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Differential inverse agonist efficacies of SB-258719, SB-258741 and SB-269970 at human recombinant serotonin 5-HT$_7$ receptors

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Abstract

Recombinant 5-hydroxytryptamine 5-HT$_7$ receptors are known to express constitutive, i.e., agonist-independent activity. Nonselective ligands, like methiothepin, ritanserin or clozapine behave as full inverse agonists at 5-HT$_7$ receptors. The aim of the present study was to evaluate the degree of inverse agonist activity of three selective 5-HT$_7$ receptor antagonists ((*R*)-3,*N*-dimethyl-*N*-[1-methyl-3-(4-methyl-piperidin-1-yl)propyl]benzene sulfonamide or SB-258719, *R*-(+)-1-(toluene-3-sulfonyl)-2-[2-(4-methylpiperidin-1-yl)ethyl]-pyrrolidine or SB-258741 and (*R*)-3-(2-(2-(4-methylpiperidin-1-yl)ethyl)-pyrrolidine-1-sulfonyl)-phenol or SB-269970) in the same model. cAMP accumulation was measured in intact Chinese hamster ovary (CHO) cells expressing human recombinant 5-HT$_{7a}$ receptors. In these cells, 5-HT stimulated cAMP levels and a series of ligands antagonized the effect of 5-HT with a 5-HT$_7$ receptor-like profile. SB-258719 had no inverse agonist activity, SB-258741 behaved as a partial inverse agonist and SB-269970 was a quasi-full inverse agonist (as compared to methiothepin). The inverse agonist effect of SB-269970 was antagonized in a concentration-dependent manner by SB-258719. The widespread spectrum of inverse agonist activities shown by these compounds should help assessing the physiological relevance of constitutive 5-HT$_7$ receptor activity in native tissues.

Keywords: 5-HT$_7$ receptor; Inverse agonism; Constitutive activity; SB-2587 19; SB-259741; SB-269970

1. Introduction

Receptors for serotonin (5-hydroxytryptamine, 5-HT) are classified into seven major groups (5-HT$_{1-7}$), based on structural, functional and pharmacological criteria (Hoyer et al., 1994). The 5-HT$_7$ receptor, cloned from mouse (Plassat et al., 1993), rat (Lovenberg et al., 1993; Ruat et al., 1993; Shen et al., 1993), guinea-pig (Tsou et al., 1994) and human (Bard et al., 1993), is positively coupled to adenylyl cyclase through the stimulatory G protein G$_s$ (Bard et al., 1993) and displays a unique pharmacological profile which is consistent across species. Sequence alignments show a high degree of inter-species homology (95%) but a low overall homology (< 40%) with other 5-HT receptors. A number of splice variants of both the human (5-HT$_{7a/b/d}$) and rat (5-HT$_{7a/b/c}$) receptor have been identified. They display similar pharmacological and functional characteristics when expressed in cell lines (Jasper et al., 1997; Heidmann et al., 1997; Krobert et al., 2001). The most abundant 5-HT$_{7a}$ isoform consists of a 445-amino acid polypeptide with a relatively short third intracellular loop and a long carboxy terminus.

5-HT$_7$ receptors have been localized in brain limbic and cortical areas, as well as in smooth muscles. There is a therapeutic potential for 5-HT$_7$ receptor ligands in depression, sleep disorders, migraine, pain and hypertension (for a review, see Vanhoenacker et al., 2000).

Constitutive activity of G-protein-coupled receptors is a relatively recent notion stemming from the observation that certain drugs previously known as classical antagonists, reduce the activity of some (mainly recombinant) receptor systems (for reviews, see Strange, 2002; Milligan, 2003;

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Kenakin, 2004). Such compounds have been known as inverse agonists. The spectrum of efficacies at a given receptor spreads from full inverse agonism to full agonism, through various degrees of inverse partial, neutral antagonism and partial agonism. In fact, very few neutral (i.e., silent) antagonists have been recognized, many compounds previously considered as antagonists acting as inverse agonists, e.g. cimetidine, haloperidol or prazosin.

This is also true for the 5-HT_7 receptor, for which several reports have described constitutive activity when expressed in human embryonic kidney (HEK)293 cells (Thomas et al., 1998; Hagan et al., 2000; Lovell et al., 2000; Krobert and Levy, 2002). In these cells, nonselective 5-HT_7 receptor ligands, like methiothepin, ritanserin or clozapine behaved as full inverse agonists whereas mesulergine can be regarded as a partial inverse agonist (Thomas et al., 1998; Krobert and Levy, 2002).

Several selective 5-HT_7 receptor antagonists have been recently introduced as research tools. The most prominent ones belong to a series of SmithKline Beecham compounds (see Fig. 1), namely SB-258719 ((*R*)-3,*N*-dimethyl-*N*-[1-methyl-3-(4-methyl-piperidin-1-yl)propyl]benzene sulfonamide) (Forbes et al., 1998), SB-258741 (*R*-(+)-1-(toluene-3-sulfonyl)-2-[2-(4-methylpiperidin-1-yl)ethyl]-pyrrolidine) (compound "13" in the work of Lovell et al., 2000) and SB-269970 ((*R*)-3-(2-(2-(4-methylpiperidin-1-yl)ethyl)-pyrrolidine-1-sulfonyl)-phenol) (Lovell et al., 2000).

SB-258719 has been reported as a partial inverse agonist at recombinant 5-HT_7 receptors expressed in HEK293 cells, as compared to ritanserin or methiothepin (Thomas et al., 1998). In a different study, SB-269970 has been shown to produce 'a small inhibition of basal adenylyl cyclase activity' in the same cells, however, its degree of inverse agonist activity was not quantified (Hagan et al., 2000). With respect to SB-258741, nothing is known of its potential inverse agonist activity, although this compound has been rather thoroughly evaluated in a variety of behavioural models (Pouzet, 2002; Pouzet et al., 2002). The aim of the present study was therefore to evaluate the degree of inverse agonist activity of these three selective 5-HT_7 receptor ligands in the same model and to compare them with a variety of known inverse agonists.

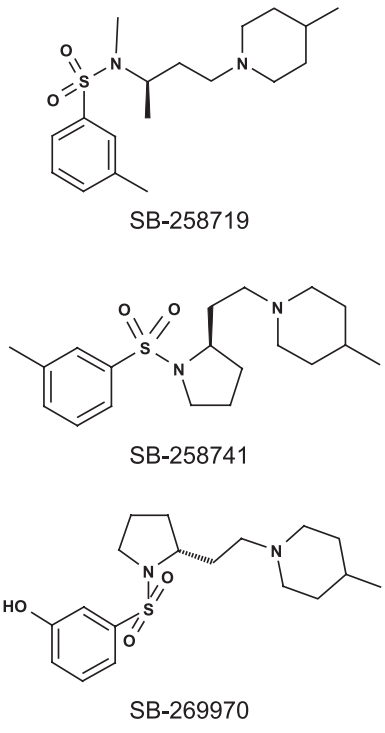

Fig. 1. Chemical structures of SB-258719, SB-258741 and SB-269970.

2. Materials and methods

2.1. Cell engineering and culture

Chinese hamster ovary (CHO)-K1 cells were grown in Dulbecco's Modified Eagle Medium (Gibco Cat. No. 31885), 10% foetal calf serum, non-essential aminoacids (Gibco Cat. No. 11140), penicillin G (100 units/ml) and streptomycin (100 μg/ml). They were transfected with 4.5 μg pXMT3-5-HT7 (containing the human 5-HT_{7a} receptor cDNA; Bard et al., 1993) and 0.5 μg pCDNA3.1hygromycin(+) (hygromycin B resistance gene; Invitrogen, Basel, Switzerland) using the Superfect Reagent Kit, according to the manufacturer's instructions (Qiagen, Hilden, Germany). Selection was started 48 h after the transfection by maintaining the cells in growth medium supplemented with 0.5 mg/ml hygromycin B. After 2 weeks, single colonies were picked with cloning rings, expanded in 6-well plates and further in 24-well plates for cAMP measurements (see below). The best clone in terms of stimulation by 5-HT was selected and propagated for the study. It is referred to as CHO/5-HT_7 cells in the following. CHO/5-HT_7 cell membranes gave a B_{max} of 3.5 pmol/mg protein in [^{3}H]mesulergine saturation binding studies, using 5-HT (10 μM) for non-specific binding.

2.2. cAMP measurements and analysis of data

cAMP accumulation was measured in intact cells seeded in 24-well plates, using the standard [^{3}H]adenine prelabelling technique, as previously described (Schoeffter et al., 1997, 1999). Cells were incubated with agonists/antagonists during 15 min in the presence of 1 mM isobutylmethylxanthine. The [^{3}H]cAMP/([^{3}H]cAMP+[^{3}H]ATP) cpm ratio (cAMP conversion rate) was calculated for each sample. Concentration–response curves were fitted to the nonlinear logistic function of the Origin 6.1 software package (OriginLab, Northampton, MA, USA). E_{max} and EC_{50} values were derived from these analyses. Results are given as means ± S.E.M. of the indicated *n* number of experiments, each performed in duplicate. Apparent K_B and pK_B values of antagonists were calculated according to Chen and Prusoff

(1973). Schild analysis was performed according to Arunlakshana and Schild (1959).

2.3. Drugs

5-Hydroxytryptamine creatinine sulphate and loxapine succinate were from Sigma-RBI (Buchs, Switzerland), pimozide from ANAWA (Wangen, Switzerland). Ritanserin was a generous gift from Janssen (Beerse, Belgium). The other compounds used, including SB-258719, SB-258741 and SB-269970, were synthesized at Novartis Pharma, Basel. Millimolar stock solutions of test compounds were made on the day of the experiment in dimethylsulphoxide or distilled water. Further dilutions were made in distilled water.

3. Results

3.1. Effect of 5-HT in CHO/5-HT$_7$ cells

The basal cAMP conversion rate in CHO/5-HT$_7$ cells was $1.52 \pm 0.07 \times 10^{-3}$ ($n = 12$). 5-HT stimulated the cAMP conversion rate in a concentration-dependent manner, with a pEC_{50} value of 8.15 ± 0.08 and an E_{max} representing 22 ± 2 times the basal levels ($n = 12$).

3.2. Effects of antagonist compounds on 5-HT-stimulated cAMP levels

A series of nonselective 5-HT$_7$ receptor ligands inhibited the effect of 5-HT (0.1 μM) on cAMP levels, in a concentration-dependent manner (Fig. 2A). Pindolol was inactive up to the concentration of 100 μM or even induced a very marginal, non-significant stimulatory effect on top of that of 5-HT. Apparent pK_B values are to be found in Table 1.

Table 1
Parameters of inhibitory effects of 5-HT$_7$ receptor ligands on 5-HT (0.1 μM)-stimulated and unstimulated cAMP levels in CHO/5-HT$_7$ cells

Compound	5-HT Stimulated levels		Unstimulated levels	
	Apparent pK_B	Max. inhibition (%)	pIC_{50}	Max. inhibition (%)
SB-269970	8.79 ± 0.03 (3)	94 ± 1	8.60 ± 0.13 (6)	46 ± 4
SB-258741	8.47 ± 0.05 (3)	94 ± 1	≈ 7.6	19 ± 6
Methiothepin	8.12 ± 0.16 (3)	98 ± 1	8.11 ± 0.13 (5)	60 ± 6
Mesulergine	8.06 ± 0.03 (3)	93 ± 1	/	0
Pimozide	7.81 ± 0.09 (3)	100	7.30 ± 0.13 (4)	64 ± 8
Clozapine	7.79 ± 0.03 (3)	98 ± 1	7.78 ± 0.23 (5)	58 ± 10
SB-258719	7.57 ± 0.05 (3)	92 ± 2	/	(stimulation)
Ritanserin	7.30 ± 0.08 (3)	100	6.88 ± 0.14 (4)	48 ± 4
Loxapine	7.16 ± 0.01 (3)	98 ± 1	7.10 ± 0.05 (3)	66 ± 8
Pindolol	<5 (3)	0	/	0

The three selective 5-HT$_7$ receptor ligands, SB-258719, SB-258741 and SB-269970 also inhibited the effect of 5-HT in a concentration-dependent manner (Fig. 2B; Table 1).

The overall rank order of antagonist potency (apparent pK_B) was: SB-269970 (8.79)>SB-258741 (8.47)>methiothepin (8.12)>mesulergine (8.06)>pimozide (7.81) = clozapine (7.79)>SB-258719 (7.57)>ritanserin (7.30)>loxapine (7.16)>pindolol (inactive).

3.3. Effects of antagonist compounds on unstimulated cAMP levels

When tested on unstimulated cAMP levels, these compounds had differential effects. Three types of behaviour

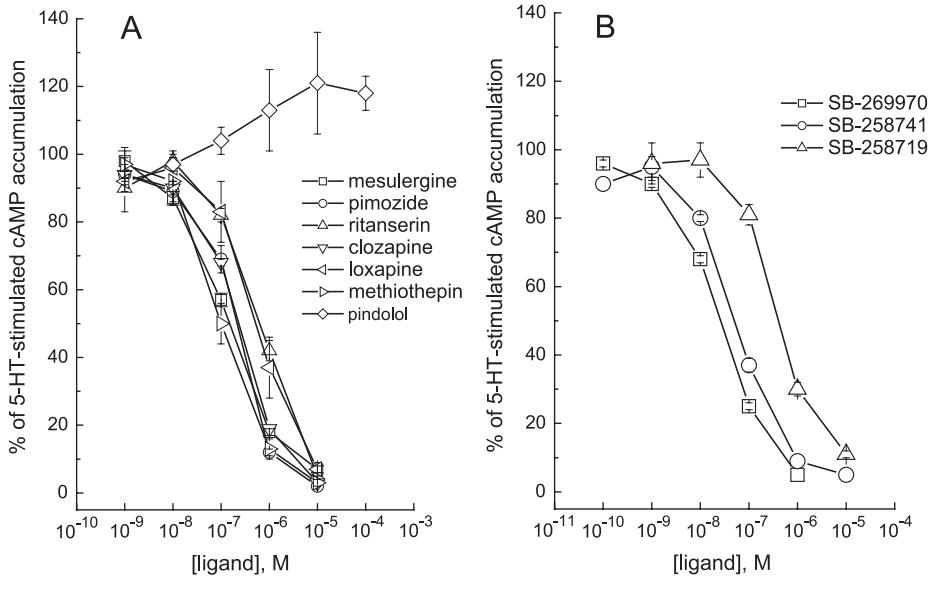

Fig. 2. Concentration–response curves of (A) a series of reference ligands and (B) SB-269970, SB-258741 and SB-258719 for inhibition of 5-HT-stimulated cAMP levels in CHO/5-HT$_7$ cells. Data are expressed as percentages of cAMP levels in the presence of 5-HT (0.1 μM) alone. Mean values ± S.E.M. from three individual experiments.

could be noticed: with a first group of compounds, including methiothepin, pimozide, clozapine, ritanserin, loxapine (Fig. 3A) and SB-269970 (Fig. 3B), concentration-dependent inhibition of unstimulated cAMP levels was observed. These inverse agonists induced maximal inhibitory effects by 46% to 66% (no statistical differences were found between compounds, using analysis of variance). The rank order of potency (pIC_{50}) was: SB-269970 (8.60)>methiothepin (8.11)>clozapine (7.78)>pimozide (7.30)>loxapine (7.10)>ritanserin (6.88). SB-258741 was particular in that it behaved as a partial inverse agonist, with maximal inhibition of unstimulated cAMP levels by 19% and a mean pIC_{50} value of 7.58 (Fig. 3B). Analysis of variance followed by post-hoc pairwise comparisons using Bonferroni adjustment showed that the maximal inhibition obtained with SB-258741 significantly differed ($P<0.05$) from the maximal inhibitions induced by the first group of compounds. A third group of compounds consisting of mesulergine, pindolol and SB-258719 was either silent (Fig. 3A) or even, in the case of SB-258719, slightly stimulated cAMP levels, i.e., had a weak positive agonist effect (Fig. 3B). The stimulatory effect of SB-258719 (147% of basal levels) was however very small as compared to that of 5-HT (22-fold or 2200% increase, see above). These data are summarized in Table 1.

3.4. Antagonism of the inverse agonist effect of SB-269970 by SB-258719

The inverse agonist effect of SB-269970 could be antagonized in a concentration-dependent manner by SB-258719 (0.1, 1 and 10 μM; Fig. 4). This resulted in rightwards shifts of the concentration–response curve to SB-269970, with no changes in the E_{max} of the inverse agonist, and this in spite of the small intrinsic activity of

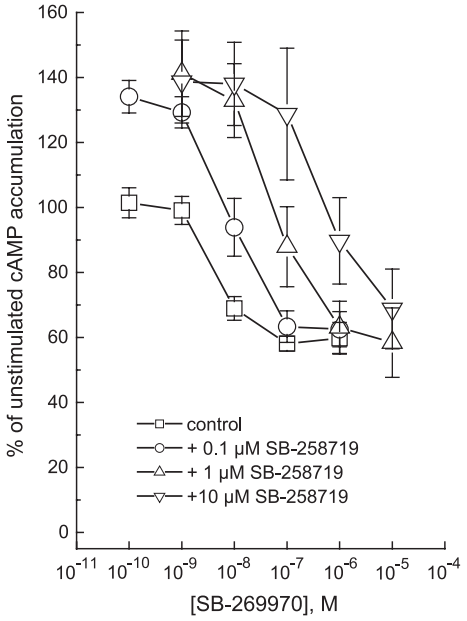

Fig. 4. Antagonism of the inverse agonist effect of SB-269970 by SB-258719 in CHO/5-HT_7 cells. Concentration–response curves of SB-269970-induced inhibition of unstimulated cAMP levels, in the absence and in the presence of increasing concentrations of SB-258719 (0.1 to 10 μM). Data are expressed as percentages of cAMP levels in the absence of compound. Mean values ± S.E.M. from three individual experiments.

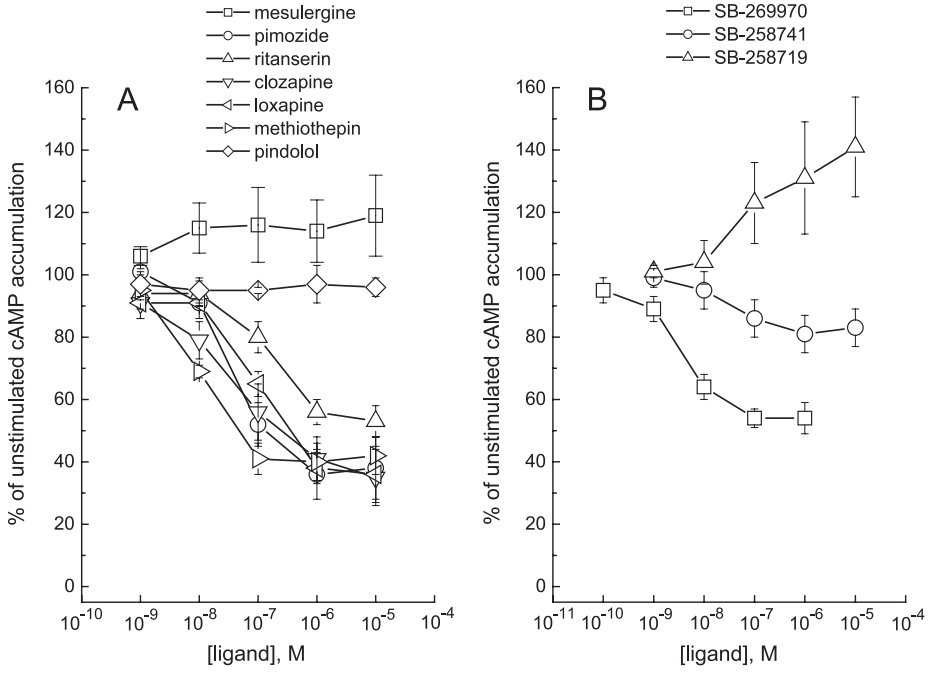

Fig. 3. Effects of (A) a series of reference ligands and (B) SB-269970, SB-258741 and SB-258719 on unstimulated cAMP levels in CHO/5-HT_7 cells. Data are expressed as percentages of cAMP levels in the absence of compound. Mean values ± S.E.M. from 3–5 (A) and 6–7 (B) individual experiments.

SB-258719. Schild analysis of this antagonism yielded a pA_2 value of 6.96 for SB-258719, with a slope factor of 1.06.

4. Discussion

The present study aimed at evaluating the degree of inverse agonist activity of the three selective 5-HT_7 receptor ligands, SB-258719, SB-258741 and SB-269970, comparing them with a variety of known inverse agonists at human recombinant 5-HT_{7a} receptors. Whereas nothing has been published on the inverse agonist potential of SB-258741, both SB-258719 (Thomas et al., 1998) and SB-269970 (Hagan et al., 2000) have independently been reported to display such activity. However, the relative inverse agonist efficacy of these compounds, as compared to each other, was still unknown.

The results show that the three antagonists are spread over a rather wide spectrum of inverse agonist activities. SB-269970 can be described as a quasi-full inverse agonist (compared to methiothepin, pimozide, clozapine, ritanserin or loxapine). Consistent with this finding, Sprouse et al. (2004) recently described SB-269970 and methiothepin as having the same inverse agonist efficacy at the rat recombinant 5-HT_{7a} receptor. SB-258741 can now be viewed as a partial inverse agonist, since it produced only ca. 50% of the maximal inhibition of unstimulated cAMP levels induced by SB-269970 and other compounds like methiothepin. At this point, it should be noticed that, chemically speaking, SB-258741 and SB-269970 are very closely related: the hydroxy group in position 3 of the aromatic group of SB-269970 is simply replaced by a methyl group in the structure of SB-258741 (see Fig. 1), with a probable change in lipophilicity (Lovell et al., 2000). As to SB-258719, it was neutral or even showed signs of a weak (positive) intrinsic activity (which, however, with a 1.4-fold stimulation, remained marginal compared to the 22-fold increasing effect of 5-HT). Thomas et al. (1998) found SB-258719 to behave as a partial inverse agonist. In that study, nevertheless, SB-258719 was the weakest inverse agonist in terms of maximal effect. Mesulergine was the second weakest one. Depending on the models, mesulergine is either a weak inverse agonist or neutral 5-HT_7 receptor antagonist (Krobert and Levy, 2002; Sprouse et al., 2004; present study). It thus appears that the inverse agonist activity of SB-258719, like that of mesulergine, may vary to some extent according to the model used (HEK293 versus CHO cells, membrane versus intact cell assay). It remains however that, of the three selective 5-HT_7 receptor antagonists used in the present study, SB-258719 is closest to a neutral antagonist. SB-258741 is a partial inverse agonist and SB-269970 the most robust, quasi-full inverse agonist (taking methiothepin as a reference).

There is little doubt that unstimulated cAMP levels correspond to constitutive activity of the 5-HT_7 receptor in the present study. The lack of inhibitory effect of mesulergine and SB-258719 on unstimulated cAMP levels, while both ligands potently and fully antagonized the 5-HT-stimulated effect, is strongly supportive of such a view. In addition, the overall pharmacological profile of the inhibition of unstimulated cAMP levels, in particular the competitive antagonism of SB-269970 by increasing concentrations of SB-258719, points to a 5-HT_7 receptor-mediated effect.

In the absence of any clear picture on the physiological importance of inverse agonism (see Kenakin, 2004), it is difficult to predict the impact of the present findings. Varying degrees of inverse agonism among ligands might translate into diverse effects in native tissues or systems only if constitutive activity of the receptor is expressed. So far, this has not been shown for the 5-HT_7 receptor. Evidence for constitutive activity of the 5-HT_7 receptor might be suggested in models in which administration of SB-269970 demonstrated effects per se. For example, SB-269970 reduced the time spent in paradoxical sleep in conscious rats (Hagan et al., 2000), increased dialysate 5-HT levels in the medial prefrontal cortex of freely moving rats (Roberts et al., 2001) and attenuated distension-evoked bladder contraction in rats (Read et al., 2003). In all these models, however, it would be difficult to dissociate putative constitutive activity and tonic, 5-HT-dependent activation of the receptor. In addition, in none of these models were the other selective 5-HT_7 receptor antagonists investigated. Conversely, SB-258741 has been evaluated in a variety of behavioural models, but never in parallel with SB-269970 (Pouzet, 2002; Pouzet et al., 2002). A strict comparison of SB-258719, SB-258741 and SB-269970 in a given model (like in the present study) would give clues on its level of constitutive 5-HT_7 receptor activity. As pointed out by Milligan (2003), ligands with ‘close to zero efficacy’ (i.e., neutral antagonists) are of great help to investigate constitutive activity in native settings. This has been exemplified by the case of histamine H_3 receptors, for which proxyfan is such a ligand. By reversing the effect of both agonists and inverse agonists, proxyfan allowed to demonstrate the relevance of constitutive activity of histamine H_3 receptors in rat brain membranes (Morisset et al., 2000). In such a way, SB-258719, which antagonized both the agonist effect of 5-HT and the inverse agonist effect of SB-269970 in the present study, should prove to be a useful tool, along with SB-269970 and SB-258741, in examining constitutive 5-HT_7 receptor activity in native tissues.

To summarize, the three selective 5-HT_7 receptor antagonists, SB-258719, SB-258741 and SB-269970, show a wide spectrum of inverse agonist activities at human recombinant 5-HT_7 receptors. SB-258719 is closest to a neutral antagonist, SB-258741 is a partial inverse agonist and SB-269970 the most robust inverse agonist. The parallel use of these compounds should help assessing the physio-

logical relevance of constitutive 5-HT_7 receptor activity in native tissues.

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