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GR127935 acts as a partial agonist at recombinant human 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors

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Abstract

In this study we have investigated the functional activity of GR127935 (2-methyl-4-(5-methyl-1,2,4 oxadiazol-3-yl)-biphenyl-[4-carboxylic acid 4-methoxy-3-(4-methyl-piperazine-1-yl)-phenyl]-amide) at human 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors which have been expressed in a Chinese Hamster Ovary (CHO) cell line. Using [^{35}S]GTPγS binding to cell membranes as a measure of receptor-G protein coupling, GR127935 showed partial agonist activity in both 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptor expressing cells (E_{max}: 29 and 31% above basal control; pEC_{50}: 8.6 and 9.7, respectively). GR127935 also acted as a potent antagonist at the 5-$HT_{1D\alpha}$ (app. pA_2 8.5) and 5-$HT_{1D\beta}$ (app. pA_2 9.1) receptors. From studies measuring cAMP accumulation in cultured CHO cells GR127935 also displayed partial agonism, as well as acting as a potent antagonist at the 5-$HT_{1D\alpha}$ receptors which stimulate cAMP levels and 5-$HT_{1D\beta}$ receptors which inhibit cAMP levels (app. pA_2 8.6 and 9.7, respectively). The 5\-HT_1-like receptor antagonist methiothepin showed negative intrinsic activity at both receptors in the [^{35}S]GTPγS binding assay only. From studies using the receptor alkylating agent EEDQ (*N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline) the 5-$HT_{1D\alpha}$ cell line displayed a lack of receptor reserve but it was evident in the 5-$HT_{1D\beta}$ cell line. In previous studies we have also shown that agonist stimulation of 5-$HT_{1D\alpha}$ receptors increases cAMP levels which may be due to high receptor expression. Further investigation using up to 1 μM EEDQ to reduce 5-$HT_{1D\alpha}$ receptor number did not reveal an underlying inhibitory adenylyl cyclase response. In conclusion, GR127935 acts as a partial agonist, aswell as a potent antagonist, at the human 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors when expressed in CHO cells.

Keywords: GR127935; 5-$HT_{1D\alpha}$ receptor; 5-$HT_{1D\beta}$ receptor; [^{35}S]GTPγS binding; cAMP accumulation; Receptor reserve

1. Introduction

The 5-hydroxytryptamine$_1$ (5-HT_1) receptor family has been classified into five different subtypes, namely 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F} (Hoyer et al., 1994). In human, the 5-HT_{1D} receptor has been subdivided into 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptor subtypes (Weinshank et al., 1992), the latter being the human homologue of the rat 5-HT_{1B} receptor. The amino-acid sequence of the two 5-HT_{1D} subtypes revealed that they were closely related, although with an overall amino acid identity of 63% the two receptors are quite distinct.

However, despite this modest homology, 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors display a very similar pharmacology. It has been reported that both 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors are negatively coupled to adenylyl cyclase and thus decrease forskolin-stimulated cAMP accumulation (Weinshank et al., 1992; Hamblin and Metcalf, 1991; Van Sande et al., 1993). However we have previously reported that 5-$HT_{1D\alpha}$ receptors, when expressed in Chinese Hamster Ovary (CHO) cells, stimulate cAMP production (Watson et al., 1994).

In the central nervous system, the 5-HT_{1D} receptor is thought to function as a presynaptic autoreceptor (Hoyer and Middlemiss, 1989; Schlicker et al., 1989) but definitive characterisation requires potent and selective antagonists. GR127935 (2-methyl-4-(5-methyl-1,2,4 oxadiazol-3-yl)-biphenyl-[4-carboxylic acid 4-methoxy-3-(4-methyl-piperazine-1-yl)-phenyl]-amide) is a potent and orally active 5-HT_{1D} receptor antagonist (Skingle et al., 1993) and has allowed characterisation of several reported 5-HT_{1D} receptor functions. GR127935 antagonises 5-HT_{1D} receptor-mediated responses such as sumatriptan-induced contractions in dog basilar artery, contralateral turning elicited

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by a unilateral intranigral infusion of the 5-HT_1 receptor agonist GR56764 (5-(3-methylamino-ethyl)-1*H*-indol-5-yl-1*H*-1,2,4 triazol-3-yl)methanol) into guinea-pig substantia nigra, stimulation of central 5-HT_{1D} receptors causing hypothermia in the guinea-pig and 5-HT-induced inhibition of 5-HT release from electrically stimulated guinea-pig brain slices (Skingle et al., 1993; Starkey and Skingle, 1993, 1994; Hatcher et al., 1995; Roberts et al., 1996). However GR127935 does not discriminate between 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors, therefore attributing functions to either receptor awaits more selective compounds.

In this study we have examined the effect of GR127935 on human 5-$HT_{1D\alpha}$ or 5-$HT_{1D\beta}$ receptors expressed by CHO cells. The receptor-G protein interaction was investigated using [^{35}S]GTPγS binding and adenylyl cyclase activity was studied by measuring cAMP accumulation. In addition, by using EEDQ (*N*-ethoxycarbonyl-2-ethoxy 1,2-dihydro-quinoline) the non-specific receptor alkylating agent (Crocker and Cameron, 1989), receptor number was reduced in both cell lines to estimate receptor reserve and to determine whether an underlying inhibitory adenylyl cyclase response exists in 5-$HT_{1D\alpha}$ expressing cells, as previously reported.

2. Materials and methods

2.1. Cell preparation

The Chinese Hamster Ovary (ACC098) cell line was stably transfected with the human 5-$HT_{1D\alpha}$ or 5-$HT_{1D\beta}$ receptor gene using electroporesis. Both cell lines of non-clonal origin were cultured, in suspension, in a SmithKline Beecham proprietary medium devoid of serum and nuleosides. Gentecin 418 (40 μg/ml) was used as a selection marker for 5-$HT_{1D\alpha}$ expressing cells. Both cell lines were subcultured for cAMP accumulation assays which involved centrifugation of the cell suspension for 5 min at $200 \times g$. The resultant pellet was resuspended in flat-bottomed flasks and plated at a density of 10^5 cells/ml. Frozen membranes were used in binding assays.

2.2. Radioligand binding assays

CHO cell membranes expressing the human 5-$HT_{1D\alpha}$ or 5-$HT_{1D\beta}$ receptors were homogenised twice in 50 mM Tris buffer containing EGTA (10 mM followed by 0.1 mM) and resuspended in 50 mM Tris buffer containing $MgCl_2$ (10 mM), ascorbate (6 mM) and pargyline (0.5 μM). Saturation studies were conducted using [^{3}H]5-HT at 5 or 6 different concentrations, ranging from 1 to 100 nM.

Competition studies were performed in single samples using 10 concentrations of unlabelled compound in the presence of 4 nM [^{3}H]5-HT and non-specific binding was determined by 10 μM 5-HT. Membranes (10^6 cells) were incubated in a final volume of 0.5 ml for 45 min at 37°C and the reaction was stopped by rapid filtration through Whatman GF/B filters followed by 5 brief washes with ice cold Tris buffer. Radioactivity was determined using liquid scintillation spectrometry.

2.3. [^{35}S]GTPγS binding studies

CHO cells expressing the human 5-$HT_{1D\alpha}$ or 5-$HT_{1D\beta}$ receptors were prepared as described by Thomas et al., 1995 and a [^{35}S]GTPγS binding assay was carried out essentially as described (Lazareno et al., 1993) with minor modifications. In brief, 10^8 cells were preincubated (30°C for 30 min) in Hepes (20 mM) (pH 7.4) in the presence of $MgCl_2$ (3 mM), NaCl (100 mM), GDP (10 μM) and ascorbate (0.2 mM) with or without test drugs. The reaction was started by the addition of 10 μl of [^{35}S]GTPγS (100 pM) followed by a further 30 min incubation at 30°C. The reaction was stopped by rapid filtration using Whatman GF/B grade filters followed by five washes with ice-cold Hepes (20 mM)/$MgCl_2$ (3 mM) buffer. Radioactivity was determined by liquid scintillation spectrometry. Non-specific binding was determined by addition of unlabelled GTPγS (10 μM), prior to the addition of cells.

2.4. cAMP accumulation studies

The cAMP accumulation studies were performed as described (Watson, 1995). Briefly, 2×10^5 CHO cells expressing the human 5-$HT_{1D\alpha}$ or 5-$HT_{1D\beta}$ receptor were incubated in the presence or absence of 10 μM forskolin with or without test compound for 10 min at 37°C. The reaction was stopped by addition of perchloric acid (3 M) and the cAMP was extracted using a 1:1 mixture of trioctylamine and trichlorotrifluoroethane. The cAMP produced by the cells was determined by radioimmunoassay (RIANEN cAMP [^{125}I]Radioimunoassay Kit, DuPont, Stevenage, UK).

2.5. EEDQ treatment

CHO cells used for binding and cAMP accumulation studies were incubated in the absence or presence of EEDQ (*N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline) ranging from 0.1 to 3 μM for 30 min at 37°C. Following incubation, the EEDQ was removed from the cells by centrifugation for 5 min at $200 \times g$ and washing the resultant pellet twice with UltraCHO medium.

2.6. Materials

GR127935 (2′-methyl-4′-5-methyl-1,2,4 oxadiazol-3-yl)-biphenyl-4-carboxylic acid [4-methoxy-3-(4-methyl-piperazin-1-yl)-phenyl]-amide) was synthesised at SmithKline Beecham Pharmaceuticals (Harlow, UK). 5-HT (5-hydroxytryptamine creatine sulphate) and EEDQ (*N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline) were obtained

from Sigma (Poole, UK), methiothepin mesylate from Research Biochemicals (Natick, MA, USA). GTPγS (Guanosine-5'-*O*-3-thiotriphosphate) was obtained from Boehringer Mannheim (East Sussex, UK), [^{35}S]GTPγS (NEG-030H, 1000–1500 Ci/mmol) from Dupont UK and [^{3}H]5-HT (59 Ci/mmol) from Amersham International plc (Buckinghamshire, UK). Gentecin (G418) was supplied by Gibco BRL and UltraCHO medium by Bio Whittaker, Walkersville, MD 21793, USA.

2.7. Data analysis

Receptor binding saturation curves were analysed using LIGAND (Elsevier Biosoft). Drug concentration response curves from binding assays and the cAMP accumulation assay were analysed by a 4-parameter logistic equation in GRAFIT (Erithacus Software). Antagonist activity of compounds was quantified by calculating their apparent pA_2 value according to the equation, apparent pA_2 = log [(IC_{50} in the presence of antagonist)/(IC_{50} in its absence) − 1] − log (molar concentration of antagonist).

3. Results

3.1. Receptor binding

[^{3}H]5-HT binding to CHO cell membranes expressing 5-$HT_{1D\alpha}$ or 5-$HT_{1D\beta}$ receptors was saturable and linear Scatchard plots indicate the presence of a single high affinity site in both cell lines (Fig. 1). The density of the 5-$HT_{1D\alpha}$ receptors (7.9 pmol/mg protein) was approximately 12 fold greater than that of the 5-$HT_{1D\beta}$ receptors (0.65 pmol/mg protein) but K_d values for [^{3}H]5-HT were similar (6.6 nM and 6.2 nM, respectively). Competition studies were performed to determine the affinity of 5-HT, the 5-HT_1-like receptor antagonist methiothepin and GR127935 for each receptor. The pK_i values are summarised in Table 1. All three compounds show high affinity for both receptors with a rank order of affinity of GR127935 > 5-HT > methiothepin.

3.2. [^{35}S]GTPγS binding studies

In CHO cells expressing the 5-$HT_{1D\alpha}$ receptor, the agonist 5-HT produced a concentration-dependent stimulation of basal [^{35}S]GTPγS binding, reaching a maximum of 51% above control levels. GR127935 acted as a partial agonist with a maximum stimulation of 30% above basal (Fig. 2a), resulting in an intrinsic activity of approximately 0.6 compared to 5-HT. The 5-HT_1-like receptor antagonist, methiothepin, produced a dose-dependent inhibition of basal binding suggesting that it acts as an inverse agonist at the 5-$HT_{1D\alpha}$ receptor. Fig. 2d illustrates a similar response of all three compounds at the 5-$HT_{1D\beta}$ receptor: 5-HT is a full agonist, GR127935 acts as a partial agonist

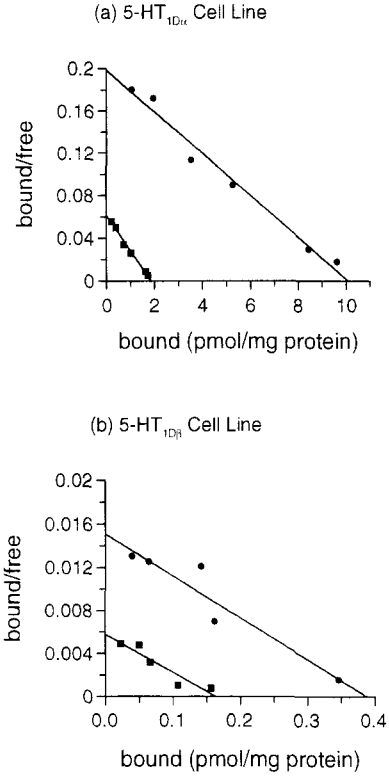

Fig. 1. Scatchard analysis of [^{3}H]5-HT binding to a membrane preparation of CHO cells expressing cloned human 5-$HT_{1D\alpha}$ receptors (a) or 5-$HT_{1D\beta}$ receptors (b). [^{3}H]5-HT binding was carried out in the absence (●) or presence (■) of EEDQ (1 μM). Curves were constructed using mean values of duplicate samples from representitive experiments.

and methiothepin shows inverse agonism. A summary of the pEC_{50} and maximum response is illustrated in Table 2. 5-HT, GR127935 and methiothepin showed no effect on basal [^{35}S]GTPγS binding in parental cells (data not shown).

Antagonist studies were carried out to determine the affinity of GR127935 and methiothepin at these receptors (Fig. 2b,c,e,f). Both compounds shifted the 5-HT concentration response curve to the right; GR127935 (10 nM) gave an apparent pA_2 of 8.5 and 9.1 for the 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptor, respectively. Methiothepin (10 nM) produced an apparent pA_2 of 8.9 and 8.7 for the 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors, respectively. The elevation of basal [^{35}S]GTPγS binding by GR127935 is due to its partial

Table 1
Receptor binding affinities of 5-HT, Methiothepin and GR127935

Receptor	Compound (pK_i)		
	5-HT	Methiothepin	GR127935
5-$HT_{1D\alpha}$	8.26 ± 0.03	7.34 ± 0.09	8.61 ± 0.19
5-$HT_{1D\beta}$	8.31 ± 0.32	7.08 ± 0.14	9.14 ± 0.06

All values are expressed as means ± S.E.M. of 3 independent experiments, each performed in single samples. pK_i values were calculated using the Cheng-Prussof equation. S.E.M.: standard error of the mean.

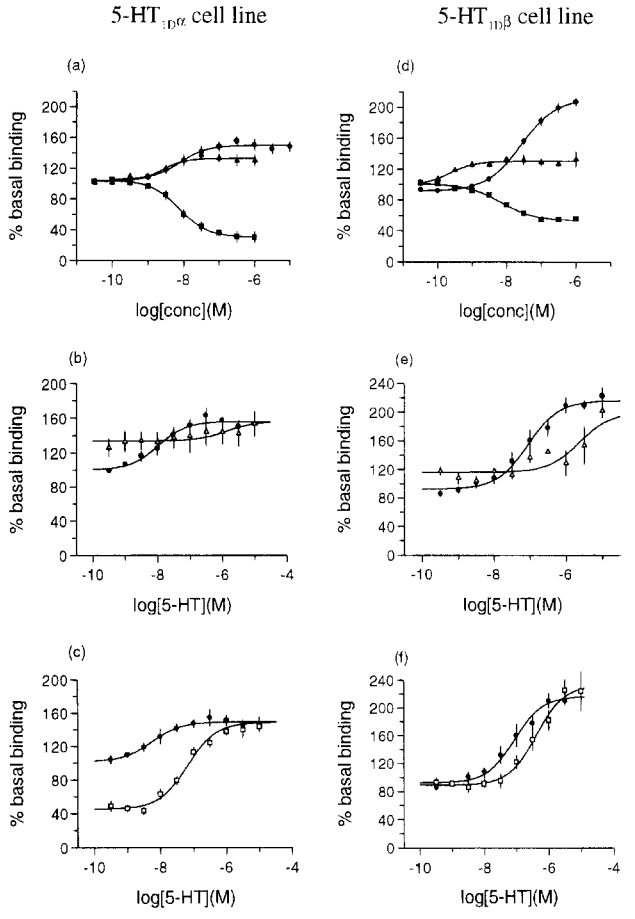

Fig. 2. [^{35}S]GTPγS binding to CHO cell membranes expressing human 5-HT$_{1D\alpha}$ receptors (a–c) or 5-HT$_{1D\beta}$ receptors (d–f). Method used as described earlier. (a,d) Increasing concentrations of 5-HT (●), GR127935 (▲) and methiothepin (■); (b,e) increasing concentrations of 5-HT in the absence (●) or presence of 10 nM GR127935 (△); (c,f) increasing concentrations of 5-HT in the absence (●) or presence of 10 nM methiothepin (□). Results are expressed as a percentage of basal [^{35}S]GTPγS binding to cell membranes in the presence of 10 μM GDP. Curves were constructed using mean ± S.E.M. values of 3–6 independent experiments, each performed in duplicate. All pEC$_{50}$, E_{max} and apparant pA$_2$ values are summarised in Table 2.

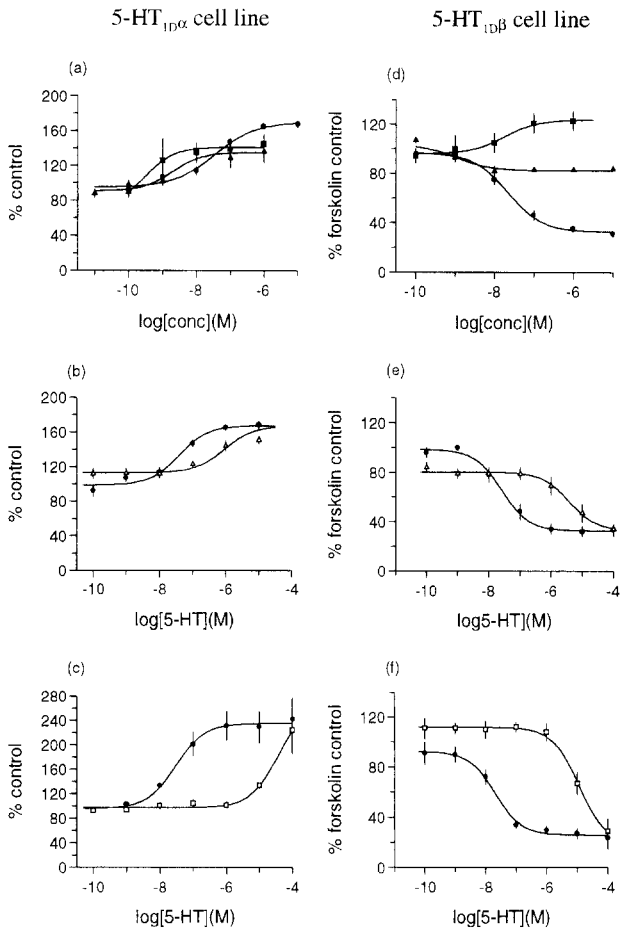

Fig. 3. cAMP accumulation in CHO cells expressing human 5-HT$_{1D\alpha}$ receptor (a–c) or 5-HT$_{1D\beta}$ receptors (d–f). Cells were cultured as described and for the 5-HT$_{1D\beta}$ expressing cells, exposed to 10 μM forskolin. (a,d) Increasing concentrations of 5-HT (●), GR127935 (▲) and methiothepin (■); (b,e) increasing concentrations of 5-HT in the absence (●) or presence of 10 nM GR127935 (△); (c,f) increasing concentrations of 5-HT in the absence (●) or presence of 1 μM methiothepin (□). For the 5-HT$_{1D\alpha}$ cell line: results are expressed as a percentage of basal cAMP levels; for the 5-HT$_{1D\beta}$ cell line: results are expressed as a percentage of forskolin-stimulated cAMP levels. Curves were constructed using mean ± S.E.M. values of 3–6 independent experiments, each performed in quadruplicate. All pEC$_{50}$, E_{max}, and apparant pA$_2$ values are summarised in Table 3.

agonist activity and suppression of basal binding by methiothepin due to inverse agonism. All curves gave slopes which did not significantly differ from unity.

Table 2
[^{35}S]GTPγS binding studies in CHO cells expressing the human 5-HT$_{1D\alpha}$ or 5-HT$_{1D\beta}$ receptor

Receptor	Compound	pEC$_{50}$	E_{max} (% basal)	pA$_2$
5-HT$_{1D\alpha}$	5-HT	8.2 ± 0.2	151 ± 6	
	GR127935	8.6 ± 0.3	129 ± 5	8.5 ± 0.2
	Methiothepin	8.1 ± 0.2 [a]	30 ± 7 [a]	8.9 ± 0.3
5-HT$_{1D\beta}$	5-HT	7.6 ± 0.03	196 ± 5	
	GR127935	9.7 ± 0.1	131 ± 10	9.1 ± 0.2
	Methiothepin	8.2 ± 0.1 [a]	56 ± 2 [a]	8.7 ± 0.2

[a] Inverse agonist. All values are expressed as means ± S.E.M of 3–6 independent experiments, each performed in duplicate.

3.3. cAMP accumulation studies

In 5-HT$_{1D\alpha}$ receptor expressing cells, 5-HT produced a concentration-dependent stimulation of basal cAMP levels reaching a maximum of 70% above control (Fig. 3a and Table 3). Methiothepin and GR127935 showed partial stimulation of cAMP levels with increasing concentrations (Table 3). However methiothepin, but not GR127935, also produced a similar response in the parental cell line suggesting this could be a non-specific effect in the former case (Table 4). In non-transfected cells, 5-HT produced a small degree of inhibition possibly due to the presence of an endogenous 5-HT receptor. GR127935 had no effect on this cell line (Table 4). Antagonist studies on the 5-HT$_{1D\alpha}$

Table 3
cAMP accumulation studies in CHO cells expressing the human 5-$HT_{1D\alpha}$ or 5-$HT_{1D\beta}$ receptor

Receptor	Compound	pEC_{50}	E_{max} (% control)	pA_2
5-$HT_{1D\alpha}$	5-HT	7.5±0.1	167± 3	
	GR127935	8.6±0.1	136±10	8.6±0.03
	Methiothepin	8.9±0.3	148± 6	9.1±0.1
5-$HT_{1D\beta}$	5-HT	7.6±0.1	32± 2	
	GR127935	8.9±0.3	83± 1	9.7±0.3
	Methiothepin	8.3±0.5	124± 8	8.9±0.1

All values are expressed as means±S.E.M of 3–6 independent experiments, each performed in quadruplicate.

Table 4
cAMP accumulation studies in parental CHO cells

Compound	% forskolin control
5-HT (1 μM)	87±4
Methiothepin (1 μM)	127±7
GR127935 (1 μM)	100±9

All values are expressed as means±S.E.M. of 3–9 independent experiments, each performed in quadruplicate.

receptor expressing cell line revealed that GR127935 (10 nM) and methiothepin (1 μM) shifted the 5-HT response curve to the right, producing an apparent pA_2 of 8.6 and 9.1, respectively (Fig. 3b,c). GR127935 elevated the baseline of the response curve by 20% by virtue of its partial agonist response, in addition to causing a rightward shift.

In the 5-$HT_{1D\beta}$ receptor expressing cells, 5-HT and GR127935 elicited an inhibition of enhanced levels of cAMP produced by forskolin (10 μM) (Fig. 3d). 5-HT was a full agonist reaching a maximum inhibition of 68% whereas GR127935 acted as a partial agonist producing 17% inhibition of forskolin-stimulated cAMP levels. Methiothepin stimulated cAMP levels to a similar extent to that seen in the parental and 5-$HT_{1D\alpha}$ receptor transfected cell lines. GR127935 (10 nM) and methiothepin (1 μM) both produce a rightward shift of the 5-HT concentration response curve giving apparent pA_2 values of 9.7 and 8.9, respectively (Fig. 3e,f). GR127935 depressed the control forskolin stimulated levels of cAMP, again emphasising a partial agonist response. The slope for all curves was not significantly different from unity.

Table 5
[^{3}H]5-HT receptor binding assay in CHO cells expressing the 5-$HT_{1D\alpha}$ or 5-$HT_{1D\beta}$ receptor

Receptor	[EEDQ] (μM)	% inhibition of specific binding	K_d (nM)	B_{max} (pmol/mg protein)
5-$HT_{1D\alpha}$	0	0	6.6±1.5	7.9 ±1.1
	0.1	28±11		
	0.3	39±14	ND	ND
	1.0	62±10	7.7±2.2	3.2 ±1.2 [b]
5-$HT_{1D\beta}$	0	0	6.2±1.8	0.65±0.12
	0.3	48±7	ND	ND
	1.0	66±4	9.2±2.2	0.31±0.07 [c]
	3.0	84±2	ND	ND

All values are expressed as a mean±S.E.M of 3 independent experiments, each performed in triplicate. [b] $P = 0.07$, [c] $P = 0.02$). Specific binding is referred to as binding at a fixed concentration of [^{3}H]5-HT where non-specific binding is defined by 10 μM 5-HT. ND: not determined.

3.4. EEDQ treatment

From radioligand binding studies 30 min exposure to EEDQ revealed a concentration-related inhibition of specific binding in both cell lines (Table 5) and a reduction in B_{max} by 1 μM EEDQ (7.9–3.2[b] pmol/mg protein for 5-$HT_{1D\alpha}$ receptors and 0.65–0.31[c] pmol/mg protein for 5-$HT_{1D\beta}$ receptors; [b]$P = 0.07$, [c]$P = 0.02$). The K_d values before and after exposure to 1 μM EEDQ were not significantly different (6.6 vs. 7.7 nM, respectively, at the 5-$HT_{1D\alpha}$ receptor and 6.2 vs. 9.2 nM, respectively, at the 5-$HT_{1D\beta}$ receptor).

From cAMP accumulation studies EEDQ treatment produced a concentration-dependent inhibition of the maximal

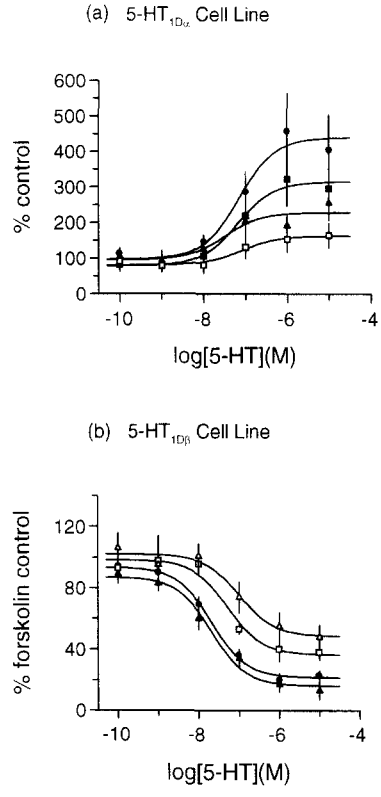

Fig. 4. Effect of EEDQ treatment on cAMP accumulation in CHO cells expressing the human 5-$HT_{1D\alpha}$ or 5-$HT_{1D\beta}$ receptor. Cells were cultured as described and incubated in the absence or presence of EEDQ (0.1–3.0 μM) for 30 min at 37°C. Subsequently, cAMP levels were measured in control and treated cells. (a) Increasing concentrations of 5-HT in the absence (●) or presence of 0.1 μM (■); 0.3 μM (▲) and 1 μM (□) EEDQ in the 5-$HT_{1D\alpha}$ cell line; (b) increasing concentrations of 5-HT in the absence (●) or presence of 0.3 μM (▲); 1 μM (□) and 3 μM (△) EEDQ in the 5-$HT_{1D\beta}$ cell line. Results are expressed as a percentage of basal cAMP levels for the 5-$HT_{1D\alpha}$ receptor expressing cells and as a percentage of forskolin-stimulated cAMP levels for the 5-$HT_{1D\beta}$ receptor expressing cells. Curves were constructed using mean±S.E.M. values of 3 independent experiments, each performed in quadruplicate. All pEC_{50} and E_{max} values are summarised in Table 6.

Table 6
Effect of EEDQ treatment on cAMP accumulation studies in CHO cells expressing the human 5-$HT_{1D\alpha}$ or 5-$HT_{1D\beta}$ receptor

Receptor	[EEDQ] (μM)	pEC_{50}	E_{max} (% difference from control)
5-$HT_{1D\alpha}$	0	7.2 ± 0.06	336 ± 104
	0.1	7.3 ± 0.06	214 ± 80
	0.3	7.3 ± 0.20	125 ± 28
	1.0	7.0 ± 0.20	65 ± 30 [d]
5-$HT_{1D\beta}$	0	7.7 ± 0.06	−78 ± 3
	0.3	7.7 ± 0.1	−73 ± 5
	1.0	7.3 ± 0.1	−63 ± 6 [d]
	3.0	6.8 ± 0.2 [d]	−57 ± 5 [d]

All values are expressed as means ± S.E.M. of 3–6 independent experiments. [d] $P \leq 0.05$.

response of 5-HT in both cell lines (Fig. 4a,b) but did not alter the potency of 5-HT in the 5-$HT_{1D\alpha}$ receptor expressing cells (Table 6). Conversly, the potency of 5-HT in the 5-$HT_{1D\beta}$ cell line decreased with increasing EEDQ concentration.

4. Discussion

The present study has investigated the effects of the 5-HT_{1D} receptor antagonist GR127935 and the non-specific 5-HT_1 receptor antagonist methiothepin on CHO cells stably expressing human 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors. The agonist 5-HT produced a concentration-dependent stimulation of [^{35}S]GTPγS binding in 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptor expressing cells, consistent with agonist stimulation of receptor-G protein coupling. GR127935 and methiothepin shifted the 5-HT response curve to the right with app. pA_2 values of 8.5, 8.9 for 5-$HT_{1D\alpha}$ receptors, respectively and 9.1, 8.7 for 5-$HT_{1D\beta}$ receptors, respectively. These values are consistent with binding affinities for GR127935 but show discrepancies for methiothepin. GR127935 and methiothepin, displayed intrinsic activities of opposite nature. GR127935 showed partial stimulation of basal [^{35}S]GTPγS binding at 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors. Methiothepin displayed inverse agonism at both receptors. Negative intrinsic activity has also been observed at other receptors including mAch and β-adrenoceptors (Matesic and Luthin, 1991; Samama et al., 1994) and could be a possible explanation for the discrepancy between binding affinities and app. pA_2 values for methiothepin at 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors.

From studies measuring cAMP accumulation, the agonist 5-HT produced a concentration-related inhibition of forskolin-stimulated cAMP accumulation at 5-$HT_{1D\beta}$ receptors consistent with negative coupling to adenylyl cyclase. In contrast, 5-HT produced a stimulation of basal cAMP levels in CHO cells expressing 5-$HT_{1D\alpha}$ receptors, suggesting positive coupling to adenylyl cyclase. This response has not been observed at this receptor by other groups to date but agonist stimulation of the RDC4 receptor, the dog homologue of the human 5-$HT_{1D\alpha}$ receptor, has been shown to cause an elevation of cAMP in several different cell lines (Maenhaut et al., 1991). GR127935 and methiothepin shifted the 5-HT-induced inhibition of forskolin-stimulated cAMP to the right in 5-$HT_{1D\beta}$ receptor expressing cells giving an app. pA_2 of 9.7 and 8.9, respectively. In the absence of agonist, GR127935 produced a partial inhibition of forskolin-stimulated cAMP at 5-$HT_{1D\beta}$ receptors and a partial elevation of basal cAMP levels at 5-$HT_{1D\alpha}$ receptors. Methiothepin elevated cAMP levels in both receptor expressing cells but produced a similar result in the parental cell line suggesting this response is non-specific. It therefore appears that inverse agonism can be observed at the level of receptor-G protein coupling but is less obvious at the receptor/effector level.

Thus we have shown that GR127935 displays agonist activity at 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors when expressed in CHO cells. This intrinsic activity has been reported by other groups where GR127935 acts as a partial agonist at the 5-$HT_{1D\alpha}$ receptor when expressed in CHO-K1 cells and as full agonist when expressed in HeLa cells (Pauwels and Palmier, 1995; Walsh et al., 1995). Walsh and colleagues have also shown GR127935 to be a full agonist at 5-$HT_{1D\beta}$ receptors but Pauwels and colleagues reported it as a silent antagonist at this receptor. Differences in the receptor expression systems, such as G protein or adenylyl cyclase complement of the cells, could be a possible explanation for such discrepancies.

Another variable which may influence functional responses in artificial systems is the level of receptor expression. For example, it has been reported that several different receptors negatively linked to adenylyl cyclase exhibit dual coupling to G_i and G_s; a response which becomes more prominent as receptor expression increases (Eason et al., 1992; Jones et al., 1991). Receptor reserve is also a consequence of high receptor number and can result in amplifying the agonist activity of a partial agonist. For example, the β-adrenoceptor antagonists propranolol and pindolol show full agonist activity in a 5-HT_{1B} receptor expressing system with a high degree of receptor reserve (Adham et al., 1993). This has been supported by other groups who have shown that the degree of intrinsic activity exhibited by these compounds depends on the degree of receptor reserve in the system (Bouhelal et al., 1988; Murphy and Bylund, 1989).

The receptor expression levels in our studies were high (~ 1 pmol/mg protein for 5-$HT_{1D\beta}$ receptors and ~ 8 pmol/mg protein for 5-$HT_{1D\alpha}$ receptors) and so it may be that (i) the 5-HT-induced elevation of cAMP levels at 5-$HT_{1D\alpha}$ receptors is due to promiscuous coupling to G_s and (ii) the partial agonist activity of GR127935 is a consequence of high receptor reserve. We therefore carried out studies using EEDQ to reduce 5-$HT_{1D\alpha}$ receptor number in an effort to reveal an underlying inhibitory cAMP response and also to determine the degree of receptor

reserve in both cell lines. EEDQ (up to 1 μM) did not reveal a 5-HT-induced inhibition of cAMP levels in 5-$HT_{1D\alpha}$ expressing cells. This may be because 5-$HT_{1D\alpha}$ receptors, when expressed in CHO cells, are positively linked to adenylyl cyclase or that we did not reach a high enough concentration of EEDQ. This must be considered as treatment with 1 μM EEDQ still resulted in a receptor density approximately 5-fold greater than non-treated 5-$HT_{1D\beta}$ receptor expressing cells. However, increasing concentrations of EEDQ did cause a dose-dependent inhibition of the maximum response elicited by agonist at 5-$HT_{1D\alpha}$ receptors. Although the inhibition of this response only reached significance at 1 μM EEDQ the trend was evident and the pEC_{50} value after EEDQ treatment was not altered. Both of these factors suggest a lack of receptor reserve in this cell line with relation to stimulation of cAMP levels but we cannot rule out the possibility of reserve being present in another functional pathway. This phenomenon is exhibited by 5-HT_{1A} receptors highly expressed in HeLa cells where reserve is present for adenylyl cyclase inhibition but not for Ca^{2+} mobilisation (Fargin and collaborators, personal communication). Conversely, EEDQ treatment did alter the pEC_{50} and maximum response of agonist curves in 5-$HT_{1D\beta}$ receptor expressing cells suggesting receptor reserve may be present in this system with regard to this functional pathway.

It therefore appears that the partial agonist activity of GR127935 at 5-$HT_{1D\alpha}$ receptors expressed in CHO cells is not related to receptor reserve but may be a true measure of the intrinsic activity of this compound. This is supported by studies on native tissue investigating a monosynaptic reflex from rat neonatal spinal cord where GR127935 acts as a partial agonist (Manuel et al., 1995). In this preparation the receptor involved has been termed 5-HT_{1D}-like and is ketanserin sensitive suggesting the involvement of 5-$HT_{1D\alpha}$ receptors. Since 5-$HT_{1D\beta}$ receptor expressing cells demonstrate receptor reserve in our studies, it may be that the intrinsic activity of GR127935 in this system is an over-estimation. This is supported by the failure to observe partial agonism in in vitro studies on native tissue expressing 5-$HT_{1D\beta}$ receptors (Skingle et al., 1995). However from microdialysis studies GR127935 has been shown to decrease 5-HT levels from guinea-pig brain (Roberts et al., 1994; Skingle et al., 1995) which may be evidence of partial agonist activity in vivo.

In conclusion, the 5-HT_{1D} antagonist GR127935 acts as a partial agonist in CHO cells stably transfected with human 5-$HT_{1D\alpha}$ or 5-$HT_{1D\beta}$ receptors. This activity should be borne in mind in any interpretation of biological studies with this drug.

5. Note added in proof

During the review of this manuscript another report has appeared on the degree of receptor reserve of human 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptor expressing cell lines. Similar conclusions were reached between the two groups (Zgombick et al., 1996).

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References

Adham, N., B. Ellerbrock, P. Hartig, R.L. Weinshank and T. Branchek, 1993, Receptor reserve masks partial agonist activity in a cloned rat 5-hydroxytryptamine$_{1B}$ receptor expression system, Mol. Pharmacol. 43, 427.

Bouhelal, R., L. Smounya and J. Bockaert, 1988, 5-HT_{1B} receptors are negatively coupled with adenylate cyclase in rat substantia nigra, Eur. J. Pharmacol. 151, 189.

Crocker, A.D. and D.L. Cameron, 1989, Receptor alkylating agents: novel tools for the study of receptor function in the central nervous system of the rat, Clin. Exp. Pharmacol. Physiol. 16, 545.

Eason, M.G., H. Kurose, B.D. Holt, J.R. Raymond and S.B. Liggett, 1992, Simultaneous coupling of α_2-adrenergic receptors to two G-proteins with opposing effects, J. Biol. Chem. 267, 15795.

Hamblin, M.W. and M.A. Metcalf, 1991, Primary structure and functional characterisation of a human 5-HT_{1D} type serotonin receptor, Mol. Pharmacol. 40, 43.

Hatcher, J.P., P.D. Slade, C. Roberts and J.J. Hagan, 1995, 5-HT_{1D} receptors mediate SKF 99101H-induced hypothermia in the guinea-pig, J. Psychopharmacol. 93, 234.

Hoyer, D. and D.N. Middlemiss, 1989, Species differences in the pharmacology of terminal 5-HT autoreceptors in mammalian brain, Trends Pharmacol. Sci. 13, 130.

Hoyer, D., D.E. Clarke, J.R. Fozard, P.R. Hartig, G.R. Martin, E.J. Mylecharane, P.R. Saxena and P.P. Humphrey, 1994, International union of pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin), Pharmacol. Rev. 46, 157.

Jones, S.V.P., C.J. Heilman and M.R. Brann, 1991, Functional responses of cloned muscarinic receptors expressed in CHO-K1 cells, Mol. Pharmacol. 40, 242.

Lazareno, S., T. Farries and N.J.M. Birdsall, 1993, Pharmacological characterisation of guanine-nucleotide exchange reactions in membranes from CHO cells stably transfected with human muscarinic receptors M1-M4, Life Sci. 52, 449.

Maenhaut, C., J. Van Sande, C. Massart, C. Dinsart, F. Libert, E. Monferini, E. Giraldo, H. Ladinsky, G. Vassart and J.E. Dumont, 1991, The orphan receptor cDNA RDC4 encodes a 5-HT_{1D} serotonin receptor, Biochem. Biophys. Res. Commun. 180, 1460.

Manuel, N.A., D.I. Wallis and H. Crick, 1995, Ketanserin-sensitive depressant actions of 5-HT receptor agonists in the neonatal rat spinal cord, Br. J. Pharmacol. 116, 2647.

Matesic, D.F. and G.R. Luthin, 1991, Atropine dissociates complexes of muscarinic acetylcholine receptor and guanine nucleotide-binding protein in heart membranes, FEBS Lett. 284, 184.

Murphy, T.J. and D.B. Bylund, 1989, Characterisation of serotonin-1B receptor negatively coupled to adenylate cyclase in OK cells, a renal epthelial cell line from the opossum, J. Pharmacol. Exp. Ther. 249, 535.

Pauwels, P.J. and C. Palmier, 1995, Functional effects of the 5-HT_{1D} receptor antagonist GR127, 935 at human 5-$HT_{1D\alpha}$, 5-$HT_{1D\beta}$, 5-HT_{1A} and opossum 5-HT_{1B} receptors, Eur. J. Pharmacol. Mol. Pharmacol. Sect. 290, 95.

Roberts, C, L. Thorn, G.W. Price, D.N. Middlemiss and B.J. Jones, 1994, Effect of the selective 5-HT_{1D} receptor antagonist, GR127935, on in vivo 5-HT release, synthesis and turnover in the guinea-pig frontal cortex, Br. J. Pharmacol. 112, 489P.

Roberts, C., J. Watson, M. Burton, G.W. Price, D.N. Middlemiss and B.J. Jones, 1996, Functional characterisation of the 5-HT terminal autoreceptor in the guinea-pig brain cortex, Br. J. Pharmacol. 117, 384.

Samama, P., P. Gang, T. Costa, S. Cotecchia and R.J. Leftkowitz, 1994, Negative antagonists promote an inactive conformation of the β-2 adrenergic receptor, Mol. Pharmacol. 45, 390.

Schlicker, E., K. Fink, M. Gothert, D. Hoyer, G. Molderings, I. Roschke and P. Schoeffter, 1989, The pharmacological properties of the presynaptic serotonin autoreceptor in the pig brain cortex conform to the 5-HT_{1D} receptor subtype, Naunyn-Schmied. Arch. Pharmacol. 340, 45.

Skingle, M., D.I.C. Scopes, W. Feniuk, H.E. Connor, M.C. Carter, J.W. Clitherow and M.B. Tyers, 1993, GR127935: a potent orally active 5-HT_{1D} receptor antagonist, Br. J. Pharmacol. 110, 9P.

Skingle, M., A.J. Sleight and W. Feniuk, 1995, Effects of the 5-HT_{1D} receptor antagonist GR127935 on extracellular levels of 5-HT in the guinea-pig frontal cortex as measured by microdialysis, Neuropharmacology 34, 377.

Starkey, S.J. and M. Skingle, 1993, 5-HT_{1D} autoreceptors modulate stimulated 5-HT release in the guinea-pig dorsal raphe nucleus, Br. J. Pharmacol. 110, C102.

Starkey, S.J. and M. Skingle, 1994, 5-HT_{1D} as well as 5-HT_{1A} autoreceptors modulate 5-HT release in the guinea-pig dorsal raphe nucleus, Neuropharmacology 33, 393.

Thomas, D.R., S.A. Faruq, J.M. Balcerek and A.M. Brown, 1995, Pharmacological characterisation of [^{35}S]-GTPγS binding to chinese hamster ovary cell membranes stably expressing cloned human 5-HT_{1D} receptor subtypes, J. Rec. Sig. Trans. Res. 15, 199.

Van Sande, J., A. Allgeier, C. Massart, A. Czernilofsky, G. Vassart, J.E. Dumont and C. Maenhaut, 1993, The human and dog 5-HT_{1D} receptor can both activate and inhibit adenylate cyclase in transfected cells, Eur. J. Pharmacol. 247, 177.

Walsh, D.M., D.T. Beattie and H.E. Connor, 1995, The activity of 5-HT_{1D} receptor ligands at cloned human 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors, Eur. J. Pharmacol. 287, 79.

Watson, J., 1995, In vitro measurement of the second messenger cAMP: ria vs flashplates, Biotech Update 10, 11.

Watson, J., M. Burton, M. Coldwell, G.W. Price, D. Cronk, S. Wilson, J. Balcarek, J. Robinson and B. Jones, 1994, The human 5-$HT_{1D\alpha}$ receptor is positively coupled to adenylyl cyclase in transfected CHO cells, Br. J. Pharmacol. 112, 301P

Weinshank, R.L., J.M. Zgombick, M.J. Macchi, T.A. Branchek and P.R. Hartig, 1992, Human serotonin 1D receptor is encoded by a subfamily of two distinct genes: 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$, Proc. Natl. Acad. Sci. USA 89, 3630.

Zgombick, J.M., L.E. Schechter, N. Adham, S.A. Kucharewicz, R.L. Weinshank and T.A. Branchek, 1996, Pharmacological characterisations of recombinant human 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptor subtypes coupled to adenylate cyclase inhibition in clonal cell lines: apparent differences in drug intrinsic efficacies between human 5-HT_{1D} subtypes, Naunyn-Schmied. Arch. Pharmacol. 354, 226.