British Journal of Pharmacology (1998) 123, 1655–1665
http://www.stockton-press.co.uk/bjp

Agonistic properties of alniditan, sumatriptan and dihydroergotamine on human 5-HT_{1B} and 5-HT_{1D} receptors expressed in various mammalian cell lines

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1 Alniditan, a novel migraine abortive agent, is a potent 5-HT_{1B}/5-HT_{1D} receptor agonist of nM affinity. We compared the agonistic properties of alniditan, sumatriptan and dihydroergotamine on the cloned human 5-HT_{1B} receptor expressed at 200 fmol mg^{-1} protein (B_{max}) in non-induced L929sA cells, at 740 fmol mg^{-1} protein in HEK 293 and at 2300 fmol mg^{-1} protein in mIFNβ-induced L929sA cells, and on the human cloned 5-HT_{1D} receptor expressed in C6 glioma cells (B_{max} 780 fmol mg^{-1} protein).

2 Sodium butyrate treatment increased the expression level of human (h)5-HT_{1B} receptors in HEK 293 cells and h5-HT_{1D} receptors in C6 glioma cells approximately 3 fold, the binding affinities of [^{3}H]-5-HT and [^{3}H]-alniditan were unaffected.

3 Agonistic properties were evaluated based on inhibition of cyclic AMP accumulation in the cells after stimulation of adenylyl cyclase by forskolin or isoproterenol. Alniditan, sumatriptan and dihydroergotamine were full agonists at the h5-HT_{1B} receptor (IC_{50} values were 1.7, 20 and 2 nM, respectively in HEK 293 cells) and h5-HT_{1D} receptors (IC_{50} values of 1.3, 2.6 and 2.2 nM, respectively). At the h5-HT_{1B} receptor the agonist potency of the compounds slightly increased with higher receptor density. The opposite was seen for antagonists (ocaperidone, risperidone and ritanserin).

4 This comparative study demonstrated that alniditan was 10 times more potent than sumatriptan at the h5-HT_{1B} receptor, and twice as potent at the h5-HT_{1D} receptor. Dihydroergotamine was more potent an agonist at the h5-HT_{1B} receptor when expressed at high and low level in L929sA cells (but not in HEK 293 cells), and was less potent at the h5-HT_{1D} receptor.

Keywords: Migraine; therapeutics; alniditan; sumatriptan; dihydroergotamine; 5-HT_{1B}; 5-HT_{1D}; signal transduction

Introduction

Alniditan((−)-**R**-*N*-[(3,4-dihydro-2*H*-1-benzopyran-2-yl)-methyl]-*N*′-(1,4,5,6-tetrahydro-2-pyrimidinyl)-1,3-propanediamine dihydrochloride) is a novel benzopyran derivative (Figure 1), and the first non-indole, non-ergolide potent agonist at 5-hydroxytryptamine$_{1B}$ (5-HT_{1B}) and 5-HT_{1D} receptors (Van Lommen *et al*., 1995; Leysen *et al*., 1996). In anaesthetized dogs, alniditan induced a dose-dependent vasoconstriction of the carotid vascular bed, with little effect on coronary, mesenteric and renal arterial blood flow (Van de Water *et al*., 1995). We recently described the receptor binding profile of alniditan and the receptor binding properties of [^{3}H]-alniditan (Leysen *et al*., 1996).

5-HT_1 receptors were proposed to play a role in the efficacy of anti-migraine agents (Moskowitz & Curter, 1993; Peroutka, 1993). Migraine is an episodic headache disorder that occurs in about 12% of the western population. The pathophysiology of the syndrome may involve vascular and neurogenic mechanisms (Moskowitz, 1992). Overactivity of trigeminal fibres which innervate cranial vessels, the trigeminovascular system, causes release of calcitonin gene-related peptide, a potent vasodilator, and substance P (Goadsby & Edvinsson, 1993). The resulting increase in cerebral blood flow, vasodilatation and neurogenic inflammation of the dura mater may transmit nociceptive signals through the trigeminal fibers to the central trigeminal nucleus caudalis, causing a pounding sensation of pain (Goadsby *et al*., 1991). Normalized cranial blood flow and reduced neurogenic inflammation may prove beneficial in migraine treatment. It was hypothesized that stimulation of 5-HT_{1B} receptors mediates vasoconstriction (Hamel *et al*., 1993b). A specific role for the 5-HT_{1B} receptors in the vascular system is suggested by the detection of mRNA in human and bovine cerebral arteries (Hamel *et al*., 1993a). Current evidence suggests that human and canine prejunctional receptors on noradrenergic neurones which innervate blood vessels and mediate inhibition of noradrenaline release, are of the 5-HT_{1B} type (Molderings *et al*., 1990; Janssens, personal communication). Stimulation of 5-HT_{1D} receptors of which the mRNA is specifically detected in the terminals of the trigeminal ganglion and the trigeminal nerve (Rebeck *et al*., 1994; Bonaventure *et al*., 1996), attenuates neuropeptide release in the rat superior sagittal sinus (Buzzi *et al*., 1991) as well as plasma extravasation from blood vessels in the dura mater (Buzzi & Moskowitz, 1990). These functional roles of both receptor subtypes are likely to be involved in the therapeutic action of anti-migraine drugs (Moskowitz, 1992).

As a potent human (h)5-HT_{1B} and h5-HT_{1D} agonist, alniditan has therapeutic potential for the acute treatment of migraine. In double-blind clinical trials, it was shown that 72% of all patients showed complete relief after 2 h when treated with 1.4 mg alniditan subcutaneously, and that only 16% suffered migraine recurrence (Goldstein *et al*., 1996). Drugs on the market for acute migraine therapy include dihydroergotamine and sumatriptan, both agonists at 5-HT_1-type receptors.

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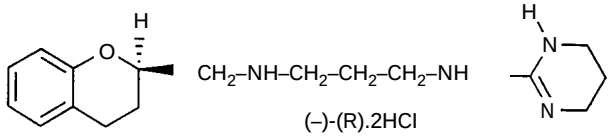

Figure 1 Chemical structure of alniditan.

The cloning and expression of human receptors has allowed us to demonstrate (Leysen *et al*., 1996) that dihydroergotamine is a broad spectrum ligand with high affinity for h5-HT_{1B}, h5-HT_{1D}, h5-HT_{1A}, rat (r)5-HT_{2A}, r5-HT_6, r5-HT_7, rat α_1 and α_2 adrenoceptors, and hD_{2L}, hD_3 and $hD_{4.2}$ receptors, whereas sumatriptan binds more selectively and with high affinity to h5-HT_{1D}, h5-HT_{1B} and h5-HT_{1F} receptors. The binding profile of alniditan revealed high affinity for h5-HT_{1B}, h5-HT_{1D}, h5-HT_{1A}, and moderate affinity for α_{2A} and α_{2B}-adrenoceptors. At the h5-HT_{1A} receptor, alniditan showed little functional activity (Leysen *et al*., 1996).

5-HT_{1B} and 5-HT_{1D} receptors belong to the family of G-protein coupled receptors with 7 transmembrane regions. Stimulation of 5-HT_{1B} and 5-HT_{1D} receptors causes activation of the G_i protein, leading to inhibition of adenylyl cyclase. The negative coupling of 5-HT_{1B} and 5-HT_{1D} receptors to adenylyl cyclase allowed investigation of the agonistic properties of compounds by measuring inhibition of forskolin-stimulated adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP formation. The objective of this study was to investigate the agonist properties of alniditan at the cloned h5-HT_{1B} and h5-HT_{1D} receptors in comparison to sumatriptan, dihydroergotamine and 5-HT. It was our aim to define whether the agonist properties of alniditan at the h5-HT_{1B} receptor changes with receptor expression level, and how this compared to changes in functional response of sumatriptan and dihydroergotamine. We also investigated whether agonist properties as derived in forskolin stimulated cells, reflected the agonist properties in a more physiological situation, such as modelled by indirect stimulation of adenylyl cyclase via a positively coupled G-protein receptor.

In order to answer these questions, the cloned h5-HT_{1B} receptor was expressed at different levels in mammalian cell lines. The h5-HT_{1B} receptor was permanently expressed in HEK 293 cells under control of a constitutive cytomegalovirus promoter. The stable expression level of the h5-HT_{1B} receptor in these cells could be elevated further by treatment with sodium butyrate. The h5-HT_{1B} receptor was also permanently expressed in L929sA cells under control of an IFNβ-inducible promoter. Non-induced cells showed low receptor expression, whereas induction with mIFNβ resulted in high receptor levels. The h5-HT_{1D} receptor was permanently expressed in C6 glioma cells under control of the constitutive simian virus SV40 early promoter. Treatment with sodium butyrate increased the expression level of the h5-HT_{1D} receptor in these cells.

For each of the aforementioned expression systems we investigated the binding properties of [^{3}H]-5-HT and [^{3}H]-alniditan. Since the affinity of these radioligands was unaffected by the expression level of the receptors, we used the high expressing cell systems (5-HT_{1B} receptor in mIFNβ-induced L929sA cells and in butyrate-treated HEK 293 cells, and h5-HT_{1D} receptor in butyrate-treated C6 glioma cells) to illustrate binding affinities of the agonists and antagonists under study. In the first signal transduction experiments with these expression systems, we investigated the effect of sodium butyrate on cyclic AMP formation. Since our results indicated that sodium butyrate interfered with accumulation of cyclic AMP, we refrained from using sodium butyrate-induced cell systems in functional studies. This implied that for the study of agonistic properties of alniditan, sumatriptan, dihydroergotamine and 5-HT we relied on two cell lines, for three h5-HT_{1B} receptor expression levels: a low and high density in non-induced and IFN-β-induced L929sA cells, and a medium density in non-treated HEK 293 cells. Agonistic properties of the anti-migraine compounds were investigated also at the human cloned 5-HT_{1D} receptor, permanently expressed in C6 glioma cells. In these cells, cyclic AMP accumulation could be stimulated either directly via forskolin or indirectly via an endogenously expressed β-adrenoceptor, activated by isoproterenol. Agonist properties of the anti-migraine compounds were analysed under both conditions, and potencies were compared. Some of our findings were further broadened with the activities of antagonists in these systems.

Methods

Cell culture

HEK 293, a human transformed embryonic kidney cell line (ATCC, CRL 1573), was grown in Dulbecco's modified Eagle's medium containing 1 mM sodium pyruvate, 2 mM L-glutamine, antibiotics and 10% foetal calf serum. The growth medium for C6 glioma, a rat glial tumour cell line (ATCC, CCL 107), and L929sA, a mouse fibrosarcoma cell line (Dr Konings, Rega Institute Leuven, Belgium) was as described previously (Leysen *et al*., 1996). The selection and culture medium for L929sA cells expressing the 5-HT_{1B} receptor contained an extra 500 μg ml^{-1} geneticin, for HEK 293 cells expressing the 5-HT_{1B} receptor it contained 800 μg ml^{-1} geneticin and for C6 glioma cells expressing the 5-HT_{1D} receptor it contained 300 μg ml^{-1} geneticin. Geneticin was left out at least 2 days before assay. Cells were grown at 37°C in humidified air with 5% CO_2.

Cloning and expression of the h5-HT_{1B} and h5-HT_{1D} receptors

The cloning and expression of the h5-HT_{1B} and h5-HT_{1D} receptors in L929sA cells and C6 glioma cells were as described previously (Leysen *et al*., 1996; Vanhoenacker *et al*., 1997). In C6 glioma cells, expression of the h5-HT_{1D} receptor was under control of the constitutive SV40 early promoter from a modified pSG5 expression vector (kindly provided by J. Naranjo, Instituto Cajal, Spain). Expression of the h5-HT_{1B} receptor in L929sA cells was under control of the interferon-β (IFNβ)-inducible murine Mx1 promoter. The same h5-HT_{1B} coding region was also subcloned into the pRc/CMV vector (Invitrogen) for transfection of HEK 293 cells. The calcium phosphate transfection method (Cullen, 1987) was used with the following modifications: cells were seeded in 10 petri dishes and transfected the next day. A 2 M $CaCl_2$ solution (final concentration 125 mM) was slowly added to 10 μg plasmid DNA in 0.5 ml transfection buffer (20 mM HEPES, 6 mM glucose, 137 mM NaCl, 5 mM KCl, 7 mM Na_2HPO_4, pH 7.05). Following this procedure, the precipitate was prepared separately for each of the 10 plates. After 20 min incubation at ambient temperature, the medium on the HEK 293 cells was aspirated, the precipitate was added onto the cells and petri dishes were incubated for 15 min. Culture medium was added and plates were further incubated for 4 h. The DNA-

containing medium was aspirated and cells were treated with 15% glycerol in phosphate buffered saline for 90 s. Cells were washed with medium and left to recover for 24 h. The medium was then switched to selection medium. Transfected cells were left to grow until confluency, after which the cells were passaged for receptor binding assays. Cells from the plate showing highest binding activity were used for isolating monoclonal cell lines via limiting dilution. Amongst the approximately 70 monoclonal HEK 293 cell lines isolated, 4 had a receptor expression level of approximately 2000 fmol mg^{-1} protein (when stimulated with sodium butyrate). One of these was used for further characterization and study (clone 41).

Cell membrane preparation and ligand binding

Cells were grown to confluence, washed and scraped from the culture plate, and membranes were prepared as described elsewhere (Leysen *et al*., 1996). Binding studies with [^{3}H]-5-HT and [^{3}H]-alniditan were essentially as described before (Leysen *et al*., 1996).

Adenylyl cyclase assay

Cells were plated in Multi-well 24 plates. The next day, cells were washed and incubated for 20 min in the presence of the appropriate concentration of test compound with controlled salt solution (composition in mM: NaCl 120, KCl 5, $MgCl_2$ 0.8, $CaCl_2$ 1.8, glucose 15 and phenol red 0.04 in 25 mM Tris-HCl pH 7.4) containing 1 mM isobutylmethylxanthine, 1 μM pargyline, 1 μM paroxetine and either 100 μM forskolin, 1 μM isoprenaline or solvent. The incubation was stopped with ice-cold $HClO_4$. The extract was neutralized to pH 7.5 with K_3PO_4/KOH solution (0.5 M K_2HPO_4 to which KOH was added till pH 13.5). After precipitation of $KClO_4$ at 4°C, plates were centrifuged, and the supernatant was assayed for cyclic AMP content with a commercial [^{125}I]-cyclic AMP radioimmunoassay kit (Immunotech International, France) according to the procedure recommended by the manufacturer. The cyclic AMP levels were expressed as percentage of forskolin- or isoprenaline-stimulated cyclic AMP production (set at 100%), and plotted against the drug concentration on a logarithmic scale. Sigmoidal curves of best fit were calculated by non-linear regression analysis by use of GraphPad software (San Diego, CA). The pIC_{50} value referred to the concentration of a compound producing half the maximum reduction in cyclic AMP seen with 5-HT.

Induction of receptor expression

Sodium butyrate (5 mM) was used to enhance receptor expression in C6 glioma and HEK 293 cell lines. Treatment occurred during the last 18 h before assay. Expression of h5-HT_{1B} receptors in L929sA cells was induced for 18 to 24 h with 1000 u ml^{-1} recombinant murine IFNβ. As a source for mIFNβ we used a crude extract from *E. coli* transformed with an expression plasmid encoding mIFNβ (Vanhoenacker *et al*., 1997).

Materials

Alniditan and [^{3}H]-alniditan (41.4 Ci $mmol^{-1}$) are original compounds of Janssen Pharmaceutica (Beerse, Belgium). [^{3}H]-alniditan was obtained by catalytic tritiation as previously described (Janssen, 1993). [^{3}H]-5-HT (88 Ci $mmol^{-1}$) was obtained from Amersham (Little Chalfont, U.K.). Dihydroergotamine-mesylate was obtained from Sigma. Sumatriptan was kindly donated by Glaxo. Other chemicals were purchased from J. T. Baker Chemicals Co, Merck, Serva, Sigma, Boehringer Mannheim or Acros Chimica. Culture media and sera were obtained from Life Technologies (Merelbeke, Belgium).

Except for 5-HT, which was dissolved and diluted in water, alniditan, sumatriptan, dihydroergotamine and forskolin were all dissolved and further diluted in 100% dimethylsulphoxide (DMSO). For binding studies, a final 200 fold dilution yielded DMSO concentrations of 0.5%. For signal transduction assays, the DMSO concentration never exceeded 0.2%.

Results

HEK 293 and L929sA cells stably expressing the h5-HT_{1B} receptor and C6 glioma cells stably expressing the h5-HT_{1D} receptor were established to evaluate the functional activity of anti-migraine compounds and to investigate relationships between agonistic effects of compounds and expression level. The expression level of the receptors was derived from radioligand concentration binding isotherms performed with [^{3}H]-5-HT or [^{3}H]-alniditan on membranes of the transfected cells (for B_{max} values, see Table 1). Transfected HEK 293 and C6 glioma cell lines were treated with sodium butyrate to enhance gene expression. Butyrate treatment of HEK 293 cells expressing the h5-HT_{1B} receptor increased the B_{max} value approximately 3 to 6 fold (Table 1). The higher expression levels were comparable to the level seen in L929sA cells after

Table 1 Maximum number of binding sites (B_{max}) and apparent equilibrium dissociation constant (K_d) measured with [^{3}H]-5-HT and [^{3}H]-alniditan

		[^{3}H]-5-HT binding[a]		*[^{3}H]-alniditan binding*[a]	
Receptor type	*Transfected cell line*	K_d (nM)	B_{max} (fmol mg^{-1} protein)	K_d (nM)	B_{max} (fmol mg^{-1} protein)
h5-HT_{1B}	HEK 293				
	Not induced	7.3±1.2 (4)	610±210 (4)	2.0±0.1 (2)	740±270 (2)
	Sodium butyrate	7.4±2.1 (3)	3500±2900 (3)	2.4±0.5 (5)	2500±670 (5)
h5-HT_{1B}	L929sA				
	Not induced	2.8±0.8 (3)	150±94 (3)	0.9±0.3 (3)	200±78 (3)
	mIFNβ	2.6±0.7 (3)	1800±100 (3)	1.7±0.4 (6)	2300±530 (6)
h5-HT_{1D}	C6 glioma				
	Not induced	2.7±0.3 (4)	730±410 (4)	1.2±0.3 (11)	780±400 (11)
	Sodium butyrate	3.3	1700	1.4±0.3 (5)	2000±220 (5)

[a]Data shown are mean±s.d. (*n*).

mIFNβ induction. When L929sA cells were not induced, the expression of the h5-HT_{1B} receptor dropped to a 10 fold lower level. The affinity of [^{3}H]-alniditan for the h5-HT_{1B} receptor in butyrate-treated HEK 293 cells (K_d 2.4 nM) and in mIFNβ-treated L929sA cells (K_d 1.7 nM) was not different from the affinity seen in untreated HEK 293 cells (K_d 2.0 nM). The h5-HT_{1D} receptor expressing C6 glioma cells revealed essentially the same: sodium butyrate treatment increased the expression level of h5-HT_{1D} receptors about 2.5 fold, without affecting the affinity of the radioligands [^{3}H]-5-HT and [^{3}H]-alniditan.

Alniditan, sumatriptan and dihydroergotamine showed high affinity for the human cloned 5-HT_{1B} and 5-HT_{1D} receptors. K_i-values, as derived from inhibition binding experiments with [^{3}H]-5-HT and [^{3}H]-alniditan on membranes from the stable cell lines are shown in Table 2. Alniditan bound with the same high affinity to h5-HT_{1B} receptors expressed in HEK 293 cells and L929sA cells. The affinity of sumatriptan for the h5-HT_{1B} receptors in these cell lines was more than 10 times lower. Dihydroergotamine showed a somewhat higher affinity than alniditan in these membrane preparations. At the h5-HT_{1D} receptors, expressed in C6 glioma cells and assayed with [^{3}H]-5-HT, alniditan bound with highest affinity, followed by dihydroergotamine, 5-HT and sumatriptan. K_i-values for h5-HT_{1B} and h5-HT_{1D} receptors, measured with [^{3}H]-alniditan, were higher for most compounds than those measured with [^{3}H]-5-HT.

Inhibition of cyclic AMP accumulation was measured to evaluate functional activity of the anti-migraine compounds. In Table 3, basal cyclic AMP content and forskolin- or isoprenaline-stimulated cyclic AMP accumulation in non-transfected and permanently transfected cell lines are summarized. mIFNβ treatment of L929sA cells did not change basal and forskolin-stimulated cyclic AMP accumulation. However, in C6 glioma cells and HEK 293 cells, butyrate treatment reduced basal cyclic AMP content as well as forskolin- and isoprenaline-stimulated cyclic AMP production by more than 50% (Table 3). Because of this apparent interference of sodium butyrate with cyclic AMP accumulation, we refrained from using sodium butyrate-stimulated cells for functional assays. In h5-HT_{1B} receptor-expressing HEK 293 cells, forskolin-stimulated cyclic AMP production was somewhat more pronounced than in non-transfected cells (a 140 fold versus a 105 fold stimulation over basal levels). A similar effect was seen in C6 glioma cells (a 130 fold versus a 109 fold stimulation over basal levels). This difference in ability of forskolin to stimulate adenylyl cyclase was not seen in L929sA cells. Although basal cyclic AMP content was not lower in L929sA cells than in other cell lines, stimulation with forskolin yielded only a 10 fold increase in cyclic AMP content; one possible explanation could be that forskolin only partially penetrated these cells.

When cyclic AMP content was measured in non-transfected HEK 293 and L929sA cells treated with 5-HT, alniditan, sumatriptan or dihydroergotamine (up to 1 μM), no effect on basal or forskolin-stimulated cyclic AMP accumulation was found (data not shown), suggestive of the absence of functional positively and negatively G-protein coupled 5-HT receptors in these parental cell lines. Expression of the human

Table 2 Binding to the cloned human 5-HT_{1B} and 5-HT_{1D} receptors: pIC_{50} values and derived apparent equilibrium inhibition constant (K_i) for anti-migraine compounds

	h5-HT_{1B}-R in HEK 293[a]		*h5-HT_{1B}-R in L929sA*[b]		*h5-HT_{1D}-R in C6 glioma*[a]	
	[^{3}H]-5-HT	*[^{3}H]-alniditan*	*[^{3}H]-5-HT*	*[^{3}H]-alniditan*	*[^{3}H]-5-HT*	*[^{3}H]-alniditan*
Alniditan	8.93±0.04 (2) 0.8	8.42±0.20 (4) 2.1	8.65±0.14 (2) 0.9	8.46±0.15 (2) 1.6	9.03±0.13 (3) 0.4	8.25±0.12 (3) 2.3
Sumatriptan	7.60±0.13 (5) 16	7.23±0.27 (4) 32	7.51±0.14 (2) 12	6.95±0.04 (3) 52	8.05±0.05 (2) 4.0	7.58±0.05 (4) 11
Dihydroergotamine	9.29±0.18 (5) 0.3	9.30±0.29 (4) 0.3	8.86+0.09 (2) 0.5	8.89±0.11 (4) 0.6	8.25±0.12 (3) 2.5	8.75±0.16 (3) 0.7
5-HT	8.11±0.07 (2) 5.0	7.63±0.23 (4) 13	8.01±0.05 (3) 3.8	7.55±0.10 (4) 13	8.17±0.15 (4) 3.1	7.62±0.06 (6) 9.9

pIC_{50} values are presented as mean±s.d. (n); K_i values (lower values) are in nM. [a]Cells were stimulated with sodium butyrate. [b]Cells were induced with mIFNβ.

Table 3 Cyclic AMP content in non-transfected and permanently transfected HEK 293, L929sA and C6 glioma cell lines

		Cyclic AMP content (pmol/well)[a]			*Stimulation factor treated versus basal level*	
Cell line	*Treatment*	*Basal*	*Forskolin*	*Iso*	*Forskolin*	*Iso*
HEK 293	No	4.4±0.8 (6)	460±95 (6)	–	105	–
	Butyrate	1.8±0.5 (4)	200±32 (4)	–	111	–
h5-HT_{1B}-R in HEK 293	No	5.5±1.0 (6)	770±230 (6)	–	140	–
L929sA	No	4.6±1.5 (4)	46±12 (4)	–	10	–
	mIFNβ	4.4±0.7 (4)	46±10 (4)	–	10	–
h5-HT_{1B}-R in L929sA	No	5.4±0.7 (6)	58±5 (6)	–	11	–
	mIFNβ	5.5±1.6 (5)	58±12 (5)	–	11	–
C6 glioma	No	4.4±0.6 (6)	480±55 (6)	490±22 (6)	109	111
	Butyrate	2.0±0.2 (4)	190±22 (4)	170±14 (4)	95	85
h5-HT_{1D}-R in C6 glioma	No	6.0±0.6 (6)	800±110 (6)	710±180 (6)	130	120

[a]Data shown are mean±s.e.mean (n). Cells were stimulated with forskolin (100 μM) and isoprenaline (Iso; 1 μM).

cloned h5-HT_{1B} receptor in these cell lines allowed the study of the agonistic properties of alniditan, sumatriptan and dihydroergotamine at this recombinant receptor. The conditions used and h5-HT_{1B} receptor levels were: non-induced L929sA cells (B_{max} 150~200 fmol mg^{-1} protein), HEK 293 cells not treated with butyrate (B_{max} 610~740 fmol mg^{-1} protein), and murine (m)IFNβ-induced L929sA cells (B_{max} 1800~2300 fmol mg^{-1} protein). The resulting concentration-response curves for 5-HT, alniditan, sumatriptan and dihydroergotamine are shown in Figure 2. Judged from the maximum inhibition of cyclic AMP formation, all compounds were full agonists at the h5-HT_{1B} receptor. The IC_{50}-values for agonism at the h5-HT_{1B} receptor are summarized in Table 4. Agonist potencies of 5-HT, alniditan and sumatriptan were

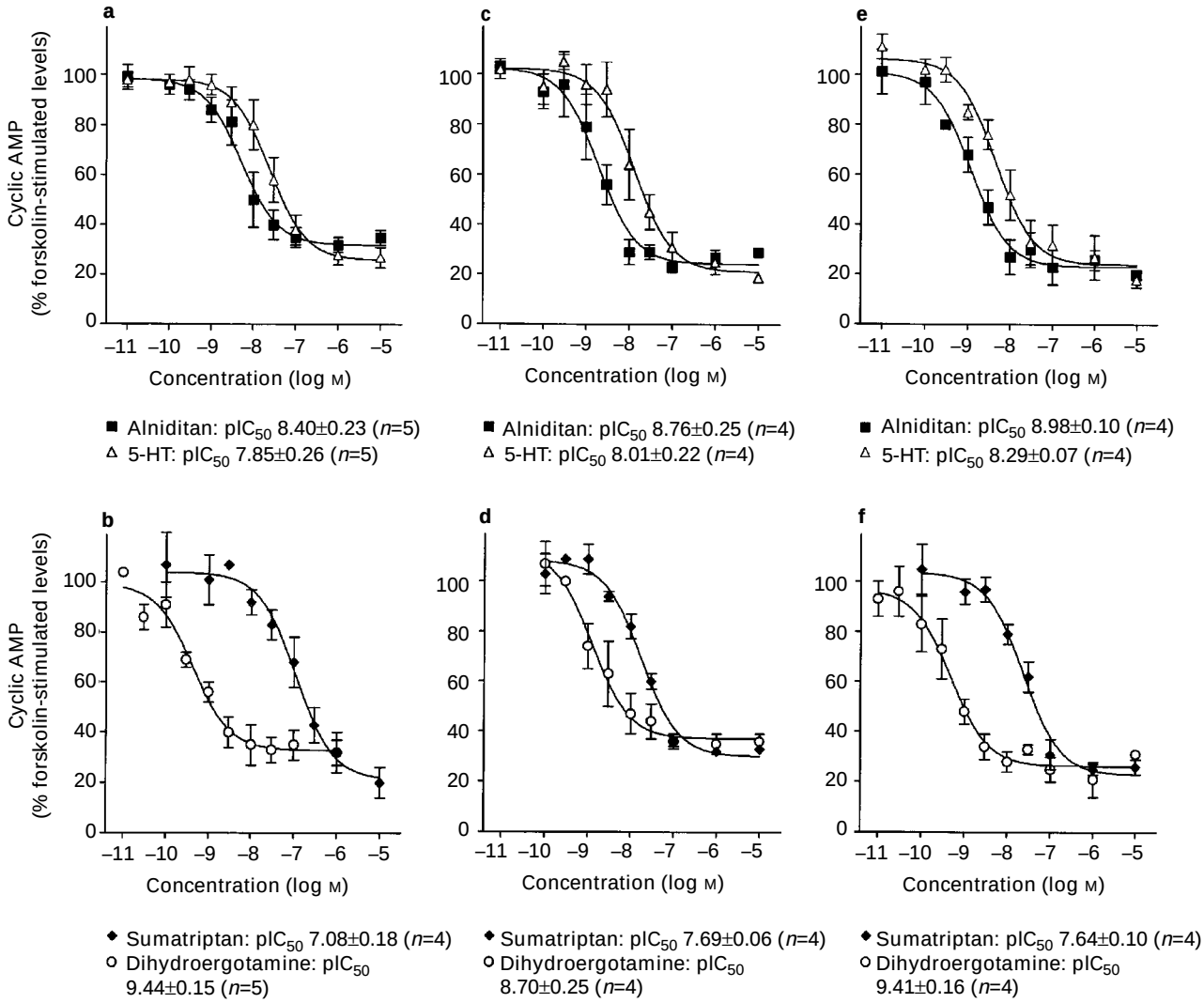

Figure 2 Signal transduction of alniditan, sumatriptan, dihydroergotamine and 5-HT mediated by the h5-HT_{1B} receptor expressed in (c and d) HEK 293 cells and (a, b, e and f) L929sA cells either induced (e and f) or not induced (a, b, c and d) with mIFNβ (1000 u ml^{-1}, 24 h). Adenylyl cyclase was stimulated by treating the cells with 100 μM forskolin in the presence of 1 mM isobutylmethylxanthine for 20 min. The resulting accumulation of cyclic AMP was measured in cell extracts by radioimmunoassay. Simultaneous treatment of the cells with alniditan, sumatriptan, dihydroergotamine or 5-HT caused a concentration-dependent decrease in forskolin-stimulated adenylyl cyclase activity. The resulting cyclic AMP accumulation was expressed as % of the forskolin-stimulated level, which was set at 100%. For absolute cyclic AMP levels, see Table 3. Sigmoidal inhibition curves were fitted by non-linear regression analysis, from which pIC_{50}-values were derived (presented in figure as mean±s.e.mean).

Table 4 Agonist potency at the cloned human 5-HT_{1B} receptor stably expressed in L929sA and HEK 293 cells, and at the cloned human 5-HT_{1D} receptor stably expressed in C6 glioma cells

	h5-HT_{1B}-R in L929sA[a] (B_{max} 200 fmol mg^{-1} protein)	*h5-HT_{1B}-R in HEK 293*[b] (B_{max} 740 fmol mg^{-1} protein)	*h5-HT_{1B}-R in L929sA*[c] (B_{max} 2300 fmol mg^{-1} protein)	*h5-HT_{1D}-R in C6 glioma*[b] (B_{max} 780 fmol mg^{-1} protein)	
	Forskolin	*Forskolin*	*Forskolin* IC_{50} (nM)	*Forskolin*	*Isoprenaline*
Alniditan	4.0	1.7	1.0	1.3	0.8
Sumatriptan	83	20	23	2.6	16
Dihydroergotamine	0.4	2.0	0.4	2.2	3.2
5-HT	14	9.8	2.4	2.4	1.1

[a]Cells were not induced with mIFNβ. [b]Cells were not treated with sodium butyrate. [c]Cells were induced with mIFNβ.

lower in the cell line expressing fewer receptors. However, this tendency was not statistically significant (overlapping 95% confidence intervals) and was not found for dihydroergotamine, which was equally potent in L929sA cells expressing high or low levels of h5-HT_{1B} receptors. Independent of the cell line or the receptor expression level, alniditan was 10 to 20 fold more potent than sumatriptan, and 2 to 6 times more active than 5-HT. In L929sA cells (but not in HEK 293 cells) alniditan was 2.5 to 10 times less active than dihydroergotamine.

When non-transfected C6 glioma cells were treated with 5-HT (up to 1 μM), no effect on basal or forskolin-stimulated cyclic AMP accumulation was observed, suggestive of the absence of functional positively and negatively G-protein coupled 5-HT receptors in the parental cell line. In h5-HT_{1D} receptor-expressing C6 glioma cells not treated with butyrate, cyclic AMP content was either raised via direct stimulation of adenylyl cyclase with forskolin or indirectly via isoprenaline acting upon the endogenous β-adrenoceptor, positively coupled to adenylyl cyclase. Concentration-response curves

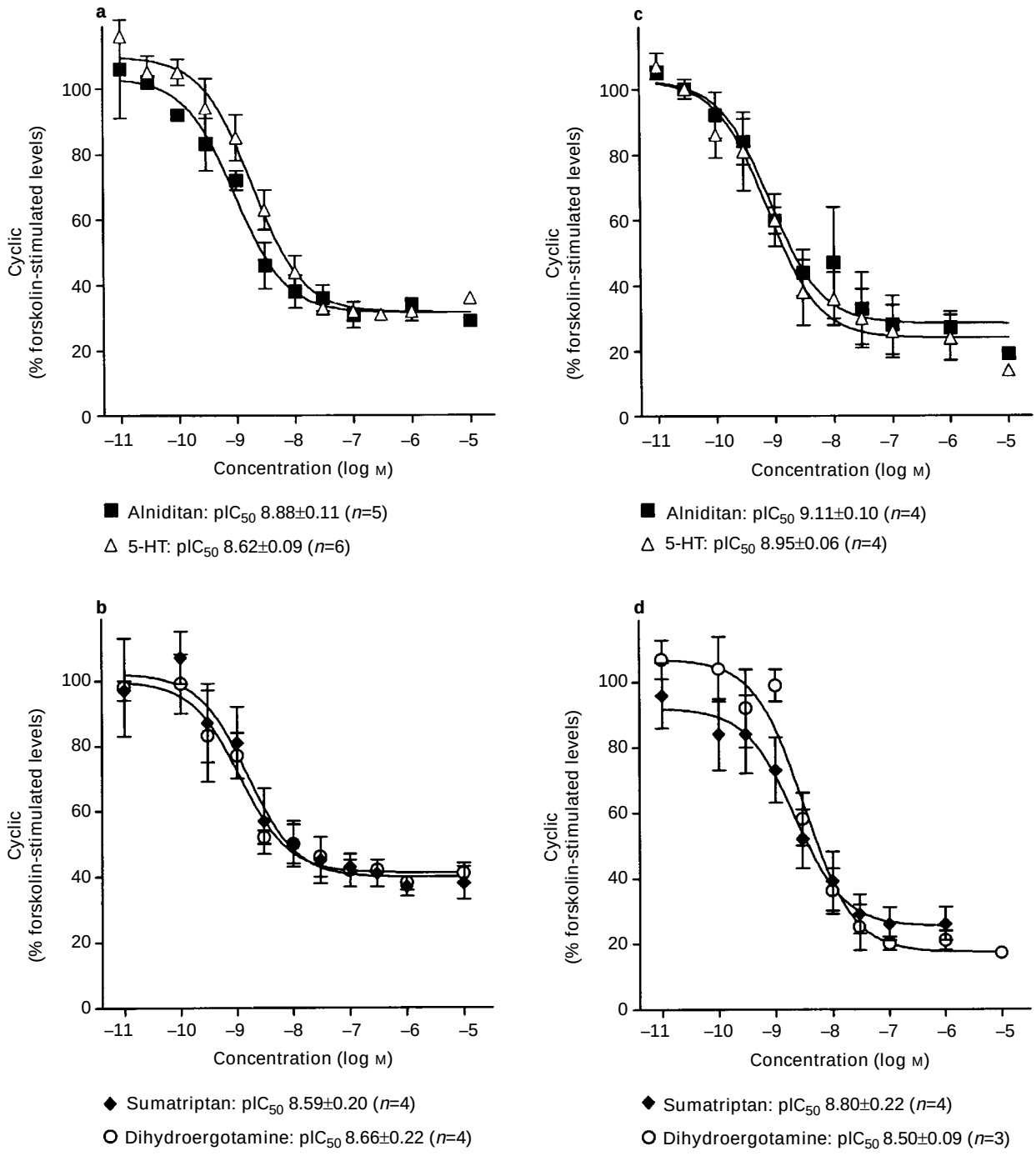

Figure 3 Signal transduction of alniditan, sumatriptan, dihydroergotamine and 5-HT mediated by the h5-HT_{1D} receptor expressed in C6 glioma cells. Adenylyl cyclase was stimulated by treating the cells either with 100 μM forskolin (a, b) or 1 μM isoprenaline (Iso; c, d) in the presence of 1 μM isobutylmethylxanthine for 20 min. The resulting accumulation of cyclic AMP was measured in cell extracts by radioimmunoassay. Simultaneous treatment of the cells with alniditan, sumatriptan, dihydroergotamine or 5-HT caused a concentration-dependent decrease in forkolin- or isoprenaline-stimulated adenylyl cyclase activity. The resulting cyclic AMP accumulation was expressed as % of the forskolin-stimulated, or isoprenaline-stimulated level, which were both set at 100%. For absolute cyclic AMP levels, see Table 3. Sigmoidal inhibition curves were fitted by non-linear regression analysis, from which pIC_{50}-values were derived (presented in figure as mean$\pm$s.e.mean).

for 5-HT, alniditan, sumatriptan and dihydroergotamine are shown in Figure 3. The maximum response of the agonists in forskolin- and isoprenaline-stimulated cells revealed that all compounds were full agonists at the h5-HT_{1D} receptor. The IC_{50}-values, as summarized in Table 4, were quite similar when obtained from forskolin- or isoprenaline-stimulated cells. This implied that potencies were not affected by the way intracellular cyclic AMP content was raised. Under both conditions, the four compounds investigated showed equally high potency (IC_{50} 1–3 nM), alniditan always showing the lowest IC_{50}-value.

To explore further the influence of receptor expression level and direct versus indirect stimulation of cyclic AMP accumulation on functional activities of ligands, we evaluated the properties of some antagonists. As shown in Table 5, the binding affinities of ocaperidone, risperidone, ritanserin and ketanserin for the h5-HT_{1B} receptor expressed in HEK 293 or L929sA cells ranged over two orders of magnitude. The rank order of affinity was similar with [^{3}H]-5-HT and [^{3}H]-alniditan as competing radioligand. Treatment of h5-HT_{1B} receptor expressing HEK 293 and L929sA cells with ocaperidone, risperidone, ritanserin and ketanserin did not affect basal cyclic AMP content (data not shown). Concentration-response curves of the compounds for reversal of 5-HT-mediated inhibition of forskolin-stimulated cyclic AMP accumulation are shown in Figure 4. Ketanserin showed no antagonism at the h5-HT_{1B} receptor up to 10 μM, reflecting its weak binding affinity. Ocaperidone and risperidone were slightly more potent as antagonists in the cell lines expressing lower receptor levels. This was even more pronounced for ritanserin. Thus, antagonist potencies appeared higher in the cell lines with lower h5-HT_{1B} receptor densities, a trend just opposite to that seen for agonists.

The h5-HT_{1D} receptors expressed in C6 glioma cells, bound ocaperidone, risperidone, ritanserin and ketanserin with high affinity (Table 5). Treatment of h5-HT_{1D} receptor expressing C6 glioma cells with these antagonists did not affect basal cyclic AMP content (data not shown). The concentration-

Table 5 Binding to the cloned human 5-HT_{1B} and 5-HT_{1D} receptors: pIC_{50} values and derived apparent equilibrium inhibition constant (K_i) of putative antagonists

	h5-HT_{1B}-R in HEK 293[a]		*h5-HT_{1B}-R in L929sA*[b]		*h5-HT_{1D}-R in C6 glioma*[a]	
	[^{3}H]-5-HT	*[^{3}H]-alniditan*	*[^{3}H]-5-HT*	*[^{3}H]-alniditan*	*[^{3}H]-5-HT*	*[^{3}H]-alniditan*
Ocaperidone	7.20±0.18 (2) 41	6.92±0.04 (2) 66	7.15±0.09 (2) 28	6.95±0.10 (3) 52	8.54±0.37 (3) 2.3	8.31±0.09 (3) 2.0
Risperidone	6.98±0.31 (3) 68	6.65±0.29 (3) 150	6.97±0.46 (2) 43	6.60±0.20 (4) 120	7.50±0.08 (3) 14	7.52±0.18 (4) 12
Ritanserin	6.04±0.04 (2) 630	6.10±0.06 (2) 430	6.51±0.14 (3) 120	6.35±0.30 (3) 210	7.21±0.08 (3) 28	7.19±0.16 (3) 27
Ketanserin	5.31±0.23 (2) 3200	5.36±0.27 (3) 2400	5.39±0.15 (6) 1600	5.19±0.20 (6) 3000	7.23±0.13 (6) 27	7.06±0.22 (6) 36

pIC_{50} values are presented as mean±s.d. (*n*); K_i values (lower values) are in nM. [a]Cells were stimulated with sodium butyrate. [b]Cells were induced with mIFNβ.

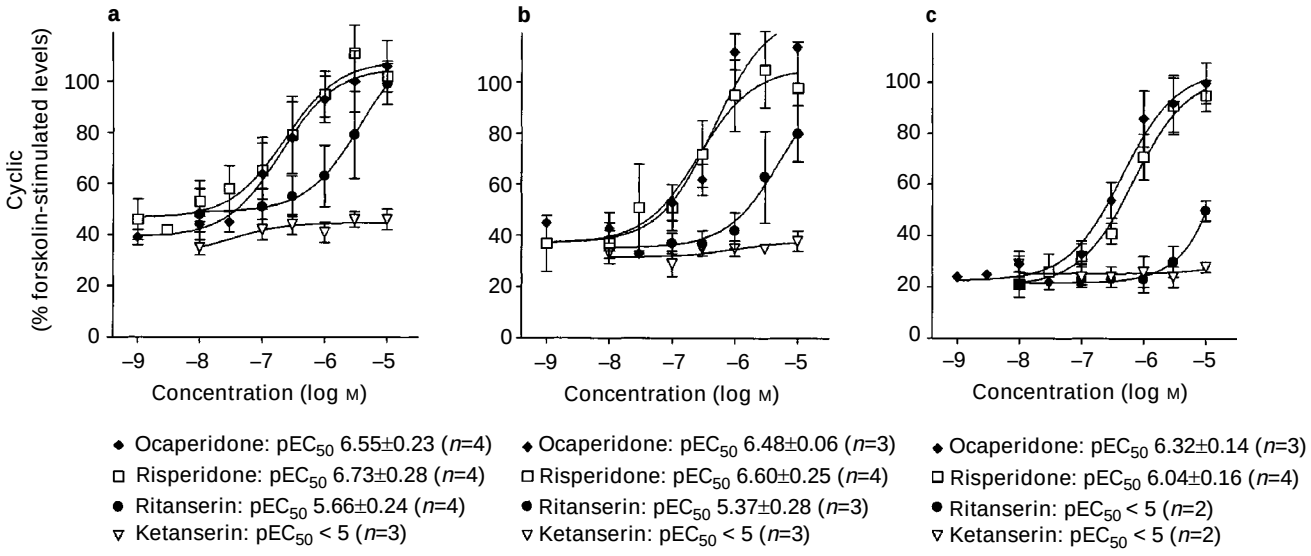

Figure 4 Antagonism by ocaperidone, risperidone, ritanserin and ketanserin of 5-HT-mediated inhibition of forskolin-stimulated adenylyl cyclase at the h5-HT_{1B} receptor expressed in (b) HEK 293 cells or (a, c) L929sA cells, either not induced (a, b) or induced (c) with mIFNβ (1000 u ml^{-1}, 24 h). Adenylyl cyclase was stimulated by treating the cells with 100 μM forskolin in the presence of 1 mM isobutylmethylxanthine for 20 min. The resulting accumulation of cyclic AMP was measured in extracts of these cells by radioimmunoassay and was set at 100%. Pretreatment of the cells for 20 min with ocaperidone, risperidone, ritanserin or ketanserin, followed by 20 min cotreatment of antagonist and 0.1 μM 5-HT (in the presence of 100 μM forskolin and 1 mM isobutylmethylxanthine) caused a concentration-dependent reversal of 5-HT-mediated inhibition of adenylyl cyclase activity. The resulting cyclic AMP accumulation was expressed as % of the forskolin-stimulated level. Sigmoidal curves were fitted by non-linear regression analysis, and the concentrations producing 50% reversal of 5-HT-mediated inhibition of adenylyl cyclase (pEC_{50} values) were derived (presented in figure as mean±s.e.mean).

response curves of these agents for restoration of 5-HT-mediated inhibition of forskolin-stimulated cyclic AMP accumulation are shown in Figure 5. Treatment of h5-HT_{1D} receptor-expressing C6 glioma cells with 5-HT (100 nM), reduced forskolin-stimulated cyclic AMP accumulation to a somewhat lesser extent than isoprenaline-stimulated cyclic AMP level (see also Figure 3), and therefore antagonist curves were more shallow in forskolin-treated cells. However, antagonist properties were not affected. Ocaperidone, risperidone, ritanserin and ketanserin were all full antagonists at the h5-HT_{1D} receptor. Furthermore, antagonist potencies were not altered by the way cyclic AMP accumulation was stimulated, except perhaps for ritanserin, which showed a slightly higher potency in forskolin-stimulated cells than in isoprenaline-stimulated cells (EC_{50}-value 0.5 versus 2 μM, respectively).

Discussion

Alniditan is a novel high affinity agonist for the h5-HT_{1B} and h5-HT_{1D} receptors (Leysen *et al*., 1996). In the present study we compared agonistic properties of alniditan with those of sumatriptan, dihydroergotamine and 5-HT at the h5-HT_{1B} and h5-HT_{1D} receptors. We used L929sA and HEK 293 cells, expressing the h5-HT_{1B} receptor at different densities, and C6 glioma cells expressing the h5-HT_{1D} receptor. Attempts to express h5-HT_{1B} receptors in C6 glioma cells had failed. In order to investigate the receptor-mediated inhibition of adenylyl cyclase, the enzyme was pre-activated directly by forskolin, or in C6 glioma cells which contain endogenous β-adrenoceptors, by stimulation with isoprenaline. These different systems allowed us to examine functional properties of agonists and antagonists at different receptor densities and under conditions of direct or indirect stimulation of cyclic AMP production.

Sodium butyrate and mIFNβ treatment of cells were used to enhance receptor expression. Sodium butyrate is a pleiotropic agent with effects on gene expression, cell growth and differentiation of cells (Prasad & Sinha, 1976; Kruh, 1982). This agent has been shown to induce several mammalian promoters, including the cytomegalovirus promoter (Cockett *et al*., 1990), which controlled h5-HT_{1B} receptor expression in HEK 293 cells, and the SV40 early gene promoter (Gorman & Howard, 1983; Goldberg *et al*., 1992) which was used for h5-HT_{1D} receptor expression in C6 glioma cells. Because sodium butyrate is also an inhibitor of histone deacetylation, treatment of cells would result in hyperacetylation of chromatin (Boffa *et al*., 1978; Sealy & Chalkley, 1978), thereby causing a relaxation of the chromatin structure and allowing overall enhanced gene transcription in the cell (Annunziato *et al*., 1988). Characterization of the cell lines revealed that receptor expression in HEK 293 cells and C6 glioma cells could be elevated approximately 3 fold by treatment with sodium butyrate (Table 1). As higher receptor density permits radioligand binding experiments with less membrane protein and because affinities of [^{3}H]-5-HT and [^{3}H]-alniditan were not affected by butyrate treatment (Table 1), we used membranes from butyrate-treated cells for inhibition studies of radioligand binding. However, our results from signal transduction experiments, suggested that butyrate may interfere with cyclic AMP production, as described in previous studies (Prasad & Sinha, 1976; Aranda *et al*., 1990; Datti & Deniss, 1993). For transfected L929sA cells, mIFNβ was used to induce the Mx promoter, controlling h5-HT_{1B} receptor expression. Receptor expression in non-induced L929sA cells was close to the detection level, suggesting little transcription from the non-

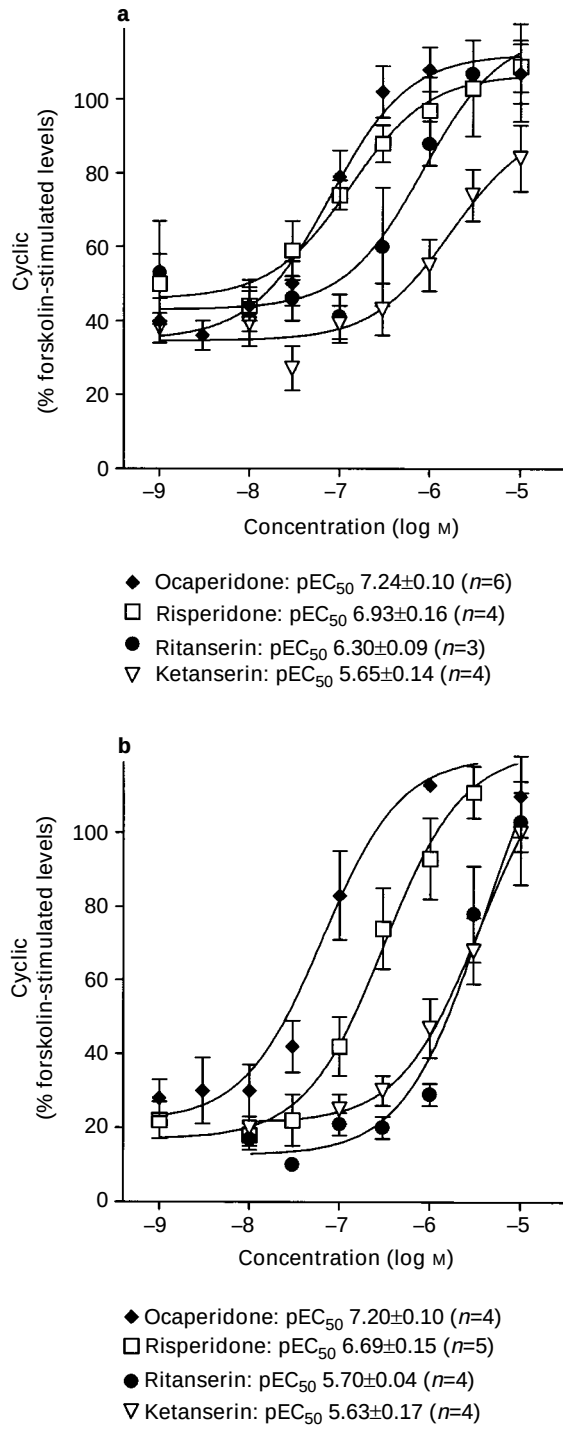

Figure 5 Antagonism by ocaperidone, risperidone, ritanserin or ketanserin of 5-HT-mediated inhibition of forskolin-stimulated adenylyl cyclase at the h5-HT_{1D} receptor expressed in C6 glioma cells. Adenylyl cyclase was stimulated by treating the cells either with 100 μM forskolin (a) or 1 μM isoprenaline (Iso; b) in the presence of 1 mM isobutylmethylxanthine for 20 min. The resulting accumulation of cyclic AMP was measured in extracts of these cells by radioimmunoassay. Pretreatment of the cells for 20 min with ocaperidone, risperidone, ritanserin or ketanserin, followed by 20 min cotreatment of antagonist and 0.1 μM 5-HT (in the presence of 100 μM forskolin and 1 mM isobutylmethylxanthine) caused a concentration-dependent reversal of 5-HT-mediated inhibition of adenylyl cyclase activity. The resulting cyclic AMP accumulation was expressed as % of the forskolin-stimulated level, or isoprenaline-stimulated level, which were both set at 100%. Sigmoidal curves were fitted by non-linear regression analysis, from which the concentrations producing 50% reversal of 5-HT-mediated inhibition of adenylyl cyclase (pEC_{50} values) were derived (presented in figure as mean $\pm$ s.e.mean).

induced promoter (Table 1). Receptor expression in mIFNβ-induced L929sA cells was approximately 10 fold higher, indicative that this promoter allowed tight control of gene expression. This was in line with previous findings (Lleonart *et al*., 1990; Vanhoenacker *et al*., 1997).

Of note is that, for this study, we grew the cells in medium containing 10% foetal calf serum, which was required for appropriate growth of the various cells. In order to check on the effect of cell exposure to 5-HT present in the serum, we performed a preliminary control study on cells grown in foetal calf serum or dialyzed calf serum. The concentration of 5-HT in medium with foetal calf serum was 730 nM and in the medium with dialyzed calf serum it was 1 nM. K_d values of the radioligands [^{3}H]-5-HT and [^{3}H]-alniditan for the h5-HT$_{1B}$ and h5-HT$_{1D}$ receptors, expressed in HEK 293 and C6 glioma cells, were exactly the same in membranes of cells cultured in 10% foetal calf serum and dialyzed calf serum. [^{3}H]-5-HT and [^{3}H]-alniditan are both full agonists and label only the high affinity, G-protein coupled state of the receptor. For the h5-HT$_{1D}$ receptor B_{max} values were the same for C6 glioma cells cultured in medium containing foetal calf serum and dialyzed calf serum, showing that the detected high affinity state of the h5-HT$_{1D}$ receptor is not sensitive to previous exposure to a high 5-HT concentration. For the h5-HT$_{1B}$ receptor, the B_{max} value was reduced by 30 to 40%, pointing to an uncoupling of part of the receptors following exposure to higher 5-HT concentrations, but a major portion of the receptor remained in the high affinity state. Hence it appeared that under the applied experimental conditions for both receptors the functionally coupled portion of the receptors could be detected reliably.

Agonistic properties of the compounds seemed to be affected by the h5-HT$_{1B}$ receptor density (Figure 2). The agonist potencies of alniditan, sumatriptan and 5-HT increased with higher receptor expression level (Table 4). This was not seen for dihydroergotamine. It is expected from the occupation theory and operational model (Kenakin, 1993) that the response and the potency of an agonist would increase with receptor level. Although our observations were in line with this, the shifts observed were smaller than would be predicted and were not statistically significant. Similar effects were described in a previous publication on 5-HT$_{1A}$ agonists (Varrault & Bockaert, 1992). It was hypothesized that receptor density may influence the efficiency of coupling between the receptor and adenylyl cyclase.

Agonist potencies of sumatriptan as we described for the different receptor expression levels, were in good agreement with several previous findings. In HEK 293 cells, expressing 740 fmol h5-HT$_{1B}$ receptor mg^{-1} protein, we found an IC$_{50}$-value for cyclic AMP inhibition of 20 nM; in LM(tk^-) cells, expressing the h5-HT$_{1B}$ receptor at a comparable level of 773 fmol mg^{-1} protein, a value of 14 nM was obtained (Zgombick *et al*., 1993). In non-induced L929sA cells, we found an IC$_{50}$-value for sumatriptan of 83 nM; in C6 glioma cells, expressing the h5-HT$_{1B}$ receptor at 359 fmol mg^{-1} protein an IC$_{50}$-value of 51 nM was found (Pauwels & Colpaert, 1995). For dihydroergotamine, data are more limited. In Chinese hamster ovary cells expressing the h5-HT$_{1B}$ receptor at 700 fmol mg^{-1} protein, an IC$_{50}$-value for dihydroergotamine of 9.8 nM was obtained (Miller *et al*., 1992), approximately 4 fold higher than that we found in HEK 293 cells. However, the same study also described an IC$_{50}$-value for sumatriptan of 317 nM, a value much higher than found by us and others.

To summarize, we found that in each of the above h5-HT$_{1B}$ receptor expression systems, alniditan was 10 to 20 times more potent an agonist than sumatriptan. Dihydroergotamine was in general slightly more potent than alniditan. A role for 5-HT$_{1B}$ receptors in migraine was suggested by their presence on human and bovine cerebral arteries (Hamel *et al*., 1993b), where they mediate the vasoconstrictive response to 5-HT$_1$-selective anti-migraine agents (Hamel *et al*., 1993a), and by their presence on human and canine noradrenergic neurones innervating the saphenous vein, where they mediate attenuation of noradrenaline release (Molderings *et al*., 1990; Janssens, personal communication). Potent agonism at the h5-HT$_{1B}$ receptor, as seen with alniditan, may thus prove beneficial in the treatment of migraine.

Agonist properties of the compounds were also evaluated at the h5-HT$_{1D}$ receptor, permanently expressed in C6 glioma cells. Both in forskolin- and isoprenaline-stimulated cells, alniditan was always twice as potent an agonist at the h5-HT$_{1D}$ receptor than sumatriptan and dihydroergotamine (Table 4). Stimulation of presynaptic 5-HT$_{1D}$ receptors was hypothesized to inhibit the release of calcitonin gene-related peptide and substance P from the perivascular trigeminal fibres, thus reducing dural neurogenic inflammation and plasma protein extravasation (Moskowitz, 1992). Supportive of this hypothesis were the findings that alniditan blocked plasma protein extravasation following electrical trigeminal stimulation in rats (F. Tegtmeier, personal communication), that sumatriptan reduced dural plasma extravasation in rats (Buzzi & Moskowitz, 1990; Shepheard *et al*., 1995), and that sumatriptan and dihydroergotamine inhibited the release of calcitonin gene-related peptide and substance P from the trigeminal nucleus (Buzzi *et al*., 1991; Goadsby & Edvinsson, 1993). Thus, potent agonistic properties at the h5-HT$_{1D}$ receptor as seen for alniditan, may aid in its therapeutic efficacy as anti-migraine drug.

To amplify on our findings with agonists in the above cell systems, we investigated the effects of antagonists such as ocaperidone, risperidone and ritanserin (which showed little selectivity for h5-HT$_{1B}$ or h5-HT$_{1D}$ receptors), and ketanserin (which bound selectively to the h5-HT$_{1D}$ receptor) (Table 5). Interestingly, we found exactly the opposite trend as seen for agonists: antagonist potency seemed increased at lower expression level. These findings are further evidence that receptor expression level may influence agonist and antagonist effects.

On the whole, we found that the agonist potencies were close to their binding affinities for the receptors (Tables 2 and 4). However, antagonist potencies (EC$_{50}$) were in general 10 fold lower than their receptor affinities (Figures 4 and 5, and Table 5). Probably, the antagonists had to counteract (the agonism of) a rather high concentration of 5-HT (100 nM), in contrast to the binding inhibition assay, where only 2 nM [^{3}H]-5-HT was used. The K_e-values, calculated from the respective EC$_{50}$-values ($K_e = EC_{50}[1 + (C_{5\text{-HT}})\,(K_{d5\text{-HT}})^{-1}]^{-1}$, corresponded indeed much more closely to the binding affinities (K_i-values).

In conclusion, we have shown that for all receptor densities evaluated, alniditan was approximately 10 times more potent an agonist than sumatriptan at the h5-HT$_{1B}$ receptor. Dihydroergotamine may be more active than alniditan at this receptor. At the h5-HT$_{1D}$ receptor, alniditan was twice as potent as sumatriptan and dihydroergotamine. In light of the hypothesis that h5-HT$_{1B}$ and h5-HT$_{1D}$ receptor agonists are efficacious in acute migraine treatment, our findings may suggest a possible therapeutic value of alniditan in migraine.

Abbreviations: 5-HT: 5-hydroxytryptamine; ATCC: American Type Culture Collection; B_{max}: maximum number of binding sites; cyclic AMP: adenosine 3′:5′-cyclic monophosphate; DMSO: dimethylsulphoxide; G-protein: guanine nucleotide binding protein; IC$_{50}$: concentration producing 50% inhibition; EC$_{50}$: concentration

producing 50% stimulation; IFN: interferon; K_d: apparent equilibrium dissociation constant; K_i: apparent equilibrium inhibition constant; K_e: apparent equilibrium excitation constant; m: murine; MW: multi-well plate; pIC_{50}: $-\log IC_{50}$

Part of this study was funded by a grant of the IWT (Vlaams Instituut voor de Bevordering van het Wetenschappelijk-Technologisch Onderzoek in de Industrie). We would like to thank Marianne De Backer for subcloning the human 5-HT_{1B} receptor cDNA, Luc Peeters for his help with adenylyl cyclase assays, Ariane Vreys for determining the receptor levels and assessing binding affinities of the compounds, and Yvette Dillen for the final editing of this manuscript. G.H. is a research director with the F.W.O. Vlaanderen (Fonds voor Wetenschappelijk Onderzoek).

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(Received July 10, 1997
Revised December 17, 1997
Accepted January 5, 1998)