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Short communication

The activity of 5-HT_{1D} receptor ligands at cloned human 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors

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Abstract

The present study has examined the functional activity of the 5-HT_{1D} receptor agonist, sumatriptan, and antagonists, 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), GR55562 (3-[3-(dimethylamino)propyl]-4-hydroxy-*N*-[4-(4-pyridinyl)phenyl]benzamide), metergoline and methiothepin in HeLa cells, stably transfected with either 5-$HT_{1D\alpha}$ or 5-$HT_{1D\beta}$ receptor subtypes. Sumatriptan, GR127935 and metergoline (each 0.01–1 μM) behaved as agonists, producing a concentration-dependent inhibition of forskolin-stimulated adenosine 3',5'-cyclic monophosphate (cAMP) production at both 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptor subtypes (mean pIC_{50} values of 8.4 and 8.3 for sumatriptan, 7.9 and 8.0 for GR127935, and 7.9 and 8.3 for metergoline, respectively). In contrast, GR55562 and methiothepin behaved as competitive 5-HT_{1D} receptor antagonists and were devoid of any agonist activity. GR55562 (10 μM) caused a rightward displacement of the GR127935 and metergoline concentration-response curves. The agonist activity of GR127935 and metergoline, observed in the present study, contrasts with their recognised 5-HT_{1D} receptor antagonist profiles in animal isolated tissue and behavioural models. Unlike GR127935, GR55562 behaved as a silent antagonist at the cloned human 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors in the study.

Keywords: GR127935; GR55562; Metergoline; Methiothepin; 5-$HT_{1D\alpha}$ receptor; 5-$HT_{1D\beta}$ receptor

1. Introduction

The human 5-HT_{1D} receptor, a member of the seven transmembrane, G-protein coupled receptor family, has been subdivided into 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ subtypes, each of which has been cloned, sequenced and shown to be negatively coupled to adenylate cyclase (Hartig et al., 1992). Although few selective compounds for either receptor subtype have been described, there are now several compounds possessing a degree of selectivity for 5-HT_{1D}, over non-5-HT_{1D} receptors. Sumatriptan, for example, is an agonist at 5-HT_{1D} receptors, and 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) is a highly selective and potent antagonist (Humphrey et al., 1988; Skingle et al., 1993). GR127935 is likely to be a more useful pharmacological tool to probe the function of 5-HT_{1D} receptors than either of the widely used compounds, metergoline or methiothepin, which lack selectivity. GR127935 displaces, with high affinity, [^{3}H]5-HT binding to HeLa cells transfected with human 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors (pK_i values of 8.9 and 9.9 respectively) and displays a good degree of selectivity over other 5-HT receptors ($pK_i < 6$) (Skingle et al., 1993). Antagonist activity of GR127935 has been demonstrated in the dog basilar artery and saphenous vein, each of which contains vascular 5-HT_1 receptors (equivalent to the 5-$HT_{1D\beta}$ receptor subtype in bovine and human cerebral arteries; Hamel et al., 1993). GR127935 also antagonises central 5-HT_{1D} receptor-mediated hypothermia and rotational behaviour in guinea-pigs (Skingle et al., 1994; Higgins et al., 1991).

The main objective of the present study was to investigate further the activity of sumatriptan and GR127935. Their effects on forskolin-stimulated production of adenosine 3',5'-cyclic monophosphate (cAMP) were characterised in HeLa cells expressing 5-$HT_{1D\alpha}$ or 5-$HT_{1D\beta}$ receptor subtypes. The activity of

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GR127935 was compared with another recently identified, selective 5-HT_{1D} receptor antagonist, GR55562 (3-[3-(dimethylamino)propyl]-4-hydroxy-*N*-[4-(4-pyridinyl)phenyl]benzamide; Connor et al., 1995), and with methiothepin and metergoline.

2. Materials and methods

2.1. Cell culture and measurement of cAMP levels

HeLa cells were stably transfected with plasmids containing either the human 5-$HT_{1D\alpha}$ (purchased from Dr M. Hamblin, Seattle Veterans Affairs Medical Centre) or 5-$HT_{1D\beta}$ receptor genes. The levels of receptor expression were 98 and 290 fmol/mg protein for the 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptor-expressing cell lines, respectively. B_{max} (maximum number of binding sites per mg of tissue) values were determined from saturation curves (using [^{3}H]5-carboxamidotryptamine as radioligand), which were analysed using the curve fitting program RADLIG (Glaxo VAX library). Levels of receptor expression remained stable for the duration of experiments. Cells were grown in an incubator at 37°C in 5% CO_2 and 92% humidity, in Ultraculture supplemented with geneticin (0.8 mg/ml) and glutamine (2 mM).

cAMP assays were conducted in duplicate. Cells (10^5/ml) were plated out into 24-well polystyrene plates 4–5 days prior to experiments. During this time, the plates were kept in an incubator at 37°C. Growth medium was removed from each of the wells using a glass Pasteur pipette connected to a vacuum pump by means of polypropylene tubing. The cells were washed with phosphate-buffered saline (0.5 ml, 37°C), which was then removed. Assay medium (0.5 ml of Dulbecco's modified Eagle's, containing 3-isobutyl-1-methylxanthine (0.3 mM)) was added to each of the wells and the plates carefully shaken. In antagonist experiments, drug or vehicle (10 μl) was added at this point. The plates were then incubated (37°C for 30 min) and agonist or vehicle (10 μl) added. A submaximal concentration of forskolin (10 μM) was added (10 μl) 2 min later. Basal levels of cAMP, in the absence of forskolin, were determined in each experiment. Reactions were terminated 10 min later by removal of the assay medium from each well and the addition of HCl (0.5 ml, 0.1 M). The plates were transferred to a refrigerator (4°C) for 1 h. Aliquots (400 μl) from each of the wells were then added to polystyrene tubes and neutralised (pH 5–7) by addition of neutralising solution (sodium acetate (0.5 M; pH adjusted to 6.2) and NaOH (2 M)). The samples were capped and returned to the fridge. cAMP levels were determined by radioimmunoassay (Amersham, UK). Protein assays were carried out on untreated samples of cells from each assay using bovine serum albumin as the standard. Results were expressed as pmol of cAMP/mg protein, or as a percentage inhibition of the forskolin-stimulated cAMP levels.

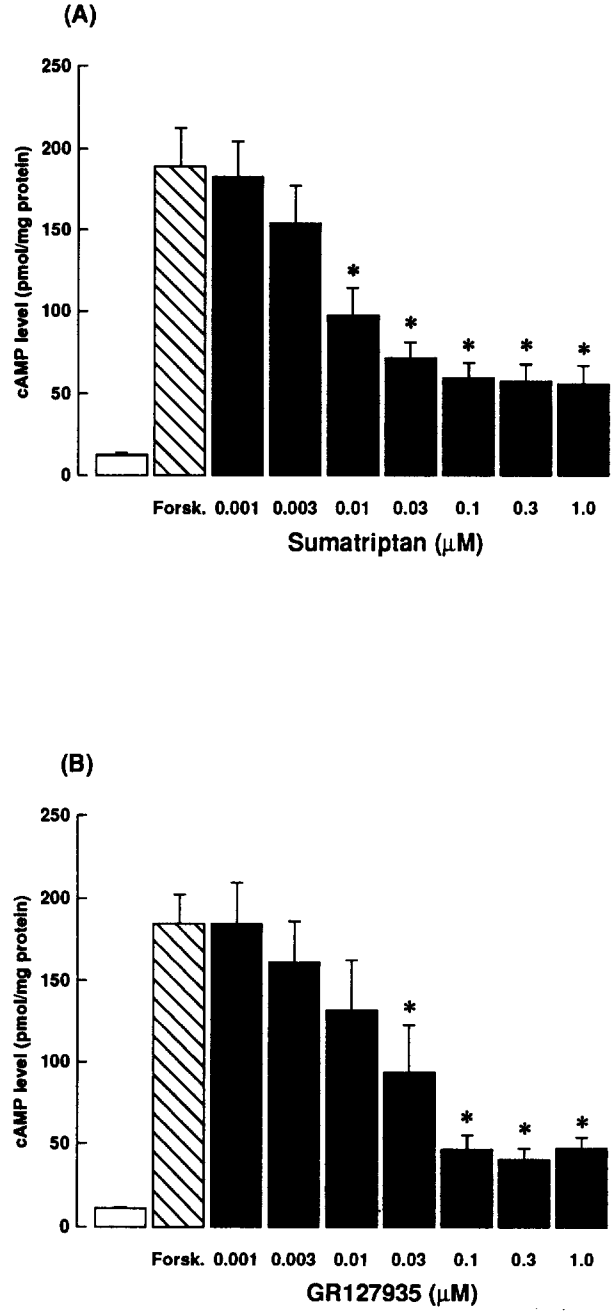

Fig. 1. Concentration-dependent inhibition of forskolin (Forsk; 10 μM)-induced increases in cAMP levels by (A) sumatriptan (0.001–1 μM) and (B) GR127935 (0.001–1 μM) in HeLa cells stably transfected with human 5-$HT_{1D\beta}$ receptors. Results are expressed as mean (±S.E.M.) cAMP/mg protein (n = 6). $^*P < 0.05$ (Student's *t*-test) compared to forskolin responses (hatched columns). The basal levels of cAMP, in the absence of forskolin, are shown by the open columns. Similar responses were produced in HeLa cells which stably expressed human 5-$HT_{1D\alpha}$ receptors (data not shown).

2.2. Calculation of results

Agonist activity was estimated by calculating the negative log of the molar concentration of compounds which produced 50% inhibition (pIC_{50} value) of the forskolin (10 μM)-induced cAMP production. Antagonist activity of compounds was quantified by calculating their pK_B value (−log dissociation constant) according to the equation, pK_B = log (IC_{50} in the presence of antagonist divided by IC_{50} in its absence − 1) − log of the molar concentration of antagonist. Results were expressed as the arithmetic means (±S.E.M.) and '*n*' values quoted refer to the number of experiments carried out.

2.3. Drugs used

The following drugs were used: GR127935 hydrochloride, GR55562 hydrochloride, sumatriptan maleate (each synthesised in the Medicinal Chemistry Department, Glaxo Wellcome, Ware, UK), methiothepin maleate (Roche), metergoline (Farmitalia), methysergide hydrogen-maleate (Sandoz), forskolin (Sigma) and 3-isobutyl-1-methylxanthine (Sigma). Drugs were dissolved in distilled water with the exception of GR127935 (dissolved initially in glacial acetic acid), metergoline (in ascorbic acid (0.04 M)), and forskolin and 3-isobutyl-1-methylxanthine (both in dimethyl sulfoxide). Subsequent dilutions were made in assay medium.

3. Results

Basal levels of cAMP were 10–20 pmol cAMP/mg protein in both 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptor-containing cell lines. Forskolin (0.1–100 μM) produced a concentration-dependent increase in cAMP levels; a submaximal concentration (10 μM), producing approximately 70–80% of the maximum response, caused a 10- to 30-fold increase in cAMP levels in both cell lines. This concentration of forskolin (10 μM) was used in subsequent 5-HT_{1D} receptor agonist/antagonist experiments.

None of the 5-HT_{1D} receptor agonists or antagonists examined had any significant effect on basal levels of cAMP in the HeLa cells. Sumatriptan (0.01–1 μM), added 2 min prior to forskolin, produced a concentration-dependent inhibition of the forskolin-stimulated cAMP levels (pIC_{50} values of 8.4 ± 0.1 and 8.3 ± 0.1 at 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors respectively (n = 6); Fig. 1A). The percentage inhibition of the forskolin (10 μM)-induced response produced by sumatriptan was similar in the 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptor-containing cell lines (66 ± 3 and 70 ± 6% (n = 6) respectively). The sumatriptan concentration-response curve was displaced to the right by pretreatment with GR55562 (1–10 μM; pK_B values of 6.2 ± 0.1 (n = 3) and 7.4 ± 0.2 (n = 4) at 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors respectively) or methiothepin (10 μM; pK_B values of 8.2 ± 0.1 and 8.3 ± 0.1 respectively (n = 3); Fig. 2A).

GR127935 (0.01–1 μM), added 2 min prior to

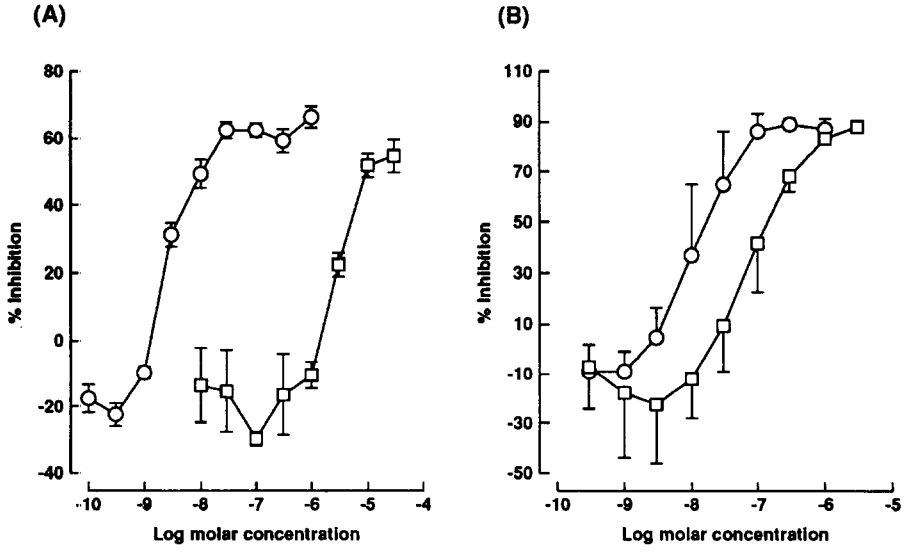

Fig. 2. Antagonism of (A) sumatriptan-induced inhibition of forskolin-stimulated cAMP production by methiothepin (10 μM) and (B) GR127935-induced responses by GR55562 (10 μM), in HeLa cells stably transfected with human 5-$HT_{1D\beta}$ receptors. Results are expressed as mean (±S.E.M.) percentage inhibition of forskolin-stimulated cAMP (n = 3). Curves in the absence of methiothepin or GR55562 are represented by the open circles, and in their presence, by the open squares.

forskolin, behaved as an agonist in HeLa cells transfected with either 5-$HT_{1D\alpha}$ or 5-$HT_{1D\beta}$ receptor subtypes, causing a concentration-dependent inhibition of forskolin-stimulated cAMP levels (pIC_{50} values of 7.9 $\pm$ 0.3 ($n = 3$) and 8.0 $\pm$ 0.3 ($n = 6$) respectively; Fig. 1B). The percentage inhibition of the forskolin (10 μM)-induced response produced by GR127935 was 58 $\pm$ 12 ($n = 3$) and 73 $\pm$ 4% ($n = 6$) in the 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptor-containing cell lines respectively. A similar profile of activity was observed with metergoline (0.01–1 μM; pIC_{50} values of 8.3 $\pm$ 0.2 and 7.9 $\pm$ 0.3 at 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors respectively; $n = 3$). GR127935 and metergoline concentration-response curves were displaced to the right by GR55562 pretreatment (10 μM; Fig. 2B). The pK_B values obtained for GR55562, against GR127935-induced inhibition of forskolin-stimulated cAMP production, were < 5 and 5.9 $\pm$ 0.2 at 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors respectively, and < 5 and 6.9 $\pm$ 0.3 against metergoline-induced responses ($n = 3$ for each). Methiothepin and GR55562 (up to 10 μM for each) were devoid of any agonist activity in either cell line.

4. Discussion

The present study has investigated the activity of the 5-HT_{1D} receptor agonist, sumatriptan (Humphrey et al., 1988) and antagonists, GR127935 (Skingle et al., 1993), GR55562 (Connor et al., 1995), metergoline and methiothepin in HeLa cells stably expressing 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors.

Sumatriptan (0.01–1 μM) produced a concentration-dependent inhibition of forskolin-stimulated cAMP production, consistent with the negative coupling of the 5-HT_{1D} receptor to adenylate cyclase. The sumatriptan concentration-response curve was displaced to the right by the 5-HT_{1D} receptor antagonists, GR55562 and methiothepin, with pK_B values in keeping with their binding affinities at 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors (6.3 and 7.3 for GR55562 and 8.2 and 7.8 for methiothepin respectively; Connor et al., 1995; Jin et al., 1992). A different profile of action was, however, observed for the 5-HT_{1D} receptor antagonists, GR127935 and metergoline. These compounds (0.01–1 μM) behaved as receptor agonists in both 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptor-containing HeLa cell lines, producing a concentration-dependent inhibition of forskolin-stimulated cAMP production. Preliminary experiments using methysergide (0.01–1 μM), a non-selective 5-HT_{1D} receptor antagonist, have indicated that it also behaves as an agonist at human 5-$HT_{1D\beta}$ receptors in the HeLa cell line (data not shown).

Although sumatriptan, GR127935 and metergoline concentration-response curves were displaced to the right by GR55562 (0.1–1 μM), the mean pK_B values obtained with GR127935 (< 5 and 5.9 at 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors, respectively) and metergoline (< 5 and 6.9 respectively) were less than expected. It is possible that a slow 'off-rate' for both GR127935 and metergoline from the 5-HT_{1D} receptors prevented the occurrence of true competitive antagonism, resulting in a low pK_B value for GR55562. This suggestion is supported by evidence from in vitro experiments, in isolated dog basilar artery and saphenous vein, where GR127935 produces an insurmountable antagonism of sumatriptan-mediated contractions (Skingle et al., 1993). In each of these isolated preparations, the antagonism is only partially reversible with prolonged washing, suggesting that GR127935 slowly dissociates from its receptors.

The identification of agonist activity for GR127935 and metergoline differs from observations in the majority of studies where antagonism has been reported (Skingle et al., 1993, 1994; Hamel et al., 1993). In addition to its antagonist activity in the dog isolated saphenous vein and basilar artery (Skingle et al., 1993), GR127935 inhibits GR46611-induced hypothermia in the guinea-pig, an action attributed to blockade of central 5-HT_{1D} receptors, while microdialysis studies have indicated that the compound blocks 5-HT_{1D} autoreceptors in the guinea-pig frontal cortex (Skingle et al., 1994, 1995). Furthermore, GR127935 inhibits rotational behaviour in guinea-pigs following intranigral administration of the 5-HT_{1D} receptor agonist, GR56764 (Higgins et al., 1991). No agonist activity was identified for GR127935 in these studies. Although metergoline is considered to be a 5-HT receptor antagonist, agonism at 5-HT_{1A} and 5-HT_{1D} receptors has been observed (Schoeffter and Hoyer, 1988; Schoeffter et al., 1988). The findings of the present investigation are more consistent with, although not identical to, results from several recent studies in which cell-based assays were used. Partial agonist activity of GR127935 has been identified in GTPγS binding and cAMP assays using Chinese hamster ovary (CHO) cells transfected with human 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors (Watson et al., 1995). More recently, full agonist activity of GR127935 was reported in C6-glial cells transfected with human 5-$HT_{1D\alpha}$ receptors, using forskolin-stimulated cAMP production as a measure of functional activity (Pauwels and Colpaert, 1995). Interestingly, however, in the same study, GR127935 behaved as an antagonist at cloned 5-$HT_{1D\beta}$ receptors (Pauwels and Colpaert, 1995). There are clear differences, therefore, in the functional activity of compounds such as GR127935 or metergoline, between cell lines expressing human 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors and animal tissue, and also between cell-based studies carried out by different groups.

Conceivably, differences in receptor reserve between recombinant cell lines and animal tissue may

explain the different profiles of compounds which can be observed. When a large receptor reserve exists, some drugs possess agonist activity, whereas with limited or no reserve, partial agonism or even antagonism can be observed (Hoyer and Boddeke, 1993). Differences in the levels of receptor expression per se have been claimed to influence functional activity. Thus, in two transfected HeLa cell lines, expressing different levels of 5-HT_{1A} receptors, ipsapirone, buspirone, spiroxatrine and MDL73005 behaved as agonists, releasing intracellular calcium in the higher receptor-expressing HA 6 cells, but as antagonists in the lower expressing HA 7 cells (Boddeke et al., 1992). In the present investigation, however, the levels of 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptor expression were similar and so this is unlikely to explain why GR127935 and metergoline behaved as agonists, and methiothepin and GR55562 as antagonists.

Another important consideration may be the nature or degree of receptor/G-protein coupling as this can influence the type of intrinsic activity which is observed (Hoyer and Boddeke, 1993). It is evident, for example, that the 5-HT_{1A} receptor, when expressed in CHO-K1 and HeLa cells, couples to multiple G-proteins to inhibit adenylate cyclase, and that the relative contributions of these G-proteins can vary (Raymond et al., 1993). If a similar phenomenon occurs with 5-HT_{1D} receptors in recombinant cell lines and animal tissue, perhaps some 5-HT_{1D} receptor ligands distinguish, more readily than others, particular receptor/G-protein combinations. It is also possible that recombinant 5-HT_{1D} receptors couple to a G-protein compliment which is unavailable in tissues, or that some compounds behave as partial or full agonists in well coupled recombinant cells, yet as antagonists in animal tissue, when 5-HT_{1D} receptors are less well coupled. These possibilities require further investigation.

While the reason for these anomalies is unclear, it is evident that this phenomenon is certainly not restricted to GR127935 and metergoline, or to 5-HT_{1D} receptors (Hoyer and Boddeke, 1993). In Y-1 cells transfected with the rat 5-HT_{1B} receptor (analogous to the human 5-$HT_{1D\beta}$ subtype), the β-adrenoceptor antagonists, propranolol and pindolol, which are reported to be 5-HT_{1B} receptor partial agonists or antagonists, were found to be full agonists (Adham et al., 1993). Moreover, depending on the functional model used, 5-HT_{1A} receptor ligands, such as buspirone, ipsapirone, spiroxatrine or BMY 7378 have each been termed either full or partial agonists, or silent antagonists (Boddeke et al., 1992).

The physiological significance of the observations made in this and similar studies requires further investigation. It has been suggested (Pauwels and Colpaert, 1995) that the 5-$HT_{1D\alpha}$ receptor agonist activity of GR127935, evident in some cell-based assays, could be relevant in vivo, particularly in the trigeminal ganglion which may selectively express this receptor subtype (Rebeck et al., 1994). However, this seems unlikely as GR127935 exhibits only antagonist activity in the trigeminovascular system, attenuating sumatriptan-induced inhibition of neurogenic dural plasma protein extravasation (M.A. Moskowitz, personal communication). It is evident that predicting the intrinsic activity of compounds, on the basis of observations in cell-based assays, requires caution.

In conclusion, the 5-HT_{1D} receptor antagonists, GR127935 and metergoline, but not methiothepin or GR55562, behaved as agonists in HeLa cell lines stably transfected with human 5-$HT_{1D\alpha}$ or 5-$HT_{1D\beta}$ receptors, in contrast to their well recognised antagonist activity in animal tissue. Variations in receptor reserve, second messenger and/or G-protein coupling systems could explain the different profiles of these compounds in cell-based assays and animal studies.

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