl₂ 2 and EDTA 1. Assays were performed on 1 ml of homogenate (approx. 225 μ g protein) in a final volume of 3 ml of Tris-Krebs buffer containing 150 pM [³H]-spiperone (35 Ci mmol⁻¹, Dupont NEN) and 50 nM ketanserin to prevent binding to 5-HT₂ sites. (+)-Butaclamol (1 μ M) was used to define specific binding. Incubation was performed at 25°C for 75 min and terminated by filtration through Whatman GF/B filters in a Brandel Cell Harvester followed by washing of the filters (3 $\times$ 5 ml buffer at 4°C).

Muscarinic acetylcholine receptors Terminal ileum was obtained from male Lister Hooded rats (200–250 g; Glaxo). Responses were recorded isometrically (Ormed Dynamometer UF1 transducer) from 2 cm lengths of tissue under 0.5 g tension in oxygenated Krebs-Henseleit solution in 10 ml organ baths at 37°C. Fluparoxan was tested at concentrations up to 1×10^{-5} M against the contractile response to muscarine with a 5 min dosing cycle.

For radioligand binding assays rat heart and cerebral cortex membranes were prepared in a HEPES buffer (20 mM HEPES + 100 mM sodium chloride + 0.5 mM EDTA at pH 7.4; buffer for heart membranes also contained 3 mM magnesium chloride). Radioligands were [³H]-N-methylscopolamine (72 Ci mmol⁻¹, Amersham International, 0.2 nM) for the heart assays and [³H]-pirenzepine (87 Ci mmol⁻¹, Dupont NEN, 0.8 nM) for cortex. Atropine (1 μ M) was used to define specific binding and assays were terminated after 90 min at 22°C by centrifugation (Lazareno & Roberts, 1989).

Histamine receptors Lengths of terminal ileum obtained from male Dunkin Hartley guinea-pigs (450–500 g; Porcellus) were set up as described above. The effect of fluparoxan on histamine H₁-receptors was assessed from concentration-response curves obtained to histamine in tissues preincubated for 30 min in fresh Krebs-Henseleit solution or Krebs-Henseleit solution containing fluparoxan. Assays for histamine

H₂-receptor activity were performed on histamine-induced tachycardia in guinea-pig atrium as described by Humphray *et al.* (1982).

5-Hydroxytryptamine (5-HT)-receptors Possible activity of fluparoxan at functional 5-HT₁-like receptors in dog saphenous vein and 5-HT₂ receptors in rabbit isolated aorta was assessed as described by Feniuk *et al.* (1985). Affinity for 5-HT_{1A} and 5-HT_{1B} binding sites was measured with rat (male hooded; Glaxo; 150–200 g) hippocampal and striatal membranes respectively. Hippocampi from 8 rats were pooled and homogenized in an Ultra-Turrax at its lowest setting for 10 s at 5°C in 10 vol of 50 mM Tris-HCl buffer, pH 7.4. The membrane preparation was centrifuged at 40 000 *g* for 15 min and the pellet was resuspended in 10 volumes of Tris-HCl buffer. A further centrifugation and re-suspension of the membranes was followed by a 10 min incubation at 37°C to remove endogenous 5-HT. Following the final centrifugation, the resulting pellet was resuspended in 10 vol of 50 mM Tris-HCl buffer, pH 7.4 at 37°C. Binding assays were performed using 100 μ l aliquots (equivalent to 0.16–0.2 mg protein). The membranes were incubated for 15 min at 37°C with [³H]-8-hydroxy-2-(di-*n*-propylamino) tetralin (85 Ci mmol⁻¹, Amersham International 0.5–1.0 mM) and the test compound in a final volume of 250 μ l. 5-HT, 1×10^{-5} M, was used to define specific binding. Assays were terminated by filtration through Whatman GF/B filters using a Brandel Cell Harvester. Affinity for 5-HT_{1B} binding sites in rat striatum was determined with [¹²⁵I]-cyanopindolol (2088 Ci mmol⁻¹, Amersham International) according to Hoyer *et al.* (1985).

Noradrenaline uptake

Male Hooded rats (250–300 g; Glaxo) were killed by stunning and decapitation. Brains were quickly removed and the occipital cortex dissected out. Occipital cortices from three rats were placed in 5.1 ml of ice-cold 0.32 M sucrose solution in a 10 ml Potter-Elvehjem glass mortar fitted with a Teflon pestle having a clearance of 0.25 mm. The tissue was homogenized twice at 840 r.p.m. each time using six complete up and down strokes of the pestle over a 30 s period with a 60 s pause with the mortar on ice in between. The homogenate was then centrifuged at 1000 *g* and 4°C for 10 min. The supernatant (crude synaptosomal preparation) was used immediately for uptake studies. Assays were performed in triplicate using 0.1 ml of the synaptosomal preparation added to 1.8 ml of an oxygenated Krebs-Henseleit buffer at 37°C. The buffer had the following composition (mM): NaCl 118, KCl 4.7, MgSO₄ 1.1, KH₂PO₄ 1.2, NaHCO₃ 25, CaCl₂ 1.3, D-glucose 10, ascorbic acid 0.5 and pargyline HCl 0.1; pH 7.5 and contained either no drug, fluparoxan or desmethylinipramine with or without ouabain (final concentration 10 mM). After 10 min, 0.1 ml of [³H]-noradrenaline (41 Ci mmol⁻¹, Dupont NEN) in Krebs-Henseleit buffer was added (final concentration 10 nM) and the tubes incubated in a shaking water-bath for 7 min. The samples were then filtered through Whatman GF/B filters using a Brandel Cell Harvester. The filters were washed with cold (4°C) buffer (2 $\times$ 5 ml) and the amount of radioactivity retained measured by liquid scintillation spectroscopy.

Specific and energy-dependent noradrenaline uptake was defined as the difference between that in the presence and absence of ouabain. The concentration of fluparoxan and desmethylinipramine inhibiting by 50% the specific noradrenaline uptake was determined from concentration inhibition-dose-response curves analysed using the logistic curve-fitting programme ALLFIT (De Lean *et al.*, 1978).

Effect on [³H]-dihydroalprenolol binding sites

Male Sprague-Dawley rats (300 g; Charles River) were given water (5 ml kg⁻¹), fluparoxan (6 or 12 mg kg⁻¹), amitriptyline (30 mg kg⁻¹) or fluparoxan (6 mg kg⁻¹) + amitriptyline (30 mg kg⁻¹) by the oral route twice daily (at 08 h 00 min and

17 h 00 min) for 6 days. Fifteen hours after the final dose the rats were killed by stunning and decapitation, their brains rapidly removed and the cerebral cortices obtained and cleared of adhering white matter. Each cerebral cortex (350–450 mg wet wt.) was rapidly frozen in liquid nitrogen and stored at -70°C for up to three weeks. Membranes for radioligand binding assays were prepared from the thawed cerebral cortices as described by Wise & Halliday (1985). The binding assays were performed on 0.2 ml aliquots of cerebral membranes incubated initially in triplicate in 50 mM Tris-HCl buffer (final volume of 1 ml, pH 7.8) at 25°C for 10 min. Thereafter, 0.1 ml [^3H]-dihydroalprenolol (1-[propyl- ^3H (N)]dihydroalprenolol HCl, 40 Ci mmol $^{-1}$, Dupont NEN) was added and the mixture incubated at 25°C for 25 min followed by filtration through Whatman GF/B filters in a Brandel Cell Harvester. The filters were washed (3×5 ml Tris-HCl buffer at 25°C) and the retained tritium measured by addition of the filters to 10 ml Pico-Fluor 30 scintillation fluid (United Technologies Packard) for approximately 15 h followed by conventional liquid scintillation spectrometry. (–)-Isoprenaline (200 μM) was included in some tubes and specific binding was calculated as the difference between binding in its absence and presence.

The density of binding sites (B_{max}) and their affinity (K_D) were determined by Scatchard analysis (Scatchard, 1949) of the binding of six concentrations (0.1–2.0 nM) of [^3H]-dihydroalprenolol.

In some studies fluparoxan, amitriptyline and (–)-isoprenaline were tested over a range of concentrations for their ability to displace specific binding of [^3H]-dihydroalprenolol (0.3 nM) from cerebrocortical membranes from untreated rats. The respective concentrations producing 50% displacement of specific binding (IC_{50}) and hence K_i values and Hill slopes were determined by log probit analysis.

Drugs and solutions

Tissues were bathed in a modified Krebs-Henseleit solution (Apperley *et al.*, 1976) unless otherwise stated. The solution was gassed with 95% O_2 and 5% CO_2 . For rat vas deferens and ileum preparations the buffer solution contained 2.5 mM CaCl_2 and for guinea-pig ileum experiments 2 μM (+)-propranolol was included. Reagents used were A.R. grade and purchased from commercial sources. The following drugs were used: fluparoxan (GR50360, *trans*-(±)-5-fluoro-2,3,3a,9a-tetrahydro-1H[1,4]-benzodioxino-[2,3-c]pyrrole hydrochloride, Glaxo Group Research Ltd); amitriptyline hydrochloride (Sigma); atropine sulphate (Sigma); (+)-butaclamol hydrochloride (Research Biochemicals Inc.-RBI); clonidine hydrochloride (Sigma); desmethylinipramine hydrochloride (Ciba-Geigy); dopamine hydrochloride (Sigma); histamine hydrochloride (Sigma); 6-hydroxydopamine hydrochloride (Sigma); 5-hydroxytryptamine creatinine sulphate (Sigma); idazoxan hydrochloride (Glaxo Group Research Ltd); (–)-isoprenaline sulphate (Sigma); ketanserin (RBI); methoxamine hydrochloride (Burroughs Wellcome); muscarine chloride (Sigma); ouabain octahydrate (Sigma); pargyline hydrochloride (Sigma); phenylbenzoquinone (Kodak); α -phenylephrine hydrochloride (Koch Light); (+)-propranolol hydrochloride (ICI); spiperone hydrochloride (RBI); UK-14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline, Pfizer); yohimbine hydrochloride (Sigma).

Drug solutions were prepared immediately before use and for *in vivo* experiments the doses refer to the free base.

Results

Antagonism of α_2 -adrenoceptor selective agonists on the rat vas deferens and guinea-pig ileum

UK-14304 and clonidine produced a concentration-dependent inhibition of the twitch response of the rat isolated field-

stimulated vas deferens. Inhibition in excess of 90% was produced by UK-14304, 2×10^{-8} M, and clonidine, 1×10^{-7} M, respectively. Fluparoxan (0.25 – 50×10^{-7} M), idazoxan (0.25 – 10×10^{-7} M) and yohimbine (1 – 100×10^{-7} M) produced concentration-dependent, parallel rightward displacements of agonist concentration-inhibitory response curves with no suppression of the maximum attainable inhibitions. The results for fluparoxan are shown in Figure 2. The Schild plots (Arunlakshana & Schild, 1959) yielded straight lines with gradients not significantly different from unity except for the antagonism of clonidine by fluparoxan where the gradient was 1.29. The pK_B values and Schild plot gradients are shown in Table 1.

Fluparoxan (0.1 – 10×10^{-6} M), idazoxan (0.25 – 10×10^{-7} M) and yohimbine (0.1 – 10×10^{-6} M) were also potent and competitive antagonists of the inhibitory action of UK-14304 on the guinea-pig isolated and field-stimulated ileum.

Figure 2 Antagonism by fluparoxan of UK-14304-induced inhibition of the twitch response of the rat isolated and field-stimulated vas deferens (a) and of phenylephrine-induced contractions of the rat isolated anococcygeus muscle (b). In (a) results are the mean of 4–6 experiments for UK-14304 alone (○) and UK-14304 in the presence of 25 nM (●), 160 nM (□), 800 nM (■) and 5000 nM (△) fluparoxan; vertical bars show s.e.mean. In (b) results are the mean of 1–6 experiments for phenylephrine alone (●) and phenylephrine in the presence of 10 μM (▲), 100 μM (▲) and 1000 μM (■) fluparoxan; vertical bars show s.e.mean. Concentration-response curves were obtained by cumulative addition of UK-14304 or phenylephrine and a separate tissue was used for each curve. Experimental data illustrated in (a) and (b) are shown plotted according to Arunlakshana & Schild (1959) in (c) and (d) respectively. In (c) and (d) each point represents the result from a separate tissue compared with the mean control response in tissues treated with either UK-14304 or phenylephrine in the absence of fluparoxan and straight lines were fitted by linear regression analysis.

Table 1 Antagonist potencies at α_2 - and α_1 -adrenoceptors in rat vas deferens and anococcygeus muscle respectively

Agonist	Tissue	Mean pK_B value $\pm$ s.e. (Schild plot slope)		
		Fluparoxan	Idazoxan	Yohimbine
UK-14304	Vas deferens	7.87 ± 0.05 (1.02)	7.73 ± 0.06 (1.02)	7.59 ± 0.04 (1.08)
Clonidine	Vas deferens	7.91 ± 0.07 (1.23)*	8.12 ± 0.06 (0.98)	7.48 ± 0.07 (1.10)
Phenylephrine	Anococcygeus muscle	4.45 ± 0.15 (1.08)	6.27 ± 0.13 (0.90)	6.25 ± 0.08 (0.91)
Methoxamine	Anococcygeus muscle	4.88 ± 0.04 (1.16)	NT	NT

* Slope significantly different from unity (linear regression analysis).

$pK_B = (\text{dose-ratio} - 1) - \log [\text{antagonist}]$ where dose-ratio is the ratio of the agonist concentration producing half maximal responses in the presence and absence of the antagonists at the molar concentration [antagonist].

The effect of increasing concentrations of fluparoxan on the inhibitory potency of UK-14304 and the resulting Schild plots are shown in Figure 3. For fluparoxan, idazoxan and yohimbine the mean pK_B values $\pm$ standard errors obtained were 7.89 ± 0.07 (Schild slope 1.04), 8.13 ± 0.04 (Schild slope 0.92) and 7.64 ± 0.06 (Schild slope 0.98) respectively. In a separate study of the antagonism of UK-14304 in the rat vas deferens, fluparoxan was compared with its two enantiomers. There were no differences in potencies. Thus for the racemate (+)-fluparoxan and (–)-fluparoxan the respective mean pK_B values ($\pm$ standard errors and Schild plot gradients) were 7.86 ± 0.07 (slope = 1.09), 7.88 ± 0.08 (slope = 0.99) and 7.68 ± 0.06 (slope = 1.04).

Antagonism of contractions produced by α_1 -adrenoceptor selective agonists on the rat anococcygeus muscle

Fluparoxan (0.1 – 10×10^{-5} M), idazoxan (0.01 – 10×10^{-5} M) and yohimbine (0.1 – 10×10^{-5} M) were also competitive antagonists of phenylephrine-induced contractions of the rat isolated anococcygeus muscle. The effects of increasing concentrations of fluparoxan are shown in Figure 2. All three compounds were weaker antagonists of α_1 -adrenoceptors than of α_2 -adrenoceptors. Fluparoxan had at least 50 times lower affinity than that of the other two antagonists. The affinity constants and Schild slopes obtained are shown in Table 1

Figure 3 Antagonism by fluparoxan of UK-14304-induced inhibition of the twitch response of the guinea-pig isolated and field stimulated ileum. The results are means of 4–5 experiments for UK-14304 alone (○), and UK-14304 in the presence of $0.1 \mu\text{M}$ (●), $0.5 \mu\text{M}$ (□), $2.5 \mu\text{M}$ (■) and $10 \mu\text{M}$ (▲) fluparoxan; vertical bars show s.e.mean. The inset shows the data plotted according to Arunlakshana & Schild (1959). Values are mean responses obtained from doses of UK-14304 producing 50% inhibition of the twitch (IC_{50}) in individual tissues compared with the mean IC_{50} value in control tissues; vertical bars show s.e.mean.

and the Schild plot for the antagonism of phenylephrine by fluparoxan in Figure 2. On this tissue preparation there were also no differences between the affinities of the enantiomers of fluparoxan and the racemate against phenylephrine concentration-response curves (mean pK_B values $\pm$ s.e. = (+)-fluparoxan: 5.00 ± 0.26 , (+)-fluparoxan: 4.57 ± 0.14 , (–)-fluparoxan: 4.95 ± 0.16).

Antagonism of phenylephrine-induced contraction of the rabbit aortic strip

Fluparoxan behaved as a competitive antagonist of phenylephrine-induced contractions of the aortic strip producing a mean ($\pm$ s.e.) pK_B value of 4.32 ± 0.06 (slope = 1.01). In separate studies with idazoxan, concentrations in excess of 1×10^{-5} M occasionally produced a weak contraction of the tissue (up to 10% of the phenylephrine maximum). Idazoxan also behaved as a competitive antagonist of phenylephrine in this tissue (mean pK_B = 5.79 ± 0.08 ; slope = 0.83).

Antagonism of clonidine-induced hypothermia and antinociception in the mouse

In the conscious mouse, fluparoxan and idazoxan were of similar potency in preventing the hypothermia produced by clonidine 0.15 mg kg^{-1} i.p. Clonidine caused a fall in oesophageal temperature of $1.77 \pm 0.33^\circ\text{C}$ (mean $\pm$ s.e.; $n = 10$). This was prevented dose-dependently by oral fluparoxan, 0.2 – 3.0 mg kg^{-1} (15%–93%).

In a second study, clonidine-induced hypothermia was $2.5 \pm 0.2^\circ\text{C}$ (mean $\pm$ s.e.; $n = 16$) which was prevented dose-dependently by oral idazoxan 0.13 – 3.0 mg kg^{-1} (16%–76%). The oral doses of fluparoxan and idazoxan required to reduce by half the clonidine induced hypothermia were 0.68 ± 0.10 and $0.84 \pm 0.09 \text{ mg kg}^{-1}$ respectively.

In another study, fluparoxan and its two enantiomers were of similar potency in producing dose-related inhibitions of the hypothermia caused by clonidine. The oral ED_{50} values for the racemate, (+)-fluparoxan and (–)-fluparoxan for inhibition of hypothermia were 0.66 ± 0.10 , 0.73 ± 0.13 and $0.46 \pm 0.06 \text{ mg kg}^{-1}$ respectively.

In the antinociception study, an intraperitoneal injection of phenylbenzoquinone caused a characteristic abdominal constriction response that was abolished by pretreatment with clonidine 0.05 mg kg^{-1} s.c. Oral doses of fluparoxan, 0.38 – 3 mg kg^{-1} , idazoxan, 0.25 – 2.0 mg kg^{-1} and yohimbine, 0.63 – 5 mg kg^{-1} , prevented this antinociceptive action of clonidine dose-dependently and with similar potencies (Figure 4). The ED_{50} values were 0.75 (0.54 – 1.07), 0.65 (0.4 – 1.03) and 1.10 (0.28 – 1.94) mg kg^{-1} orally respectively.

Antagonism of UK-14304 induced hypothermia and impaired rotarod performance in the rat

In the rat UK-14304, 2 mg kg^{-1} s.c., produced hypothermia of 4 – 5°C and a marked impairment of rotarod performance.

Figure 4 Antagonism of clonidine-induced antinociception (phenylbenzoquinone-induced abdominal constriction) in the mouse. All animals, except the saline controls, received clonidine, 0.05 mg kg⁻¹ s.c., and either vehicle, fluparoxan, idazoxan or yohimbine orally at one of the stated doses. The proportion of animals exhibiting at least one abdominal constriction response over the following 10 min is indicated by the shaded histograms.

Both effects of the α_2 -adrenoceptor agonist were prevented dose-dependently by fluparoxan and idazoxan (Figure 5). Fluparoxan was of similar potency by either route of administration unlike idazoxan which, although similar in potency to fluparoxan following intravenous dosing, was much weaker when given orally.

Antagonism of UK-14304-induced sedation and bradycardia in the dog

UK-14304 (0.05 mg kg⁻¹ s.c.) caused marked sedation and bradycardia in beagles that was apparent after 30 min, peaked at 2–3 h and persisted for more than 5 h.

Fluparoxan, 0.22–6 mg kg⁻¹ orally, prevented both effects of UK-14304 in a dose-related fashion and the antagonism

Figure 5 Antagonism of UK-14304-induced hypothermia and impaired rotarod performance by fluparoxan (○) and idazoxan (●) in the rat. Values show mean temperature changes (a, b) or median rotarod scores (c, d) of treated rats compared with vehicle/saline or vehicle/UK-14304 treated (horizontal lines) controls using the oral (a, c) or intravenous (b, d) routes of administration; vertical bars show s.e.mean. Antagonism of rotarod impairment was significant where indicated (* P < 0.05, ** P < 0.01 by Mann-Whitney U-test).

Figure 6 Antagonism of UK-14304-induced sedation (a) and bradycardia (b) in the dog. Values are means (n = 10) at the indicated times for dogs given UK-14304 (0.05 mg kg⁻¹ s.c.) 30 mins after placebo (●), or fluparoxan 0.22 (○), 0.67 (■), 2 (□), or 6 (▲) mg kg⁻¹ orally. Behaviour was scored subjectively, a reduced score representing sedation. Heart rate was obtained by palpating the femoral artery. Pre-dose values were obtained before giving either treatment.

was maintained over the 5 h testing period (Figure 6). Analysis of the relationship between peak effect and dose of fluparoxan showed no differences in the ED₅₀ values against UK-14304-induced sedation and bradycardia (ED₅₀ values of 2.5 ± 0.1 mg kg⁻¹ and 1.9 ± 0.6 mg kg⁻¹ orally, respectively).

Actions of fluparoxan at non- α -adrenoceptor sites

Fluparoxan, $1-10 \times 10^{-5}$ M, was either inactive or weakly active at most non- α -adrenoceptor sites investigated (Table 2). At functional muscarinic sites in rat ileum there was no activity at 1×10^{-5} M and only weak affinity for muscarinic M₁ binding sites in rat cerebral cortex and M₂ sites in rat heart. No antihistamine activity was detected at either H₁- or H₂-histamine receptors at concentrations up to 3×10^{-5} M in rat atria. There was a five fold displacement of the isoprenaline dose-response curve preincubated with 3×10^{-5} M fluparoxan indicating weak β_1 -adrenoceptor antagonism but no activity at β_2 -adrenoceptors in guinea-pig ileum at 3×10^{-5} M. Fluparoxan was found to have some affinity for some 5-HT sites. There was weak affinity for 5-HT_{1A} (pIC₅₀ = 5.9) and 5-HT_{1B} (pK_i = 5.5) binding sites in rat brain but no agonist or antagonist activity at functional 5-HT₁-like and 5-HT₂ receptors in dog saphenous vein and rabbit aorta respectively.

At dopamine receptors fluparoxan was more than 200 times weaker than haloperidol in preventing [³H]-spiperone binding to rat striatal membranes. At D₂-receptors in the anaesthetized cat, fluparoxan, 0.1, 1.0 and 10.0 mg kg⁻¹ i.v. caused 1.2, 1.3 and 1.4 fold displacements respectively of dopamine-induced falls of electrically-stimulated increases in heart rate. Fluparoxan and desmethylimipramine inhibited the accumulation of [³H]-noradrenaline by rat occipital cortex synaptosomes in a concentration-dependent manner

Table 2 Activity of fluparoxan at receptors other than α -adrenoceptors

Receptor type	Tissue (response)	Competing drug	Fluparoxan (max. conc. tested, μM)	Comments
Muscarinic M ₃	Rat ileum (contraction)	Muscarine	10	Inactive
Muscarinic M ₂	Rat heart (binding)	[³ H]-N-methylscopolamine	100	pIC ₅₀ = 4.4
Muscarinic M ₁	Rat cerebral cortex (binding)	[³ H]-pirenzepine	100	pK _i = 4.0
5-HT ₁ -like	Dog saphenous vein (contraction)	5-HT	25	Inactive
5-HT _{1A}	Rat hippocampus (binding)	[³ H]-8-OH-DPAT	100	pIC ₅₀ = 5.9
5-HT _{1B}	Rat striatum (binding)	[³ H]-cyanopindolol	100	pK _i = 5.5
5-HT ₂	Rabbit aorta (contraction)	5-HT	50	Inactive
Histamine H ₁	Guinea-pig ileum (contraction)	Histamine	30	Inactive
Histamine H ₂	Guinea-pig atria (rate)	Histamine	30	Inactive
β_1 -Adrenoceptor	Rat atria (force of contraction)	Isoprenaline	30	pK _B $\approx$ 5
β_2 -Adrenoceptor	Guinea-pig trachea (relaxation)	Isoprenaline	30	Inactive
Dopamine D ₂	Rat striatum (binding)	[³ H]-Spiperone	1	13% displacement of binding

In functional models fluparoxan was tested for both agonist and antagonist activity. In binding assays pIC₅₀ represents the negative logarithm of the molar concentration of fluparoxan preventing by 50% the specific binding of the competing drug. In some studies a pK_i value was obtained by the method of Cheng & Prusoff (1973). pK_B is as defined in Table 1.

giving IC₅₀ values of $8.8 \times 10^{-5} \text{ M}$ and $5 \times 10^{-9} \text{ M}$ respectively.

Effects of rat cerebrocortical [³H]-dihydroalprenolol binding sites

Both fluparoxan and amitriptyline were very weak displacers of [³H]-dihydroalprenolol binding to cerebrocortical membranes from untreated rats with pK_i values (Hill slopes) of 4.05 (0.71) and 3.64 (1.05) respectively. For isoprenaline, the pK_i was 6.38 and the Hill slope was 0.69. The effect of twice daily, oral dosing with fluparoxan 6 and 12 mg kg⁻¹, amitriptyline 30 mg kg⁻¹ or a combination of both drugs for 6 days on the density of binding sites is shown in Table 3. The number of binding sites (B_{max}) was reduced by fluparoxan (9% and 21%) and amitriptyline (31%) without affecting their affinity (K_D) for the radioligand. The greatest reduction (40%) was produced in rats treated with the combination of fluparoxan, 6 mg kg⁻¹, and amitriptyline 30 mg kg⁻¹.

Discussion

Fluparoxan behaves as a novel, competitive antagonist having very high selectivity for α_2 -adrenoceptors. The pK_B values against the α_2 -adrenoceptor responses induced by the selective agonists UK-14304 and clonidine in rat vas deferens and guinea-pig ileum (Brown *et al.*, 1979; Drew, 1978) ranged from 7.86–7.91. Agonist concentration-response curves were displaced in a parallel fashion and the data gave straight line Schild plots which, with one exception, had slopes which were not significantly different from unity indicating competitive antagonism. The Schild-plot slope for the antagonism of clonidine by fluparoxan in the rat vas deferens was 1.23. The reason for the high value is not clear and requires further investigation. However, the pK_B values obtained for idazoxan and yohimbine were in agreement with generally quoted liter-

ature values. Fluparoxan was a much weaker competitive antagonist of the α_1 -adrenoceptor selective agonists phenylephrine and methoxamine in rat anococcygeus muscle and rabbit aorta preparations. The pK_B values obtained in rat vas deferens and anococcygeus muscle showed fluparoxan to have at least 1000 fold α_2 - α_1 -adrenoceptor selectivity making it one of the most selective α_2 -adrenoceptor antagonists so far described.

Many α -adrenoceptor antagonists with an imidazoline moiety display partial agonist activity (Ruffolo, 1983). For example, idazoxan displays partial α_1 -adrenoceptor agonism in some tissues including rabbit isolated aorta (Dalrymple *et al.*, 1983). In the present study, idazoxan, at high concentrations, sometimes caused a weak contraction of the rabbit aorta preparation. In contrast, fluparoxan, which does not have an imidazoline group, did not show any α -adrenoceptor agonist activity in this or any other preparation examined.

In studies to measure the α_2 -adrenoceptor antagonism produced *in vivo*, fluparoxan was effective by the oral route and of similar potency to idazoxan in preventing clonidine-induced hypothermia and antinociception in the mouse. Similarly, in the rat, fluparoxan prevented the hypothermia and impaired rotarod performance caused by UK-14304. Fluparoxan showed similar potency by both the oral and intravenous routes of administration indicating good oral bioavailability.

To interpret these antagonist effects it is important to consider the nature of the agonist-induced response. The hypothermia produced by clonidine, although in part a consequence of stimulation of α_2 -adrenoceptors in the brain, could have a histamine H₂-receptor mediated component (Bugajski *et al.*, 1980). While amphetamine-like drugs and β -adrenoceptor agonists also prevent clonidine-induced hypothermia (unpublished data) fluparoxan does not have histamine H₂-receptor antagonist, β -adrenoceptor agonist or amphetamine-like activity.

The prevention of clonidine- and UK-14304-induced hypothermia is therefore most probably attributable to blockade of

Table 3 Effect of twice daily dosing of fluparoxan and amitriptyline for 6 days on rat cerebrocortical [³H]-dihydroalprenolol binding sites

Compound	Dose (mg kg ⁻¹ twice daily)	B_{max} (fmol mg ⁻¹ protein)	% change	K_D (nM)	n
Water		149 $\pm$ 8		0.49 $\pm$ 0.02	7
Fluparoxan	6	136 $\pm$ 6	-9	0.53 $\pm$ 0.07	7
Fluparoxan	12	111 $\pm$ 10*	-26	0.48 $\pm$ 0.05	7
Amitriptyline	30	103 $\pm$ 7*	-31	0.56 $\pm$ 0.12	8
Amitriptyline + fluparoxan	30 6	89 $\pm$ 7*	-40	0.42 $\pm$ 0.04	8

* Significant decrease in B_{max} compared to placebo-treated group ($P < 0.05$, unpaired t test).

central α_2 -adrenoceptors. The antinociceptive activity of clonidine also appears to be α_2 -adrenoceptor-mediated (Paalzow & Paalzow, 1976; Skingle *et al.*, 1982), but could have been produced both at peripheral and at central sites (Bentley *et al.*, 1977; Spaulding *et al.*, 1979; Reddy *et al.*, 1980). Impairment of rotarod performance appears to reflect the sedative effects of α_2 -adrenoceptor agonists and is believed to be a centrally-mediated action (Drew *et al.*, 1979). In the dog, fluparoxan prevented both the sedative and cardiovascular responses to UK14304 with a sustained duration of action. The *in vivo* findings with fluparoxan are therefore indicative of potent and effective antagonism of α_2 -adrenoceptors at sites in the CNS. Further studies would be needed to show whether the compound displays differential activity at pre- and postsynaptic α_2 -adrenoceptors in the CNS, as could be shown by comparing potencies against clonidine induced mydriasis and hypoactivity (Heal *et al.*, 1989), or for α_{2A} and α_{2B} subtype binding sites (Bylund, 1988).

Fluparoxan is a racemate. Dabire *et al.* (1983) reported significant differences in the selectivity and potency of the enantiomers of idazoxan. In contrast, there were no significant differences between the enantiomers of fluparoxan in terms of α_2 - and α_1 -adrenoceptor antagonism in rat tissues *in vitro* and for their oral potency in preventing clonidine-induced hypothermia in the mouse. This was the anticipated result because fluparoxan is essentially a planar molecule and the two enantiomers are virtually superimposable. This contrasts with the enantiomers of idazoxan where the imidazoline side chain lies outside of the plane of the benzodioxan moiety.

In specificity studies, fluparoxan showed no appreciable affinity or activity at histamine H_1 - or H_2 -receptors, muscarinic acetylcholine receptors, β_2 -adrenoceptors, dopamine D_1 or D_2 receptors or functional 5-HT₁-like receptors in the dog saphenous vein. The antagonist action of fluparoxan at β_1 -adrenoceptors was about 1000 times less than that at α_2 -adrenoceptors. Fluparoxan displaced [³H]-8-OH-DPAT and [³H]-cyanopindolol binding to 5-HT_{1A} and 5-HT_{1B} sites respectively, but only at concentrations between 100 and 1000 times its affinity for α_2 -adrenoceptors.

Twice daily administration of fluparoxan to rats for six days resulted in a significant reduction in the number of cerebro-

cortical [³H]-dihydroalprenolol binding sites with no reduction in affinity. Other α_2 -adrenoceptor antagonists have been shown to produce this change (Swann *et al.*, 1981; Brown *et al.*, 1990) which could reflect an adaptation to a chronically increased synaptic concentration of noradrenaline since α_2 -adrenoceptor antagonists are known to increase noradrenaline turnover (Anden *et al.*, 1982; Walter *et al.*, 1984; Scheinin & Virtanen, 1986). Uptake inhibitors and monoamine oxidase inhibitors also bring about this adaptive change (Sellinger-Barnette *et al.*, 1980).

Inhibition of uptake is unlikely to explain why fluparoxan decreases [³H]-dihydroalprenolol binding site density because it has only very weak affinity for the uptake site. Furthermore, in separate unreported studies we have found that, unlike known monoamine oxidase inhibitors, fluparoxan does not prevent the depressant effects of tetrabenazine or the pressor and mydriatic effects of orally administered tyramine in rodents. The greatest reduction in the density of [³H]-dihydroalprenolol binding sites occurred in rats given both fluparoxan and amitriptyline. This has been found with other α_2 -adrenoceptor antagonists when coadministered with uptake inhibitors (Johnson *et al.*, 1980) and is consistent with a synergistic effect on noradrenaline turnover (Halliday & Ford, 1983).

In conclusion, fluparoxan is a novel, competitive α_2 -adrenoceptor antagonist in isolated tissues with a very high α_2 - to α_1 -adrenoceptor selectivity ratio. The results of *in vivo* studies indicate good bioavailability and a potent action mediated at α_2 -adrenoceptors in the CNS. The most likely therapeutic use for fluparoxan is in the treatment of depression, and in common with known antidepressant drugs fluparoxan reduces the density of rat cerebrocortical [³H]-dihydroalprenolol binding sites (Banerjee *et al.*, 1977).

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