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European Journal of Pharmacology 432 (2001) 9–17

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In vitro activity of LY393558, an inhibitor of the 5-hydroxytryptamine transporter with 5-$HT_{1B/1D/2}$ receptor antagonist properties

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Received 9 August 2001; received in revised form 10 October 2001; accepted 16 October 2001

Abstract

1-[2-[4-(6-fluoro-1*H*-indol-3-yl)-3,6-dihydro-1(2*H*)-pyridinyl]ethyl]-3-isopropyl-6-(methylsulphonyl)-3,4-dihydro-1*H*-2,1,3-benzothiadiazine-2,2-dioxide (LY393558) is a potent inhibitor of [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT) uptake into rat cortical synaptosomes ($pIC_{50} = 8.48 \pm 0.12$). It produces a dextral shift of the 5-HT dose–response curves for the binding of GTPγ[^{35}S] to human 5-HT_{1B} ($pK_b = 9.05 \pm 0.14$) and 5-HT_{1D} ($pK_b = 8.98 \pm 0.07$) receptors and inhibits the contractile response of the rabbit saphenous vein to the 5-$HT_{1B/D}$ receptor agonist, sumatriptan ($pK_b = 8.4 \pm 0.2$). In addition, it is an antagonist at the 5-HT_{2A} ($pK_i = 7.29 \pm 0.19$) and 5-HT_{2B} ($pK_i = 7.35 \pm 0.11$) receptors. Presynaptic autoreceptor antagonist activity was demonstrated by its ability to potentiate the K^+-induced outflow of [^{3}H]5-HT from guinea pig cortical slices ($pEC_{50} = 7.74 \pm 0.05$ nM) in which the 5-HT transporter had been inhibited by a maximally effective concentration of paroxetine. It is concluded that LY393558 should be an effective antidepressant with the potential to produce an earlier onset of efficacy than selective serotonin uptake inhibitors.

Keywords: 5-HT transporter; 5-HT_{1B} receptor; 5-HT_{1D} receptor; 5-HT_2 receptor; Autoreceptor; Antidepressant

1. Introduction

In common with other antidepressants, selective serotonin reuptake inhibitors take 2–3 weeks to produce a clear therapeutic improvement (Montgomery, 1995). Although this delay has been attributed to the requirement for an adaptive change to take place prior to the onset of efficacy, there is not universal agreement as to the nature of the change. Blier et al. (1990) have shown that long-term administration of a selective serotonin reuptake inhibitor results in desensitisation of 5-HT_{1A} receptors leading to tolerance to the reduction in 5-hydroxytryptamine (5-HT, serotonin) cell firing obtained after the acute administration of the selective serotonin reuptake inhibitor. The time-course of this desensitisation has led to the suggestion (Blier and De Montigny, 1994) that it underlies the antidepressant effect of selective serotonin reuptake inhibitors. In support of this claim, Perez et al. (1997) have shown that the 5-HT_{1A} receptor antagonist, pindolol, increases the clinical effectiveness of the selective serotonin reuptake inhibitor, fluoxetine.

Another adaptive change that has been implicated in the antidepressant effects of selective serotonin reuptake inhibitors has been the down-regulation of the 5-$HT_{2B/2C}$ receptor. Prisco and Esposito (1995) have shown that the acute administration of the selective serotonin reuptake inhibitor, fluoxetine, results in a dose-dependent inhibition of the firing rate of dopaminergic cells in the ventral tegmental area. After chronic fluoxetine administration, however, no such inhibition is observed. They claim that this is due to the desensitisation of the 5-$HT_{2B/2C}$ receptor as the 5-$HT_{2B/2C}$ receptor agonist, meta-chlorophenylpiperazine, inhibits the firing of ventral tegmental area dopaminergic neurones in control animals but not in those receiving long-term fluoxetine treatment. As dopaminergic neurones arising from the ventral tegmental area have been implicated in reward and incentive motivation (Fibiger, 1995), Bonhomme and Esposito (1988) have argued that the short-term inhibitory effect of fluoxetine on mesolimbic dopaminergic systems would mask its clinical efficacy in the early stages of treatment.

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0014-2999/01/$ - see front matter
PII: S0014-2999(01)01468-6

A third possibility that has been proposed (Rollema et al., 1996; Marcoli et al., 1999; Middlemiss et al., 1999) is that the clinical efficacy of short-term selective serotonin reuptake inhibitor administration is limited by the resulting stimulation of terminal presynaptic autoreceptors leading to a decreased 5-HT release. Therapeutic improvement is achieved only after these autoreceptors down-regulate and higher synaptic concentrations of 5-HT are achieved. In the rat (Engel et al., 1986), guinea pig (Selkirk et al., 1998; Bühlen et al., 1996) and human (Fink et al., 1995; Marcoli et al., 1999; Middlemiss et al., 1999) the majority of 5-HT autoreceptors are of the 5-HT_{1B} subtype. The fact that the ability of an acute dose of a selective serotonin reuptake inhibitor to elevate extracellular concentrations is limited by presynaptic autoreceptor stimulation has been shown by Rollema et al. (1996). They demonstrated, using microdialysis techniques, that the co-administration of a selective serotonin reuptake inhibitor (sertraline) and a 5-$HT_{1B/1D}$ receptor antagonist, *N*-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl) [1,1-biphenyl]-4-carboxamide (GR127935), led to a greater elevation in the concentration of 5-HT in the dialysate than can be achieved by the acute administration of the selective serotonin reuptake inhibitor alone. Although similar results were obtained using the selective serotonin reuptake inhibitor fluoxetine (Mitchell et al., in press), another study failed to confirm these effects (Sharp et al., 1997).

In addition to a delayed onset of efficacy, selective serotonin reuptake inhibitors produce a number of side effects ranging from sexual dysfunction, sleep disturbances, nausea, anxiety and reduced appetite (Montgomery, 1995; Davis et al., 1997). A number of these side effects have been attributed to stimulation of post-synaptic 5-HT_{2A} receptors (Davis et al., 1997). These claims are supported by the observations that stimulation of 5-HT_2 receptors disrupted sexual function (Foreman et al., 1992) and that the 5-HT_{2A} antagonist, SR 46349B ({*trans*,4-[(3*Z*)3-(2-dimethylaminoethyl)oxyimino-3(2-flurophenyl) propen-1-yl]phenol hemifumarate}), produced increases in both slow wave sleep and slow wave activity in male volunteers (Landolt et al., 1999).

In this paper, we describe the in vitro pharmacology of LY393558 (1-[2-[4-(6-fluoro-1*H*-indol-3-yl)-3,6-dihydro-1(2*H*)-pyridinyl]ethyl]-3-isopropyl-6-(methylsulphonyl)-3,4-dihydro-1*H*-2,1,3-benzothiadiazine-2,2-dioxide) (Fig. 1), a potent inhibitor of the 5-HT transporter and antagonist at the 5-HT_{1B}, 5-HT_{1D} and 5-HT_2 receptors.

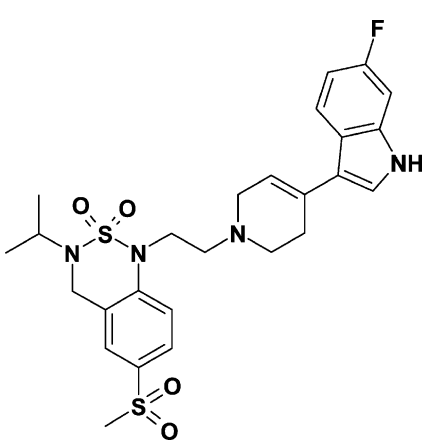

Fig. 1. Chemical structure of LY393558.

2. Materials and methods

2.1. Materials

Radiolabelled 5-HT (5-hydroxy[G-^{3}H]-tryptamine creatinine sulphate), noradrenaline (*l*-[7,8-^{3}H]noradrenaline), 8-OH-DPAT (8-hydroxy-2-(di-*n*-propylamino)tetraline) (8-hydroxy-[^{3}H]-DPAT), GR125743 (*N*-[4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]-3-methyl-4-(4-pyridyl)benzamide) ([*N*-methyl-^{3}H]-GR125743), anti-rabbit IgG and wheatgerm agglutinin treated polyvinyltoluene Scintillation Proximity Assay beads were obtained from Amersham Pharmacia Biotechnology (Buckinghamshire, UK and Piscataway, NJ, USA) and DOI (2,5-dimethoxy-4-iodoamphetamine) ([^{125}I]-(±)DOI) and GTPγ[^{35}S] from NEN Life Science Products (Boston, MA, USA). NP-40 was obtained from Boehringer-Mannheim (Indianapolis, IN, USA), rabbit polyclonal anti-Gαq/11antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and imipramine and GDP from Sigma (Poole, UK). Indalpine, fluoxetine, sumatriptan, SB224289 (1′-methyl-5-[[2′-methyl-4′(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7-tetrahydrospiro[furo[2,3-f]indole-3,4′-piperidine] oxalate) and GR127935 were provided by the Eli Lilly Research Laboratories (Surrey, UK). Paroxetine was a gift from SmithKline Beecham (Harlow, UK).

2.2. 5-HT receptor selectivity

Standard receptor-binding assay methods were used to evaluate the ability of LY393558 to interact with human 5-HT_1, 5-HT_2 and 5-HT_7 receptors as well as with the rat 5-HT transporter. The source of the receptors, the ligands and the methods used are shown in Table 1.

2.3. [^{3}H]5-HT and [^{3}H]noradrenaline uptake

The method used was the same as that described previously (Pullar et al., 2000b). Synaptosomes prepared from the cerebral cortex of male Lister–Hooded rats were incubated at 37 °C for 15 min in assay buffer pH 7.4 (10 mM HEPES, 133 mM NaCl, 4.85 mM KCl, 1.2 mM KH_2PO_4, 1.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 11.1 mM glucose, 10 μM pargyline) with either the test compound, assay buffer (total uptake) or 100 μM paroxetine (non-specific 5-HT

Table 1
The receptor type, tissue and ligands used in the receptor binding assays

Receptor	Reference	Species	Tissue	Radioligand	pK_i
5-$HT_{transporter}$	Pullar et al., 2000b	Rat	Cerebral cortex	[^{3}H]citalopram	8.67 ± 0.07
5-HT_{1A}	Zgombick et al., 1991	Human	Transfected L-M (tk-)	[^{3}H]5-HT	27 ± 8%[a]
5-HT_{1B}	Pullar et al., 2000b	Human	Transfected L-M (tk-)	[^{3}H]GR125743	7.05 ± 0.07
5-HT_{1D}	Pullar et al., 2000b	Human	Transfected L-M (tk-)	[^{3}H]GR125743	6.83 ± 0.29
5-HT_{1E}	Zgombick et al., 1991	Human	Transfected L-M (tk-)	[^{3}H]5-HT	9 ± 5%[a]
5-HT_{1F}	Adham et al., 1993	Human	Transfected L-M (tk-)	[^{3}H]5-HT	18 ± 2%[a]
5-HT_{2A}	Pullar et al., 2000b	Human	Transfected Hm2.3	[^{3}H]Ketanserin	9.06 ± 0.06
5-HT_{2B}	Wainscott et al., 1996	Human	Transfected AV12	[^{3}H]5-HT	8.09 ± 0.20
5-HT_{2C}	Wainscott et al., 1996	Human	Transfected AV12	[^{125}I]DOI	7.91 ± 0.01
5-HT_7	Gustafson et al., 1996	Human	Transfected L-M (tk-)	[^{3}H]5-HT	6 ± 4%[a]

Data expressed as mean ± S.E.M. of at least three independent experiments.
[a] Percent inhibition of binding at 1000 nM.

uptake) or 100 μM nomifensine (non-specific noradrenaline uptake). Following the addition of [^{3}H]5-HT or [^{3}H]noradrenaline (final concentrations; 50 nM) to each well, the incubation was continued for a further 15 min and the uptake of the [^{3}H]5-HT or [^{3}H]noradrenaline was terminated by filtration. The filters were dried and the radioactivity determined by scintillation spectroscopy. The results were analysed using an automatic spline-fitting program and IC_{50} values determined.

2.4. GTPγ[^{35}S] binding to human 5-HT_{1B} and 5-HT_{1D} receptors

2.4.1. Membrane preparation

LM(tk-) or AV12 cells stably transfected with human 5-HT_{1B} or 5-HT_{1D} receptors, respectively, were washed with and then homogenised in Tris buffer (20 mM Tris–HCl, 5 mM EDTA pH 7.4). After centrifugation at 1500 × *g* for 10 min at 4 °C, the supernatant was recentrifuged at 40,000 × *g* for 18 min at 4 °C and the resultant pellet washed with Tris buffer and finally resuspended in assay buffer (100 mM NaCl, 10 mM $MgCl_2$, 1.0 mM EDTA and 20 mM HEPES, pH 7.4). The protein concentration was measured (Lowry et al., 1951) adjusted with assay buffer to 400 μg ml^{-1} for the 5-HT_{1B} containing membranes and 600 μg ml^{-1} for the 5-HT_{1D} before storage at −70 °C for up to 6 weeks.

2.4.2. 5-HT_{1B} GTPγ[^{35}S] assay

All reagents were diluted in assay buffer containing 167 μg ml^{-1} dithiothreitol. Membranes from the LM(tk-) cells (10 μg), in a final volume of 250 μl, were preincubated for 30 min at 30 °C with GDP (10 μM) and 5-HT (0 to 10 μM) in the presence and absence of 300 nM test compound. Following the addition of GTPγ[^{35}S] (0.2 nM) and 1.5 mg wheatgerm agglutinin treated polyvinyltoluene Scintillation Proximity Assay beads, the incubation was continued for a further 1 h before centrifugation at 650 × *g* for 10 min at 4 °C. The radioactivity in close proximity to the beads was assessed by scintillation spectroscopy. K_B values for the test compounds were calculated from the dextral shift of the 5-HT dose–response curve.

2.4.3. 5-HT_{1D} GTPγ[^{35}S] assay

This assay was essentially the same as that for 5-HT_{1B} GTPγ[^{35}S] with the following exceptions. Each incubation contained 15 μg membrane protein from the 5-HT_{1D} expressing AV12 cells in 250 μl of assay buffer which did not contain dithiothreitol.

2.5. GTPγ[^{35}S] binding to human 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors

The GTPγ[^{35}S] immunoadsorption scintillation proximity assay has been described previously for the cloned muscarinic acetylcholine receptors (DeLapp et al., 1999) and the 5-HT_2 subfamily of receptors (Conway et al., 1999). Briefly, frozen AV12 cells stably transfected with human 5-HT_{2A}, 5-HT_{2B} or 5-HT_{2C} receptors (Lucaites et al., 1996) were thawed, washed with Tris buffer (50 mM Tris–HCl, 100 mM NaCl, 10 mM $MgCl_2$, 0.2 mM EDTA, pH 7.4) and incubated for 10 min at 37 °C. After a further wash in Tris buffer, they were suspended to give 3–4 × 10^6 cell equivalents per well of the micro titre plate. GTPγ[^{35}S] (final concentration, 0.25 nM) and GDP (final concentration, 100 nM), in the presence or absence of 5-HT, were added to the plates and, following the addition of LY393558, the guanine nucleotide exchange reaction was initiated by the addition of cell membranes. For evaluation of LY393558 as an agonist, its activity was assessed over a concentration range of 10 pM to 10 μM in the absence of 5-HT. When LY393558 was assayed for antagonist activity, 5-HT was added to give a final concentration of 100 nM for the 5-HT_{2A} and 5-HT_{2B} assays, or 10 nM for the 5-HT_{2C}. The assay volume was 200 μl. Plates were incubated at room temperature for 30 min and NP-40 detergent was added (final concentration, 0.27% w/v) to stop the reaction by solubilizing the membranes. After the addition of 1–2 μg anti-Gα q/11 rabbit polyclonal antibody to each well, the microtitre plate was incubated at room

Table 2
Inhibition of [^{3}H]5-HT and [^{3}H]noradrenaline uptake (pIC_{50}) into rat brain synaptosomes

Compound	Uptake into synaptosomes (pIC_{50})	
	[^{3}H]5-HT	[^{3}H]noradrenaline
LY393558	8.48 ± 0.12	5.76 ± 0.11
Fluoxetine	7.46 ± 0.06	5.45 ± 0.05
Paroxetine	9.15 ± 0.10	6.74 ± 0.04
Indalpine	8.19 ± 0.08	< 6.0
Imipramine	7.44 ± 0.09	6.85 ± 0.09

Data expressed as mean ± S.E.M. of at least four independent experiments.

temperature for 1 h to immunoadsorb the GTPγ[^{35}S] bound to the Gα q subunit. Finally, goat anti-rabbit IgG coated wheatgerm agglutinin treated polyvinyltoluene Scintillation Proximity Assay beads (1.25 mg per well) were added (final volume, 300 μl), the plates sealed and incubated for an additional 3 h at room temperature before centrifugation and scintillation spectroscopy. Inhibition curves were analysed by nonlinear regression curve fitting using GraphPad Prism software (San Diego, CA), and K_i values were calculated from the IC_{50} by the method of Cheng and Prusoff (1973).

2.6. Rabbit saphenous vein

Male New Zealand White rabbits (1.5–3.0 kg) (Myrtle Rabbitry, Thompson Station, TN) were sacrificed by a lethal dose of sodium pentobarbital (200 mg) injected into the ear vein. The saphenous vein was dissected free from connective tissue, cannulated in situ with polyethylene tubing (PE #50), placed in a petri dish containing modified Krebs' bicarbonate buffer (118.2 mM NaCl, 4.6 mM KCl, 1.6 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10.0 mM dextrose and 24.8 mM $NaHCO_3$), and ring preparations were obtained.

Tissues were mounted in organ baths containing 10 ml of modified Krebs' solution maintained at 37 °C and aerated with 95% O_2/5% CO_2 to give a pH of 7.4. An initial optimum resting force of 4 g was applied to the rabbit saphenous vein as determined in preliminary length tension studies using a KCl (67 mM) challenge. Isometric contractions were recorded as changes in grams on a Beckman Dynograph with Statham UC-3 transducers or with a Macintosh-Compatible Data Acquisition System (BIOPAC Systems, Santa Barbara, CA). Tissues were allowed to equilibrate for 1–2 h before exposure to compounds.

Cumulative concentration–response curves were generated to LY393558, sumatriptan or vehicle, and no tissue was used to generate more than one agonist concentration–response curve. In other experiments, tissues were pre-exposed to vehicle or LY393558 (10 or 100 nM) for 1 h prior to initiating a response to sumatriptan. The antagonist equilibrium dissociation constant (K_B) for LY393558 versus sumatriptan was determined according to Furchgott (1972) and was expressed as its negative logarithm (pK_b). All results are expressed as mean ± S.E.M.. The data are expressed as a percentage of the response to a maximal contractile concentration of KCl (67 mM) administered initially in each tissue.

2.7. [^{3}H]5-HT outflow from guinea pig cortical slices

The assay was essentially similar to that described previously (Pullar et al., 2000b). Male guinea pigs (350–400 g) were killed by asphyxiation and their brains rapidly removed. Cortical slices (350 × 350 μm) were prepared, washed once in basal buffer (10 mM HEPES, 133 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 11.1 mM glucose, 10 μM pargyline, pH 7.4) and incubated in basal buffer at 25 mg ml^{-1} wet weight with [^{3}H]5-HT (50 nM) for 30 min at 37 °C. The slices were washed three times in basal buffer and transferred to baskets (10 mm i.d. polypropylene tubes with 150 μm nylon mesh bases) at approximately 5 mg wet weight per basket. The baskets were used to transfer the tissue between the washing and release buffers.

In order to obtain a stable baseline release, the slices were incubated for 11 min in basal buffer (0.5 ml), transferred for 4 min to a second tube containing basal buffer (0.5 ml) and then, for a further 4 min, to basal buffer (0.5 ml) or to a buffer in which NaCl had been substituted with KCl, on an equimolar basis, to give a KCl concentration of 30 mM (release sample). All buffers used in the 11 min and the two 4 min incubations contained 1 μM paroxetine. Following the incubations, the tissue was digested with Soluene-350 (0.7 ml) and the baskets rinsed with propan-2-ol (0.7 ml). The tritium label in the tissue samples and in the buffers from the three incubation periods was estimated by liquid scintillation spectroscopy. The compounds being tested were present throughout the three incubation periods and were each tested in six replicates. The basal release was measured in four replicates and the control release in eight replicates.

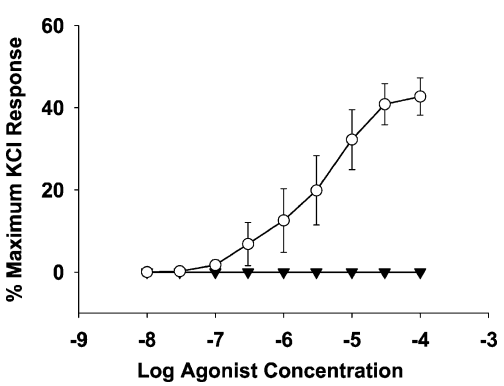

Fig. 2. Contractile concentration–response curves for sumatriptan (○) and LY393558 (▼) in the rabbit saphenous vein. Points are the mean values and vertical bars represent the S.E.M. of at least three independent experiments.

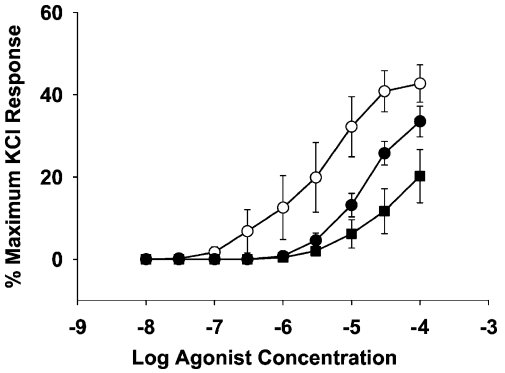

Fig. 3. Contractile concentration–response curves for sumatriptan in the rabbit saphenous vein in the presence of vehicle (○), 10 nM LY393558 (●) or 100 nM LY393558 (■). Points are the mean values and vertical bars represent the S.E.M. of at least three independent experiments.

The tritium label in the release sample was expressed as the percentage of the total tritium in the tissue at the time the sample was collected (percent fractional release). Stimulated release was calculated as the percent fractional release produced by the high potassium buffer minus that produced by the basal buffer over the same time interval. The percentage increase in release produced by the compound was calculated as the increase over the control stimulated release, where the control release is 100%. For individual experiments the mean of the replicate data was calculated. The results are the means and standard errors of at least three separate experiments.

3. Results

3.1. 5-HT receptor selectivity

As can be seen from Table 1, LY393558 has a high affinity for the rat 5-HT transporter as well as for the 5-HT_2 receptors. It has a lower affinity for the 5-HT_{1B} and 5-HT_{1D} receptors and is relatively inactive at the 5-HT_{1A}, 5-HT_{1E}, 5-HT_{1F} and 5-HT_7 receptors.

3.2. [^{3}H]5-HT and [^{3}H]noradrenaline uptake

LY393558 is a potent inhibitor of the uptake of [^{3}H]5-HT into synaptosomes prepared from rat cerebral cortex (Table 2) and like the selective serotonin reuptake inhibitors fluoxetine, paroxetine and indalpine it has marked selectivity for the 5-HT transporter over that for [^{3}H]noradrenaline. The tricyclic antidepressant, imipramine, also inhibits the 5-HT transporter with a potency similar to that of fluoxetine but does not show selectivity for this transporter over that for noradrenaline.

Table 3
pK_b value for the inhibition of 5-HT-stimulated GTPγ[^{35}S] binding to cells stably transfected with human 5-HT_{1B} and 5-HT_{1D} receptors

Compound	5-HT_{1B} (pK_b)	5-HT_{1D} (pK_b)
LY393558	9.05 ± 0.14	8.98 ± 0.07
GR127935	9.72 ± 0.12	10.00 ± 0.06
SB224289	9.11 ± 0.29	< 6.0

Data expressed as mean ± S.E.M. of at least four independent experiments.

Table 4
pEC_{50} and pK_b values for GTPγ[^{35}S] binding to cells stably transfected with human 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors

Receptor	pEC_{50}	pK_b
Human 5-HT_{2A}	< 5.00	7.29 ± 0.19
Human 5-HT_{2B}	< 5.00	7.35 ± 0.11
Human 5-HT_{2C}	< 5.00	< 5.52

Data expressed as mean ± S.E.M. of at least three independent experiments.

3.3. Rabbit saphenous vein

Sumatriptan (100 nM–100 μM) produced a marked contraction of the rabbit saphenous vein (Fig. 2). In contrast, LY393558 (in concentrations up to 100 μM) did not contract the rabbit saphenous vein. Because contraction was not observed, LY393558 (10 and 100 nM) was examined for its ability to inhibit the contraction produced by sumatriptan.

LY393558 produced a concentration-dependent dextral shift in the contractile response to sumatriptan indicating that LY393558 was an antagonist at the serotonergic contractile receptor in the rabbit saphenous vein (Fig. 3). The

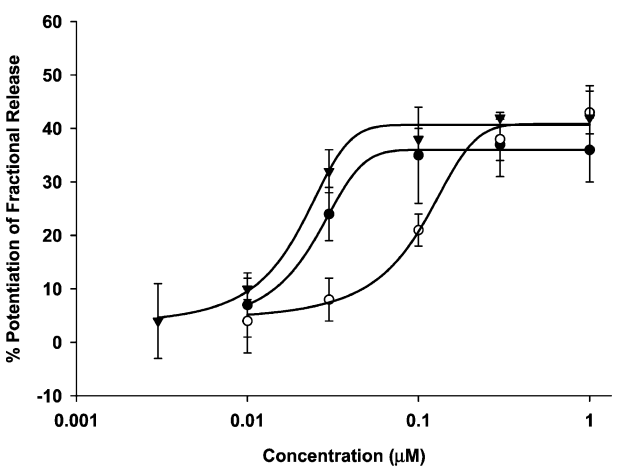

Fig. 4. Potentiation of K^+-induced [^{3}H]5-HT outflow from guinea pig cortical slices in the presence of 1.0 μM paroxetine. The potentiation in each experiment is expressed as the percent increase in fractional release above that obtained in the absence of compound. GR127935 (●), SB224289 (○) and LY393558 (▼) were tested in log concentration increments and the points are the mean values and vertical bars represent the S.E.M. of at least five independent experiments. All curves are fitted with a four-parameter logistic equation.

negative logarithm of the antagonist equilibrium dissociation constant (pK_b) for the interaction of LY393558 with a contractile serotonergic receptor in the saphenous vein was 8.4 ± 0.2 ($n = 9$). Thus, LY393558 was a potent antagonist of the contraction to sumatriptan.

3.4. GTPγ[^{35}S] binding to human 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors

LY393558 is a potent inhibitor of the 5-HT-induced binding of GTPγ[^{35}S] to both the human 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors (Table 3). The pK_b values obtained are very similar to that obtained for the inhibition of sumatriptan-mediated contraction of the rabbit saphenous vein. It is about 10-fold less active at either of these receptors than is the mixed 5-HT$_{1B/1D}$ antagonist GR127935. SB224289 shows the expected selectivity for the 5-HT$_{1B}$ receptor.

3.5. GTPγ[^{35}S] binding to human 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors

LY393558 was assessed for agonist and antagonist activity at human 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors (Table 4). At concentrations up to the highest tested (10 μM) it did not show any agonist activity. The pK_i values at the 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors are in good agreement with those obtained in the receptor-binding assays. At the 5-HT$_{2C}$ receptor, the pK_i (< 5.52) was less than that in the binding assay but in each case the compound had the lowest level of activity at this receptor.

3.6. [^{3}H]5-HT outflow from guinea pig cortical slices

The concentration–response curve and pEC_{50} values for the potentiation of K^+-induced [^{3}H]5-HT outflow from guinea pig cortical slices are shown in Fig. 4 and Table 5, respectively. In this preparation, the 5-HT$_{1B}$ receptor antagonist, SB224289 and the mixed 5-HT$_{1B/1D}$ receptor antagonist, GR127935, potentiate release. The pEC_{50} value obtained with LY393558 was similar to that for GR127935 but was greater than that for SB224289. The potentiation of outflow produced by LY393558 was not due to inhibition of the 5-HT transporter as fluoxetine, at a concentration of 1 μM, was inactive in this test (3% potentiation, data not shown).

Table 5
pEC_{50} values for the potentiation of potassium-stimulated [^{3}H]5-HT release from guinea pig cortical slices in the presence of 1 μM paroxetine

Compound	Potentiation of release pEC_{50} values
LY393558	7.74 ± 0.05
GR127935	7.59 ± 0.05
SB224289	6.89 ± 0.03

Data expressed as mean ± S.E.M. of at least five independent experiments.

4. Discussion

The high affinity of LY393558 for the 5-HT transporter and potent inhibition of [^{3}H]5-HT uptake into rat cortical synaptosomes indicate that it may possess antidepressant activity. In keeping with the selective serotonin reuptake inhibitors fluoxetine, paroxetine and indalpine it is greater than 100 times more potent at inhibiting the uptake of [^{3}H]5-HT than [^{3}H]noradrenaline into rat brain synaptosomes. In contrast, imipramine has less than 4 fold selectivity for the [^{3}H]5-HT transporter.

In addition to being a potent inhibitor of the 5-HT transporter, LY393558 is an antagonist at the 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors. Although the affinity for these receptors is low ($pK_i = 7.05$ and 6.83, respectively), it is a potent inhibitor of 5-HT stimulated GTPγ[^{35}S] binding to cloned human 5-HT$_{1B}$ ($pK_b = 9.05$) and 5-HT$_{1D}$ ($pK_b = 8.98$) receptors. The reason for this discrepancy between affinity and function at the 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors is unknown. In the receptor-binding assay, LY393558 competes with the 5-HT$_{1B/1D}$ receptor antagonist, [^{3}H]GR125743, for binding sites on the cloned receptors whilst in the functional, GTPγ[^{35}S] binding assay competition is with the natural agonist, 5-HT. It is possible that the binding sites for GR125743 and 5-HT are not identical leading to their differential displacement by LY393558. Matzen et al. (2000) have described a similar dissociation of affinity and function for compounds structurally related to LY393558. Further evidence that LY393558 is a potent functional inhibitor of these receptors is obtained from the observation that it produces a dextral shift of the contractile response curve of the rabbit saphenous vein to the 5-HT$_{1B/1D}$ receptor agonist, sumatriptan with a potency ($pK_b = 8.61$) similar to that obtained in the 5-HT stimulated GTPγ[^{35}S] binding to human 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors. The contractile response of the rabbit saphenous vein to sumatriptan is mediated via a 5-HT$_{1B/1D\text{-like}}$ receptor (Cohen and Schenck, 1999). It has been suggested that the vascular receptor most likely to be responsible for sumatriptan-induced contraction of the rabbit saphenous vein is the 5-HT$_{1B}$ receptor (Kaumann et al., 1994; Bouchelet et al., 1996; Ullmer et al., 1995; De Vries et al., 1999; Verheggen et al., 1998).

Middlemiss et al. (1999) have demonstrated that the 5-HT$_{1B}$ receptor antagonist, 1′-ethyl-5-(2′-methyl-4′-(5-methyl-1,3,4-oxadiazol-2-yl)biphenyl-4-carbonyl)-2,3,6,7-tetrahydrospiro[furo[2,3-f]indole-3,4′-piperidine] (SB23 6057), reverses 5-HT-induced suppression of electrically evoked [^{3}H]5-HT release from guinea pig cortical slices and have ascribed this activity to the inhibition of presynaptic autoreceptors. In keeping with this observation, LY393558, like the mixed 5-HT$_{1B/1D}$ receptor antagonist, GR127935, and the 5-HT$_{1B}$ antagonist, SB224289, potentiates the K^+ stimulated outflow of [^{3}H]5-HT from guinea pig cortical slices. This is not due to the potent 5-HT transporter inhibition by LY393558 as the assay is carried

out in the presence of a maximally effective concentration of the selective serotonin reuptake inhibitor, paroxetine. Under these conditions, the selective serotonin reuptake inhibitor fluoxetine has been shown to be inactive (data not shown). The increase in K^+-induced outflow of [^{3}H]5-HT resulting from the presence of 1 μM paroxetine (> 150%, Pullar et al., 2000b) will increase the tone of the presynaptic autoreceptor enabling antagonist activity to be more easily detected.

It is generally recognised that the 5-HT_{1B} receptor is a presynaptic autoreceptor controlling transmitter release in the rat (Engel et al., 1986), guinea pig (Selkirk et al., 1998; Bühlen et al., 1996) and human (Fink et al., 1995; Marcoli et al., 1999; Middlemiss et al., 1999). It is of interest that, even though SB224289 has a similar pK_b value to LY393558 and GR127935 in the 5-HT_{1B} GTPγ[^{35}S] binding assay, it is less active at potentiating K^+-stimulated [^{3}H]5-HT release from guinea pig cortical slices. SB224289 differs from the other two compounds in that it has very little activity at the 5-HT_{1D} receptor. We have shown, previously, that the 5-HT_{1D} receptor can function as a presynaptic autoreceptor in the guinea pig (Pullar et al., 2000a). The greater effect of GR127935 and LY393558 at increasing potassium-stimulated [^{3}H]5-HT release may, therefore, be due to the combined activity at the 5-HT_{1B} and 5-HT_{1D} receptors.

It has been postulated that the ability of the acute administration of a selective serotonin reuptake inhibitor to elevate synaptic concentrations of 5-HT is limited by the concomitant stimulation of inhibitory presynaptic autoreceptors and that clinical efficacy is obtained only after down regulation of these receptors (for review, see Moret and Briley, 2000). It follows, therefore, that the acute administration of a compound having functional 5-HT presynaptic autoreceptor inhibitory activity of a similar potency to its ability to block 5-HT reuptake should produce a greater increase in synaptic 5-HT concentrations than is possible with a selective serotonin reuptake inhibitor. In microdialysis studies, this has been shown to be the case with LY393558 (Mitchell et al., in press).

Evidence that the chronic selective serotonin reuptake inhibitor administration required for clinical efficacy down-regulates presynaptic 5-HT autoreceptors is provided by the studies of Moret and Briley (1990). They showed that the amount of [^{3}H]5-HT released by electrical stimulation of hypothalamic slices from rats chronically treated with citalopram was enhanced and the inhibitory effects of the agonist, lysergic acid diethylamide, reduced. In addition, these authors (Moret and Briley, 1996) observed that, in microdialysis studies, the nonselective 5-HT_{1B} receptor antagonist methiothepin, has a greater maximal effect on 5-HT outflow from rats treated acutely with citalopram than those treated chronically. In the rat dorsal raphé nucleus, chronic treatment with the selective serotonin reuptake inhibitor paroxetine, reduces the ability of the 5-HT_{1B} receptor agonist to inhibit electrically evoked 5-HT release (Davidson and Stamford, 2000) indicating a desensitisation of the 5-HT_{1B} receptor. Chronic administration of the tricyclic antidepressant clomipramine, which has a high affinity for the 5-HT transporter, also has been shown to reduce the sensitivity of presynaptic 5-HT_{1B} receptors as measured by the response to the 5-$HT_{1B/1D}$ receptor antagonist GR127935 (Newman et al., 2000).

The observation (Foreman et al., 1992; Klint and Larsson, 1995; Watson and Gorzalka, 1991) that 5-HT_2 receptor antagonists improve sexual function in rats has led to the suggestion that the sexual side effects obtained on the administration of selective serotonin reuptake inhibitors are due to an increased stimulation of this receptor. This is supported by the clinical observation that, in contrast to the selective serotonin reuptake inhibitor sertraline, the antidepressant nefazodone, which is a 5-HT_{2A} receptor antagonist, does not adversely affect sexual function. In addition, the antidepressant, mirtazapine, which is a potent 5-HT_2 receptor antagonist, has a beneficial effect on previously untreated depressed patients with sexual difficulties (Boyarsky et al., 1999).

Increased 5-HT_{2A} receptor stimulation has been implicated, also, in the sleep disturbances produced by selective serotonin reuptake inhibitor treatment. Dugovic et al. (1989) have shown that in the rat, 5-HT_2 agonists increase wakefulness and decrease slow-wave sleep and that this can be reversed by 5-HT_2 antagonists. This is probably due to an effect on the 5-HT_{2A} receptor as Landolt et al. (1999) have shown that the selective 5-HT_{2A} receptor antagonist, SR46349B, produces an increase in slow wave sleep in humans with no influence on the amount and latency to rapid eye movement sleep.

Although LY393558 has a high affinity for the 5-HT_2 receptors, its functional activity, as indicated by its ability to inhibit 5-HT stimulated GTPγ[^{35}S] binding to the cloned human receptors is low. There remains the possibility, therefore, that it may not be an effective 5-HT_2 antagonist at doses which inhibit the 5-HT_{1B}, 5-HT_{1D} receptors and block the 5-HT transporter.

In conclusion, LY393558 is a potent inhibitor of the 5-HT transporter and antagonist of the 5-$HT_{1B/1D}$ receptors. As such it should be an effective antidepressant with the potential for producing an early onset of efficacy. Its antagonist activity at the 5-HT_{2A} receptor may result in fewer sleep disturbances and less sexual dysfunction than obtained with selective serotonin reuptake inhibitors.

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