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Efficacy of selective 5-HT6 receptor ligands determined by monitoring 5-HT6 receptor-mediated cAMP signaling pathways

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1 Two novel selective 5-HT6 receptor ligands E-6801 (6-chloro-*N*-(3-(2-(dimethylamino)ethyl)-1*H*-indol-5-yl)imidazo[2,1-*b*]thiazole-5-sulfonamide) and E-6837 (5-chloro-*N*-(3-(2-(dimethylamino)ethyl)-1*H*-indol-5-yl)naphthalene-2-sulfonamide) were investigated and compared to the putative 5-HT6 receptor antagonists SB-271046 (5-chloro-*N*-(4-methoxy-3-(piperazin-1-yl)phenyl)-3-methylbenzo[*b*]thiophene-2-sulfonamide) and Ro 04-06790 (*N*-(2,6-bis(methylamino)pyrimidin-4-yl)-4-aminobenzenesulfonamide) using a cAMP-mediated pathway.

2 Forskolin stimulation, to increase the magnitude of agonist cAMP responses, and site-directed mutagenesis of the 5-HT6 receptor, in order to yield constitutively active receptor, were applied.

3 5-HT (E_{max}, % over basal: 200), E-6801 (120) and E-6837 (23) induced cAMP formation at the rat 5-HT6 receptor. In the copresence of forskolin, cAMP responses were more potent and enhanced to 294 (5-HT, % over forskolin), 250 (E-6801) and 207 (E-6837), respectively. 5-HT-mediated cAMP formation was dose-dependently blocked by SB-271046 (pA_2: 8.76 ± 0.22) and Ro 04-6790 (pA_2: 7.89 ± 0.10) and not affected by the copresence of forskolin. Both E-6801 and E-6837 yielded partial antagonism of the 5-HT response in the absence of forskolin, whereas antagonism was either completely absent (E-6801) or attenuated (E-6837) in the copresence of forskolin. Intrinsic activity of these 5-HT6 receptor ligands at a constitutively active human S267K 5-HT6 receptor in Cos-7 cells indicated similar efficacy (E_{max}, % over basal) for 5-HT (97), E-6801 (91) and E-6837 (100), while Ro 04-6790 (-33) and SB-271046 (-39) were equi-efficacious inverse agonists.

4 The use of either forskolin or a constitutively active S267K 5-HT6 receptor enhances the resolution for monitoring the efficacy of 5-HT6 receptor ligands. E-6801 and E-6837 are potent partial agonists at the 5-HT6 receptor. Ro 04-6790 and SB-271046 appear to act as inverse agonists/antagonists.
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Abbreviations: E-6801, 6-chloro-*N*-(3-(2-(dimethylamino)ethyl)-1*H*-indol-5-yl)imidazo[2,1-*b*]thiazole-5-sulfonamide; E-6837, 5-chloro-*N*-(3-(2-(dimethylamino)ethyl)-1*H*-indol-5-yl)naphthalene-2-sulfonamide; EMDT, 2-(2-ethyl-5-methoxy-1*H*-indol-3-yl)-*N,N*-dimethylethanamine; LY-483518/SGS-518, 1-methyl-3-(1-methylpiperidin-4-yl)-1*H*-indol-5-yl 2,6-ifluorobenzenesulfonate; MS-245, 2-(5-methoxy-1-(phenylsulfonyl)-1*H*-indol-3-yl)-*N,N*-dimethylethanamine; Ro 04-06790, *N*-(2,6-bis(methylamino)pyrimidin-4-yl)-4-aminobenzenesulfonamide; Ro 63-0563, *N*-(2,6-bis(methylamino)pyridin-4-yl)-4-aminobenzenesulfonamide; Ro 65-7199, 4-amino-*N*-(6-bromo-1*H*-indol-4-yl)benzenesulfonamide; SB-271046, 5-chloro-*N*-(4-methoxy-3-(piperazin-1-yl)phenyl)-3-methylbenzo[*b*]thiophene-2-sulfonamide; SB-331711, 5-chloro-3-methyl-*N*-(4-(piperazin-1-yl)quinolin-6-yl)benzo[*b*]thiophene-2-sulfonamide; SB-357134, *N*-(2,5-dibromo-3-fluorophenyl)-4-methoxy-3-(piperazin-1-yl)benzenesulfonamide; SB-399885, *N*-(3,5-dichloro-2-methoxyphenyl)-4-methoxy-3-(piperazin-1-yl)benzenesulfonamide; SB-742457, 3-(phenylsulfonyl)-8-(piperazin-1-yl)quinoline; WAY-181187/SAX187, 2-(1-(6-chloroimidazo[2,1-*b*]thiazol-5-ylsulfonyl)-1*H*-indol-3-yl)ethanamine

Introduction

The 5-hydroxytryptamine6 (5-HT6) receptor is probably exclusively localized in the central nervous system (CNS), highest in limbic and cortical regions, and has been postulated to modulate CNS acetylcholine and glutamate function (Woolley *et al*., 2004). It may have a primary role in memory processes and learning, and 5-HT6 receptor ligands may be of benefit to improve cognitive function as suggested by a list of interesting studies (see Woolley *et al*., 2004). This receptor is also likely to play a role in obesity (Vickers & Dourish, 2004). Early studies demonstrated that chronic administration of 5-HT6 antisense oligonucleotides produced a significant reduction in food intake and body weight in rats (Bourson *et al*., 1995; Bentley *et al*, 1997). Furthermore, 5-HT6 receptor knockout mice are also resistant to weight gain when exposed to a high-fat diet (Caldirola, 2003). In addition, the 5-HT6 receptor has also been suggested to be involved in psychotic and affective disorders, anxiety and epilepsy (Woolley *et al*., 2004). The 5-HT6 receptor belongs to the G-protein-coupled receptor family and is coupled to the Gs-family of G proteins

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Dedicated to the memory of Dr Gonzalo Romero.

and has been demonstrated to increase cAMP formation in recombinant expression systems (Ruat *et al*., 1993; Kohen *et al*., 1996; Boess *et al*., 1997), cultured mouse striatal neurones (Sebben *et al*., 1994) and pig caudate membranes (Schoeffter & Waeber, 1994).

In order to elucidate functional roles of the 5-HT6 receptor, potent and selective ligands with defined properties are required. During the past few years, synthesis of novel ligands has been reported, introducing various new classes of compounds as potent and selective ligands for the 5-HT6 receptor subtype (Slassi *et al*., 2002; Davies *et al*., 2005; Holenz *et al*., 2006). Historically, the first 5-HT6-selective molecules were found by high-throughput-screening as, for example, the benzene-sulfonamide antagonists Ro 04-06790 (*N*-(2,6-bis (methylamino)pyrimidin-4-yl)-4-aminobenzenesulfonamide) and Ro 63-0563 (4-amino-*N*-(6-bromo-1*H*-indol-4-yl)benzene-sulfonamide) (Sleight *et al*., 1998) and the phenyl-piperazine antagonist SB-271046 (5-chloro-*N*-(4-methoxy-3-(piperazin-1-yl)-phenyl)-3-methylbenzo[*b*]thiophene-2-sulfonamide) (Bromidge *et al*., 1999). Later, by employing medicinal chemistry approaches starting from the endogenous ligand 5-HT, selectivity could be introduced by chemical modifications resulting in powerful agonists (i.e., EMDT (2-(2-ethyl-5-methoxy-1*H*-indol-3-yl)-*N,N*-dimethylethanamine) (Glennon *et al*., 2000) and WAY-181187/SAX-187 (Cole *et al*., 2005)) as well as antagonists (i.e. MS-245 (2-(5-methoxy-1-(phenylsulfonyl)-1*H*-indol-3-yl)-*N,N*-dimethylethanamine) (Russell *et al*., 2001), Ro 65-7199 (4-amino-*N*-(6-bromo-1*H*-indol-4-yl)benzenesulfonamide) (Bös *et al*., 2001), and LY-483518/SGS-518 (1-methyl-3-(1-methylpiperidin-4-yl)-1*H*-indol-5-yl 2,6-ifluorobenzene-sulfonate) (Piñeiro-Núñez *et al*., 2005)). In parallel, optimization of the phenyl-piperazine motif resulted in antagonists, such as SB-357134 (*N*-(2,5-dibromo-3-fluorophenyl)-4-methoxy-3-(piperazin-1-yl)benzenesulfonamide) (Bromidge *et al*., 2001a), SB-399885 (*N*-(3,5-dichloro-2-methoxyphenyl)-4-methoxy-3-(piperazin-1-yl)benzenesulfonamide) (Hirst *et al*., 2003b) and SB-742457 (3-(phenylsulfonyl)-8-(piperazin-1-yl)-quinoline) (Ahmed *et al*., 2003). Analyzing a variety of 5-HT6 reference compounds from a medicinal chemistry point of view, as described in a former publication (Holenz *et al*., 2005), we detected common structural motifs and converted this information into a hypothetical pharmacological framework model which allowed us to design and synthesis selective and high-affinity 3-aminoalkylindole sulfonamides, such as E-6801 (6-chloro-*N*-(3-(2-dimethylamino)ethyl)-1*H*-indol-5-yl)imidazo-[2,1-*b*]thiazole-5-sulfonamide) and E-6837 (5-chloro-*N*-(3-(2-(dimethylamino)ethyl)-1*H*-indol-5-yl)naphthalene-2-sulfonamide). In terms of functionality, these ligands have been found to comprise antagonists, as well as agonists and partial agonists.

Little is known about most 5-HT6 receptor antagonists with regard to their intrinsic efficacy properties. For instance, are these compounds silent antagonists or do they possess either negative or positive efficacy? The degree and kind of efficacy may ultimately differentiate these compounds at the 5-HT6 receptor, in particular, when it is constitutively active or under the tonic control of 5-HT (Woolley *et al*., 2004). Recombinant expression systems have the advantage that they allow differentiation of efficacies of closely related compounds (i.e., Pauwels & Colpaert, 1995). In the present paper, we report a comparative study of the recently reported (Holenz *et al*., 2005) 3-aminoalkylindolyl sulfonamide derivatives: E-6801 and E-6837

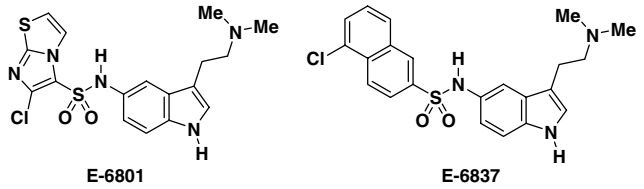

Figure 1 Chemical structures of E-6801 and E-6837.

(see Figure 1), and the presumed 5-HT6 receptor antagonists SB-271046 and Ro 04-6790 using a cAMP-mediated pathway as a functional read-out. Two experimental approaches were used to analyze the efficacy of these 5-HT6 compounds. First, the use of forskolin stimulation (Litosch *et al*., 1982) to increase the magnitude of agonist responses and second, site-directed mutagenesis of the 5-HT6 receptor in order to yield constitutively active receptor (Purohit *et al*., 2003). It appears that E-6801 and E-6837 are potent partial 5-HT6 receptor agonists, whereas SB-271047 and Ro 04-6790 possess negative efficacy and are as such inverse agonists/antagonists.

Methods

Construction of rat 5-HT6 receptor plasmid

PCR was performed with cDNA from Wistar rat striatum as a template using Expam High Fidelity polymerase (Roche). Flanking primers were: 5′-TGCAGTCACCATATCCCGTC TT-3′ and 5′-ACGGGGGAACTGAGTGGATGC-3′.

Sequential PCR conditions were: initial denaturation at 94°C for 2 min, 10 cycles at 94°C for 10 s, 65°C for 30 s and 72°C for $1\frac{1}{2}$ min. Then, 20 cycles at 94°C for 15 s, 55°C for 30 s and 72°C for $1\frac{1}{2}$ min. Finally, an elongation step was performed at 72°C for 7 min. PCR products were visualized on 0.8% agarose gel and visualized with ethidium bromide (10 μg ml^{-1}) staining. PCR products were purified using Microcon PCR columns (Millipore, Billerica, MA, U.S.A.) following the manufacturer's instructions. The purified fragments were ligated into pCR3.1 vector (Invitrogen, Frederick, MD, U.S.A.) using T4 DNA ligase and after overnight incubation at 15°C, ligated fragments were introduced in competent *Escherichia coli* TOP-F10 cells. Fifty μl of cells were mixed with 0.4 μg of the ligation product and set in ice for 30 min. Mixture was incubated for 30 s at 42°C (heat shock) and replaced at 37°C for 1 h with additional 250 μl sodium bactotryptone (SOC) medium. Afterwards, cells were seeded into Luria-Bertani (LB)-agar medium with ampicillin (100 μg ml^{-1}) and the culture was grown at 37°C overnight. Single colonies were selected and put into 3 ml LB with 100 μg ml^{-1} ampicillin and incubated at 37°C overnight. cDNA was obtained using a Qiaprep Spin Miniprep kit (Qiagen). Plasmids from selected colonies were sequenced and one corresponding to the original rat 5-HT6 receptor DNA sequence (Ruat *et al*., 1993) was taken for stable expression in HEK-293F cells.

Construction of human mutant S267 K 5-HT6 receptor plasmid

The S267K mutation was performed on native human 5-HT6 receptor cDNA (Kohen *et al*., 1996) and constructed

by site-directed mutagenesis (Purohit *et al*., 2003). Two flanking primers (sense 5′-GGAAGGCCCTGAAGGCCAAG CTTACGCTGGGCATCCTGC-3′ and antisense 5′-GCAGG ATGCCCAGCGTAAGCTTGGCCTTCAGGGCCTTCC-3′) were designed for mutagenesis of serine (AGC)-267 to lysine (AAG). PCR was performed using PFU Turbo DNA polymerase enzyme and the pCR3.1-mutant 5-HT6 fragment was transformed in DH5α cells. A *Hin*dIII restriction site was added by changing the wobble base of the adjacent leucine from CTG to CTT in order to verify its orientation and the sequence was confirmed by DNA sequencing.

Construction of HEK-293F cell line stably expressing rat 5-HT6 receptor

HEK-293F cells (Gibco) were grown in Dulbecco's modified Eagle's medium with GlutaMAX and pyruvate (DMEM: Gibco), and supplemented with 10% foetal bovine serum (Gibco), penicillin (50 U ml^{-1}) and streptomycin (50 U ml^{-1}) (Gibco). Cells were transfected with pcDNA3.1 containing 5-HT6 cDNA using FuGENE 6 Transfection Reagent (Roche) according to the manufacturer's protocol: 2 μl with 30 μg cDNA: 3 μl FuGENE per well of a six-well plate. At 24 h after transfection, cells were seeded by serial dilutions and plated in 384-well plates containing G418 (geneticin, Gibco) at 0.5 mg ml^{-1}. Isolated single colonies of cells of the geneticin-resistant phenotype were expanded and assayed for their 5-HT-mediated cAMP response using a FlashPlate technique. One clone, number #5, was selected for further work. Stably transfected cells were always grown in the presence of 0.5 mg ml^{-1} geneticin except during the cAMP experiment.

Transient transfection of human wild-type and mutant S267 K 5-HT6 receptor in Cos-7 cells

Cos-7 cells were cultured in DMEM supplemented with 40 mM glutamine and 10% of foetal bovine serum. At 24 h before transfection, cells were seeded at a subconfluent state in 150-mm Petri dishes for cAMP assay by homogeneous time resolved fluorescense (HTRF). Cells were transfected with pCR3.1-vector containing either a wild-type or mutant h5-HT6 receptor cDNA using 150 μl of lipofectamine and 37.5 μg of plasmid DNA per plate. cAMP experiments was performed at 48 h after transfection.

Radioligand-binding assay

Expression of the 5-HT6 receptor was measured by radioligand-binding assay (Hirst *et al*., 2003a) in a 96-well plate with a total reaction volume of 200 μl, containing 100 μl of membrane suspension (25 μg protein well^{-1}), 10 μl of [^{3}H]-LSD (2.5–10.0 nM) in either the absence or presence of 90 μl of either buffer or methiothepin (5 μM) for total and nonspecific binding, respectively. Binding buffer contained 50 mM Tris-HCl, 10 mM $MgCl_2$ and 0.5 mM EDTA at pH 7.4. Plates were incubated at 37°C for 60 min, filtered and plates were washed three times with ice-cold 50 mM Tris-HCL (pH 7.4). Filters were dried and counted at approximately 40% efficiency in a MicroBeta scintillation counter (Perkin-Elmer) using 25 μl per well of EcoScint liquid scintillation cocktail. To investigate binding properties of 5-HT6 ligands to h5-HT6 receptor, transfected HEK-293 membranes (35 μg protein assay^{-1}) from Perkin-Elmer (Boston, MA, U.S.A.) and [^{3}H]-LSD were used.

Measurement of cAMP responses by FlashPlate

Activation and inhibition of adenylyl cyclase activity was monitored by measuring levels of cAMP in 96-well plates by FlashPlate method (Perkin-Elmer). Briefly, HEK-293F cells expressing rat 5-HT6 receptor were grown to 80% confluency. At 2 h before the assay cells were kept on serum-free medium and subsequently dissociated with trypsin and centrifuged. The resulting pellet was resuspended (25,000 cells well^{-1}) in buffer provided with the Perkin-Elmer kit and containing 1 mM 3-isobutyl-1- methyl-xanthine (IBMX) and 20 μM pargyline. Test compounds were added either in the absence or presence of forskolin (either 1, 3 or 10 μM) and different concentrations of 5-HT, and after 30 min the reaction was stopped by addition of 100 μl of detection mix solution per well containing [^{125}I]-succinyl-cAMP tracer according to the supplier's instructions. When antagonists were used these were added 15 min before 5-HT. After 2 h incubation at room temperature, counting was performed using a MicroBeta scintillation counter (Perkin-Elmer).

Measurement of cAMP responses by Homogeneous Time Resolved Fluorescense

After overnight serum-free medium incubation, cAMP measurements on Cos-7 cells that transiently expressed either human wild-type or mutant 5-HT6 receptor were performed by HTRF (Gabriel *et al*., 2003). Cell suspension (20,000 cells per well) was added in 96-well culture plate in incubation buffer composed of Ham's F12 medium plus 1 mM IBMX and 20 μM pargyline. Forty μl of cell suspension and 10 μl of either compound in the absence or presence of forskolin or vehicle was added to each well at indicated concentrations for 30 min at 37°C. The reaction was stopped with 25 μl of cryptate and 25 μl of XL-665. Plates were incubated for 1 h at room temperature and read at 665/620 nm using a RubyStar Plate reader (BMG LabTech).

Materials

pCR3.1 plasmid and other reagents for molecular biology experiments were purchased from either Invitrogen (Frederick, MD, U.S.A.), Qiagen (Germantown, MD, U.S.A.) or Roche (Penzberg, Germany) as indicated above. Site-directed mutagenesis kit was obtained from Stratagene (La Jolla, CA, U.S.A.). Cell culture media and reagents were purchased from Gibco (Paislay, U.K.). Adenylyl cyclase activation FlashPlate kit was supplied by Perkin-Elmer Life Science (Brussels, Belgium). HTRF cAMP kit was purchased from CisBio (Bagnols, France). [^{3}H]-LSD was purchased from NEN (Boston, MA, U.S.A.). 5-hydroxytryptamine, dimethyl sulfoxide (DMSO), 3-isobutyl-1-methyl-xanthine (IBMX), forskolin and pargyline were obtained from Sigma (Poole, U.K.). Methiothepin was obtained from Tocris (Bristol, U.K.). SB-271046 and Ro 04-0670 were prepared *intramuros*. E-6801 and E-6837 are described in WO 2003/042175 A1 (Merce-Vidal *et al*., 2003). Stock compound solutions were prepared in DMSO and diluted with phosphate buffer solution (PBS) not exceeding 2% of DMSO at final concentration.

Data analysis

cAMP data are reported as mean ± s.e.m. of at least four independent experiments, each of which was performed in duplicate. cAMP data are presented in pmol 10^6 cells^{-1}. The response to modulate basal cAMP formation (E_{max}) by compounds was determined from the maximal stimulation or inhibition value that corresponded to a plateau value. The concentration of compound that produced a half-maximal response is represented by a pEC_{50} value and was calculated by nonlinear regression using XLfit (IDBS) and GraphPad Prism Version 3 programs. In experiments using antagonists, concentration ratios were calculated and used to obtain estimates of apparent pA_2 values using the following equation: apparent pA_2 = log (concentration ratio − 1) − log (antagonist concentration). The concentration dependence of each compound in the absence or presence of forskolin was analyzed by a one-way ANOVA statistical analysis. To analyze possible differences between either E_{max} or pEC_{50} values of compounds in the absence and presence of forskolin, Tukey's test within a two-way (forskolin and compound) ANOVA statistical analysis was performed using SAS program (SAS Institute Inc., Cary, NC, U.S.A.). A similar approach was used to estimate differences between either E_{max} or pEC_{50} values at the wild-type and mutant S267K 5-HT6 receptor.

Results

Intrinsic activity of 5-HT6 receptor ligands at rat 5-HT6 receptor

In contrast to nontransfected HEK-293F cells, 5-HT dose-dependently (pEC_{50}: 6.98 ± 0.08) induced cAMP formation in HEK-293F cells stably transfected with a rat 5-HT6 receptor in accordance with its pK_i value (6.96 ± 0.18). Coincubation with 1 μM forskolin enhanced not only basal cAMP formation, but also both the amplitude and potency of 5-HT (Figure 2a). Higher concentrations of forskolin did not affect the amplitude of 5-HT response (Figure 2a, Table 1). The ligand SB-271046 was free of intrinsic activity up to 10 μM and not affected by the copresence of forskolin (Figure 1b). Similar observations were made with Ro 04-6790 (Table 2). The 3-aminoalkylindolyl sulfonamide derivative E-6837 displayed a trend for intrinsic activity which was significantly enhanced in the copresence of forskolin (Figure 3) although its maximal effect was less than 5-HT (Table 2). A similar forskolin-effect was observed with the 3-aminoalkylindolyl sulfonamide derivative E-6801. It displayed potent partial agonism in the absence of forskolin and a pEC_{50} value similar to its pK_i value as was observed for 5-HT (Figure 3 and Table 2). Interestingly, E-6801 displayed an E_{max} value indistinguishable to 5-HT when measured in the copresence of forskolin.

Table 1 Effect of forskolin on basal, E_{max} and pEC_{50} values of 5-HT-induced cAMP formation in stably transfected HEK-293F/rat 5-HT6 cells

	cAMP formation		
	Basal (pmol 10^6 cells^{-1})	*5-HT* E_{max} *(pmol 10^6 cells^{-1})*	*5-HT* pEC_{50}
w/o Forskolin	4.6 ± 0.4	15.2 ± 0.4	6.98 ± 0.08
1 μM Forskolin	6.4 ± 0.4[a]	26.1 ± 0.5[b]	8.06 ± 0.08[c]
3 μM Forskolin	8.8 ± 0.7	26.8 ± 0.5	8.22 ± 0.09
10 μM Forskolin	12.7 ± 0.8	27.3 ± 0.6	8.60 ± 0.13[d,e]

E_{max} and pEC_{50} values were derived from cAMP-mediated 5-HT response curves as illustrated in Figure 1a. Data correspond to mean ± s.e.m. values of eight to ten independent experiments performed in duplicate. a: $P<0.05$ *versus* basal cAMP level w/o forskolin; b: $P<0.001$ *versus* E_{max} 5-HT w/o forskolin; c: $P<0.001$ *versus* pEC_{50} 5-HT w/o forskolin; d: $P<0.001$ *versus* pEC_{50} 5-HT 1 μM forskolin; e: $P<0.05$ *versus* E_{max} 5-HT 3 μM forskolin.

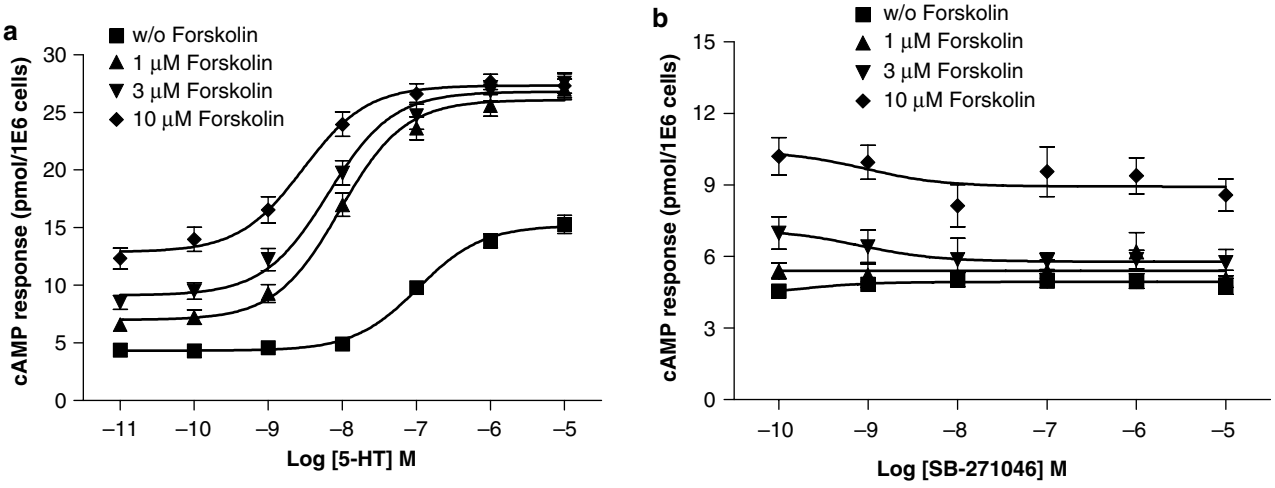

Figure 2 Effect of forskolin on 5-HT and SB-271046-mediated cAMP formation in stably transfected HEK-293F/rat 5-HT6 cells. HEK-293F cells were stably transfected with rat 5-HT6 receptor as described in Methods. cAMP formation was determined after a 2 h serum-free incubation in the presence of either 5-HT or SB-271046 in the absence or copresence of indicated concentrations of forskolin using FlashPlate technology. pEC_{50} and E_{max} values related to 5-HT are summarized in Table 1. Data points correspond to mean values ± s.e.m. from eight to ten independent experiments performed in duplicate. Specific [^{3}H]-LSD binding (2.5–10 nM) to membranes of this cell line indicated 0.40 ± 0.01 pmol mg protein^{-1}. a: 5-HT, b: SB-271046.

Table 2 E_{max} and pEC_{50} values of several 5-HT6 receptor ligands for inducing cAMP formation in stably transfected HEK-293F/rat 5-HT6 cells in either absence or copresence of forskolin, and corresponding pK_i values

	cAMP formation				
Forskolin (1 μm)	*w/o*		+		*5-HT6 receptor binding*
Compound	E_{max} *(pmol 10^6 cells^{-1})*	p*EC*$_{50}$	E_{max} *(pmol 10^6 cells^{-1})*	p*EC*$_{50}$	p*K*$_i$
5-HT	17.4±0.8	7.29±0.21	27.6±0.7[a]	8.23±0.12[b,c]	6.96±0.18
E-6801	12.7±0.5	8.02±0.23	24.5±0.6[a]	9.58±0.15[b,c]	8.46±0.13
E-6837	7.1±0.7	6.52±0.91[d]	21.5±0.7[a,e]	8.85±0.23[b]	9.13±0.17
Ro 04-6790	5.3±1.3	<5	7.3±0.6	<5	6.91±0.04
SB-271046	5.2±0.5	<5	5.9±0.5	<5	8.68±0.09
Basal	5.8±0.4	—	7.0±0.4	—	

E_{max} and pEC_{50} values were derived from cAMP-mediated agonist curves as illustrated in Figure 3. Data correspond to mean±s.e.m. values of five to six independent experiments performed in duplicate. pK_i values for h5-HT6 receptor were obtained as described in Methods. a: $P<0.001$ *versus* E_{max} w/o forskolin; b: $P<0.001$ *versus* pEC_{50} w/o forskolin; c: $P<0.01$ and d: $P<0.001$ *versus* pK_i value; e: $P<0.05$ *versus* E_{max} 5-HT with forskolin.

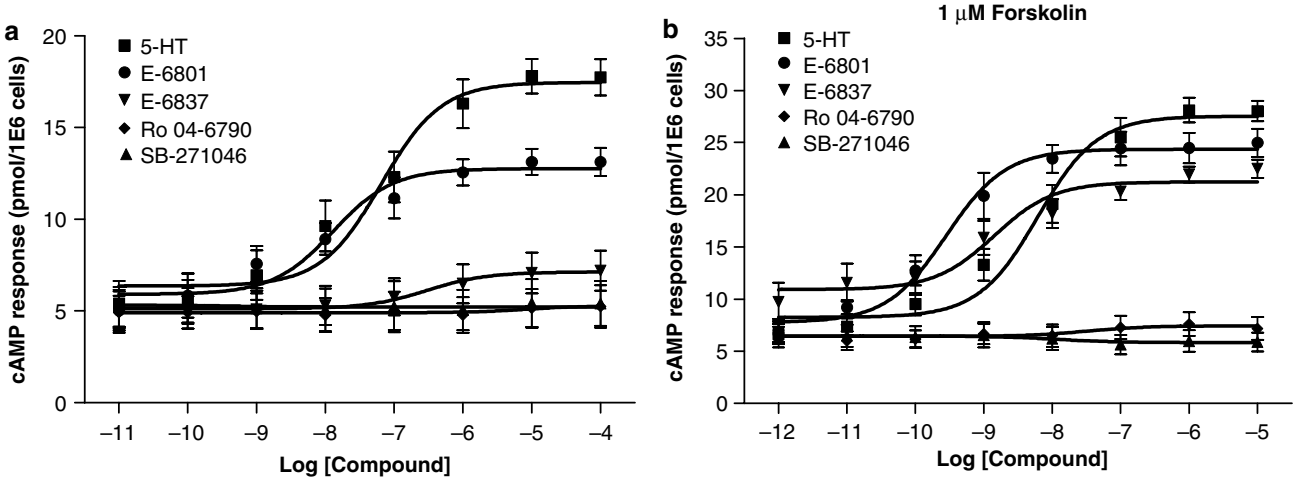

Figure 3 5-HT6 receptor ligand-mediated cAMP formation in stably transfected HEK-293F/rat 5-HT6 cells in either the absence or copresence of forskolin. HEK-293F cells were stably transfected with rat 5-HT6 receptor as described in Methods. cAMP formation was determined after a 2 h serum-free incubation in the presence of indicated ligands in either the absence (a) or presence (b) of 1 μM forskolin using FlashPlate technology. Mean dose–response curves±s.e.m. are shown from five to six independent experiments performed in duplicate. Mean E_{max} and pEC_{50} values±s.e.m. are summarized in Table 2.

Antagonist activity of 5-HT6 receptor ligands at rat 5-HT6 receptor

E-6801 (1 μM) displayed partial antagonism of the 5-HT response in the absence of forskolin as illustrated in Figure 4a. Otherwise, the combination of 5-HT and E-6801 in the copresence of 1 μM forskolin maintained maximal cAMP production (Figure 4b). E-6837 (1 μM) almost fully blocked the 5-HT response in the absence of forskolin (Figure 4c). Antagonism of 5-HT response by E-6837 (1 μM) was attenuated in the copresence of 1 μM forskolin (Figure 4d). SB-271046 and Ro 04-6790 antagonized in a dose-dependently manner the 5-HT response, both in the absence and presence of forskolin (Figures 5 and 6). The antagonist effects, as illustrated in Figure 5a and c, to a lesser extent 6a, appear not to be surmountable for the compounds SB-271046 and Ro 04-6790 at the highest agonist concentration. Otherwise, Ro 04-6790 behaved as a competitive antagonist in the copresence of forskolin (Figure 6c). pA_2 values derived from Schild plot analysis indicate SB-271046 is a more potent antagonist than Ro 04-6790, and pA_2 values are not significantly affected by the copresence of forskolin. A trend for both Ro 04-6790 and SB-271046 to attenuate cAMP values was apparent in the copresence of forskolin at low concentrations of 5-HT (Figures 5c and 6c) notwithstanding the lack of inhibition of basal receptor activity by SB-271046 in the copresence of 1 μM forskolin (Figure 2b).

Intrinsic activity of 5-HT6 receptor ligands at human wild-type and S267K 5-HT6 receptor

Basal cAMP formation was enhanced by 38±4% at the S267K mutant 5-HT6 receptor transiently expressed in Cos-7 cells as compared to nontransfected and wild-type human 5-HT6 receptor expressed in Cos-7 cells. Furthermore, the expression level of the mutant 5-HT6 receptor (0.52±0.01 pmol mg protein^{-1}) was lower than wild-type 5-HT6 receptor (1.08±0.01 pmol mg protein^{-1}). Such decreased expression is common for a constitutively active receptor, especially when mutated (S267 K) in or close to its $B^{261}BXXB^{265}$ motif in the junction of the third intracellular loop with the sixth transmembrane domain (Pauwels & Wurch, 1998). Interestingly, SB-271046 and Ro-046790 displayed negative intrinsic activity at the mutant S267K

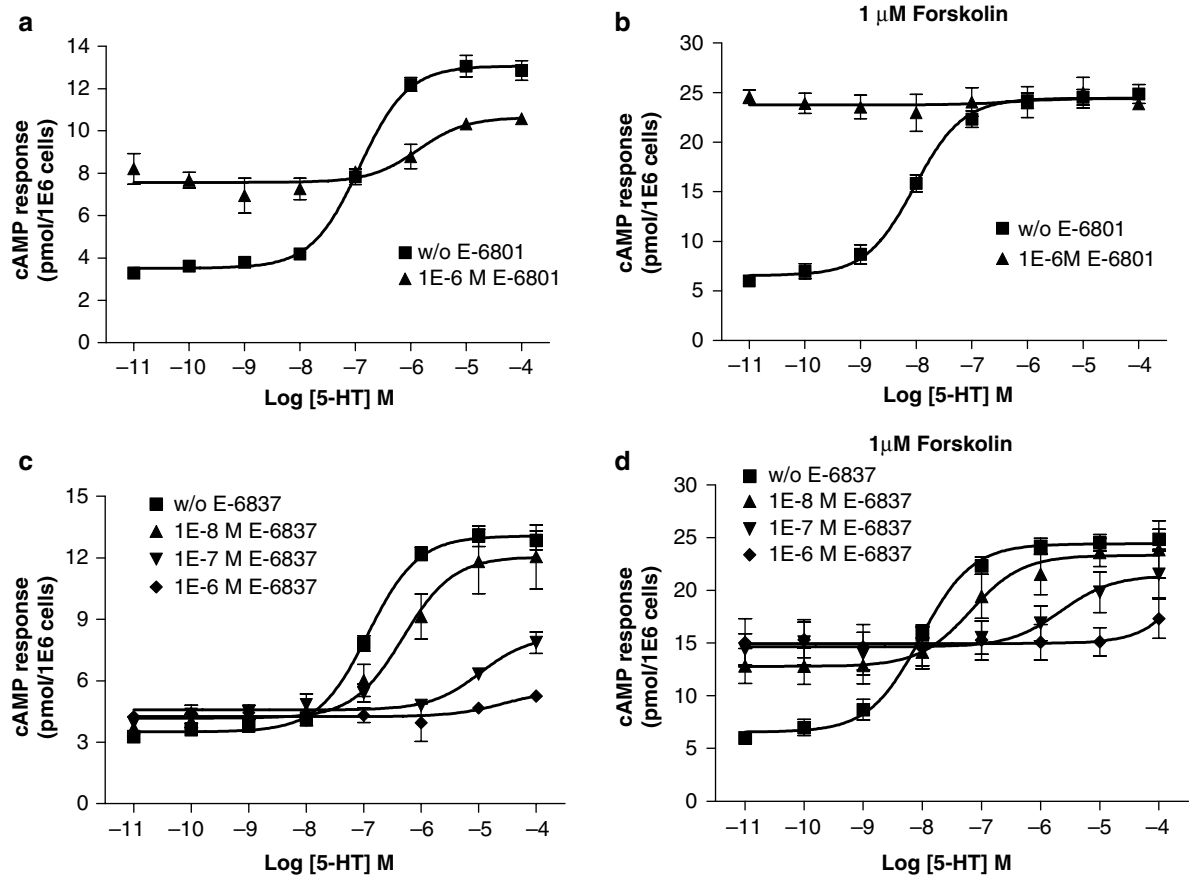

Figure 4 Antagonism of 5-HT-mediated cAMP formation by E-6801 and E-6837 in stably transfected HEK-293F/rat 5-HT6 cells in either the absence or copresence of forskolin. HEK-293F cells were stably transfected with rat 5-HT6 receptor as described in Methods. Antagonism of 5-HT-dose-dependent cAMP formation was determined after a 2 h serum-free incubation in the presence of 1 μM E-6801 or increasing concentrations of E-6837 in either the absence or copresence of 1 μM forskolin using FlashPlate technology. Mean 5-HT dose–response curves $\pm$ s.e.m. are shown from four to seven independent experiments performed in duplicate. a, c: without forskolin; b, d: with 1 μM forskolin; a, b: with E-6801; c, d: with E-6837.

5-HT6 receptor (Figure 7b) with potencies in agreement with their $p\mathrm{K_i}$ values (Tables 2 and 3). In contrast, SB-271046 and Ro 04-6790 appeared silent at the human wild-type 5-HT6 receptor (Figure 7a). E-6801 and E-6837 displayed both at wild-type and mutant 5-HT6 receptor positive efficacy with an E_{max} value indistinguishable from 5-HT (Figure 7, Table 3). Copresence of forskolin (1–10 μM) altered neither the 5-HT nor the SB-271046 potency at either wild-type or mutant 5-HT6 receptors in Cos-7 cells (Figure 8, Table 4).

Discussion

Recombinant expression systems are well known for their capacity to differentiate between closely related compounds with respect to their intrinsic efficacy properties (i.e., Pauwels & Colpaert, 1995). Although there is no doubt that these model systems are useful to differentiate between functional properties of compounds, some caution should be taken to extrapolate findings from such recombinant systems to *in vivo* integrated systems. Indeed, a compound with partial agonist properties in a recombinant expression system may demonstrate antagonist activity in *in vivo* integrated systems (Hoyer & Boddeke, 1993). Partial agonism will be observed probably in most systems for such compounds although full agonism can be seen in very efficiently coupled systems and silent, competitive antagonism in very poorly coupled receptor systems. Therefore, a molecular pharmacological characterization of new ligands can only be based on several observations as made in different expression systems and using different read-outs. In the present report we have analyzed the ability of several compounds to modulate the Gs-coupled-cAMP pathway linked to the 5-HT6 receptor. Two experimental conditions, copresence of forskolin and/or a constitutively active mutant 5-HT6 receptor, were applied to enhance the resolution to monitor either positive or negative efficacy. In the copresence of forskolin, agonist features such as E_{max} and $p\mathrm{EC_{50}}$ values of 5-HT, E-6801 and E-6837 were significantly enhanced at the rat 5-HT6 receptor. Modulation of adenylyl cyclase activation by forskolin as mediated by wild-type β2-adrenergic receptors in adipocytes (Litosch *et al*., 1982) and constitutively active Gs-coupled receptors, such as histamine H2 and 5-HT7 receptors in HEK-293 cells, has previously been reported (Alewijnse *et al*., 1997; Krobert & Levy, 2002). Moreover, the increased forskolin-mediated cAMP response was inhibited by either H2 receptor inverse agonists or 5-HT7 receptor inverse agonists. We did not observe agonist-independent rat 5-HT6 receptor activation in HEK-293F cells, neither in the absence nor in the copresence of forskolin. Constitutive activation of the human 5-HT6 receptor was,

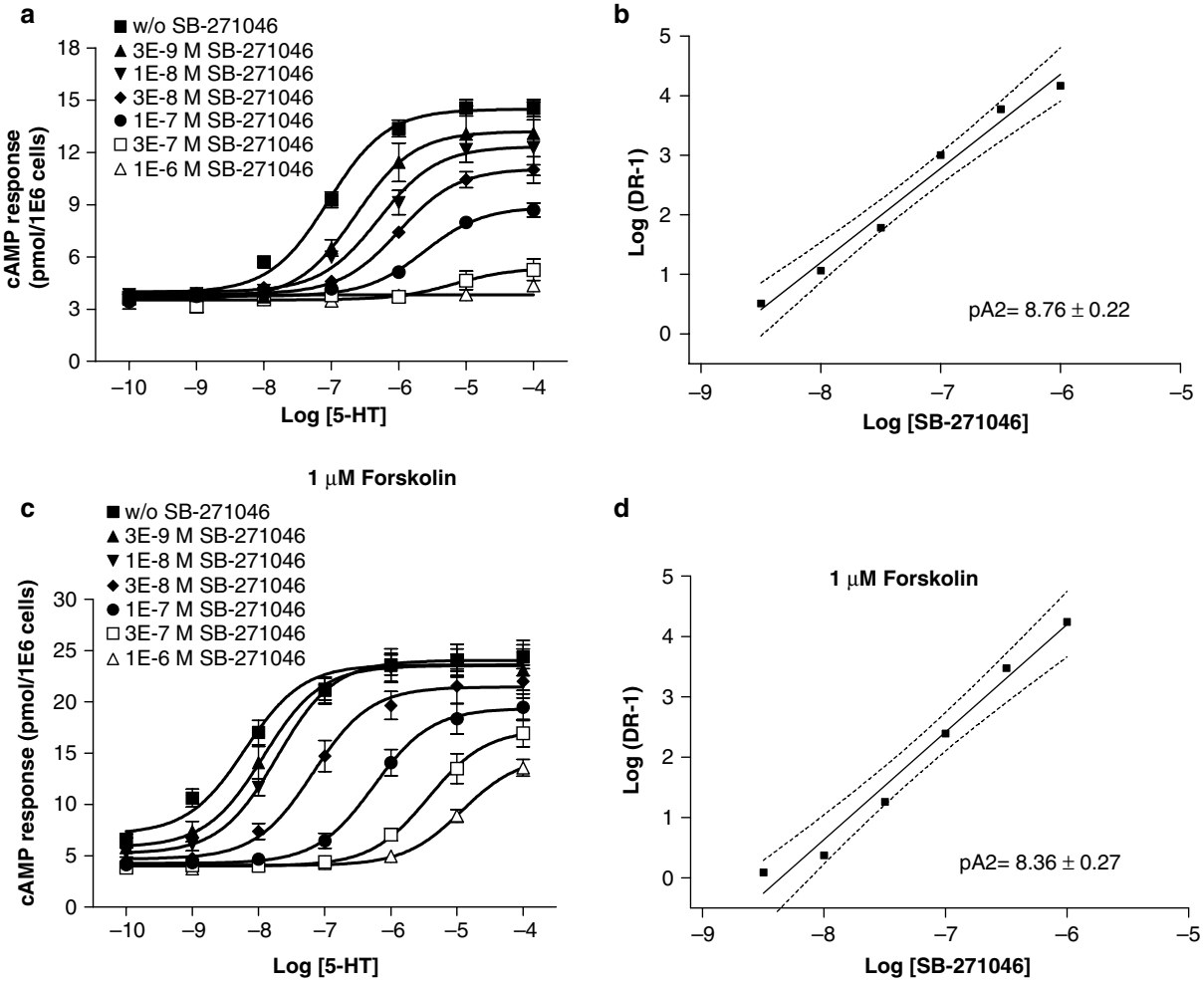

Figure 5 Antagonism of 5-HT-mediated cAMP formation by SB-271046 in stably transfected HEK-293F/rat 5-HT6 cells in either the absence or copresence of forskolin. HEK-293F cells were stably transfected with rat 5-HT6 receptor as described in Methods. Antagonism of 5-HT-dose-dependent cAMP formation was determined after a 2 h serum-free incubation in the presence of increasing concentrations of SB-271046 in either the absence (a, b) or copresence of 1 μM forskolin (c, d) using FlashPlate technology. Mean 5-HT dose–response curves ± s.e.m. are shown from five to ten independent experiments performed in duplicate. Schild plots (b, d) were constructed using mean values ± s.e.m. The gradient of the best-fit straight line was determined by linear regression. Slope (95% confidence intervals) was between 1.28–1.87 (b) and 1.42 to 2.14 (d).

however, observed by mutation of its Ser267 to a Lys in accordance with previous observations (Teitler *et al*., 2002). Copresence of forskolin did not modify the potency of agonist/inverse agonist-dependent cAMP formation in Cos-7 cells. Therefore, the effect of forskolin seems to be mainly determined by the host cell type and likely the receptor subtype. The S267K mutant receptor expressed in Cos-7 cells described here provides a sensitive system to monitor both inverse agonism and agonism in 5-HT6 receptor ligands.

Several major conclusions can be drawn with regard to the 5-HT6 receptor ligands investigated in this report. Firstly, SB-271046 behaved as a 5-HT6 antagonist at the human 5-HT6 receptor in accordance with the report of Routledge *et al*. (2000). This compound was reported as virtually free of intrinsic activity but it was measured under presumably silent 5-HT6 receptor conditions. The present study demonstrates SB-271046 displays negative efficacy at a constitutively active human S267K 5-HT6 receptor. Similar negative efficacy has been previously reported at this constitutively active mutant receptor for the atypical antipsychotic clozapine (Teitler *et al*., 2002; Purohit *et al*., 2003; 2005) and typical antipsychotic fluphenazine (Purohit *et al*., 2005). This strongly suggests SB-271046 is an inverse agonist and this property, although its physiological role is not well-defined (see Kenakin, 2004), may be of importance under both acute and chronic constitutively active 5-HT6 receptor conditions. In these instances in which negative efficacy is expressed, there may be conditions in which this is a useful property (i.e., reduce pathologically-induced constitutive activity) or an undesired property (tolerance to antagonism). Ro 04-6790 also behaved as a 5-HT6 receptor inverse agonist/antagonist. Sleight *et al*. (1998) reported this compound had no effect on basal cAMP accumulation in HeLa cells stably expressing human 5-HT6 receptor, suggesting Ro 04-6790 is neither an agonist nor an inverse agonist. The present study suggests that Ro 04-6790 is an inverse agonist/antagonist. Secondly, partial to full agonism was observed both with E-6801 and E-6837. Full and highly potent agonism was observed for E-6801 and E-6837 in the Cos-7 expression systems, both at human wild-type and mutant S267 K 5-HT6 receptors. Nonetheless, it is unlikely

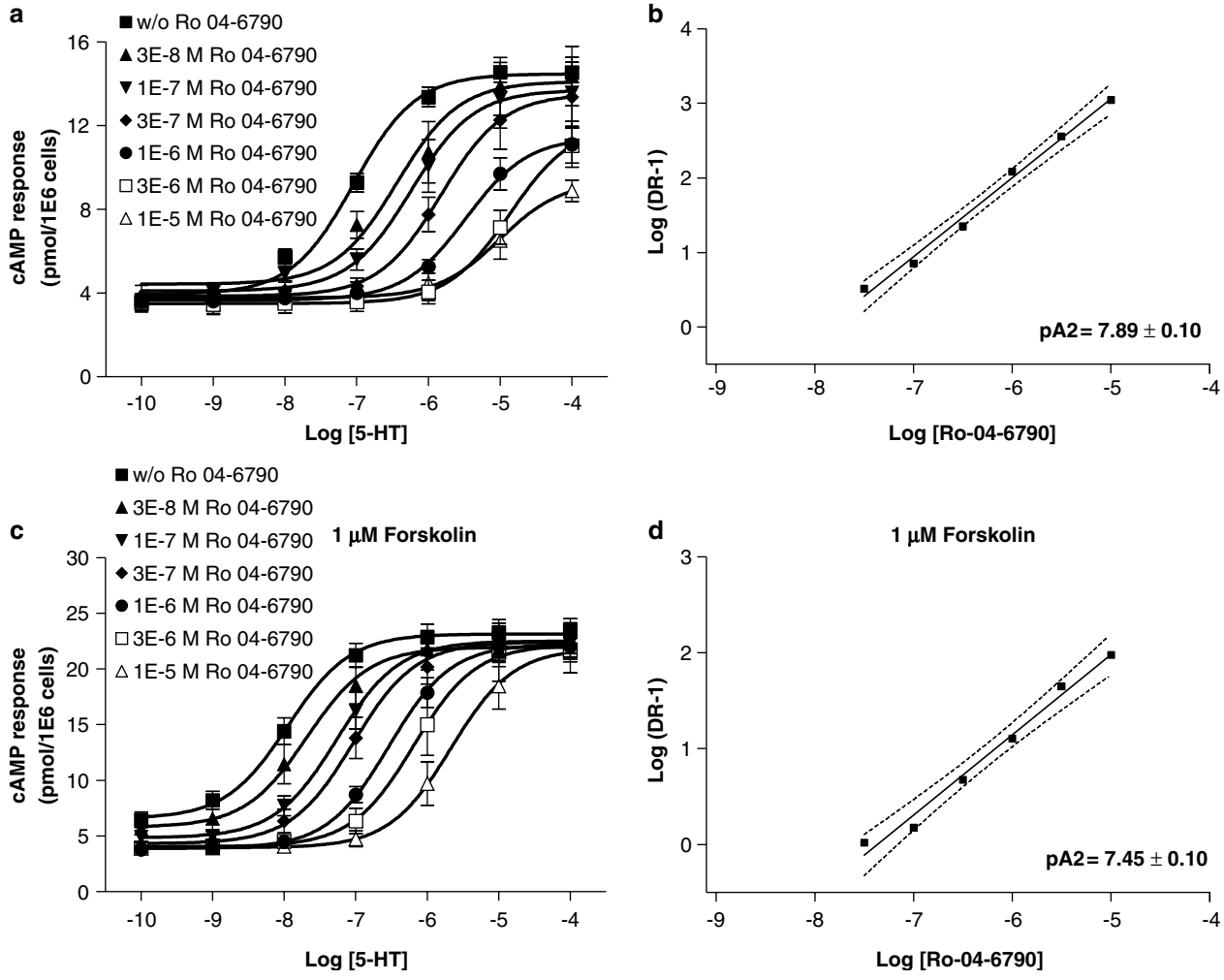

Figure 6 Antagonism of 5-HT-mediated cAMP formation by Ro 04-6790 in stably transfected HEK-293F/rat 5-HT6 cells in either the absence or copresence of forskolin. HEK-293F cells were stably transfected with rat 5-HT6 receptor as described in Methods. Antagonism of 5-HT-dose-dependent cAMP formation was determined after a 2 h serum-free incubation in the presence of increasing concentrations of Ro 04-6790 in either the absence (a, b) or copresence of 1 μM forskolin (c, d) using FlashPlate technology. Mean 5-HT dose-response curves ± s.e.m. are shown from five to ten independent experiments performed in duplicate. Schild plots (b, d) were constructed using mean values ± s.e.m. The gradient of the best-fit straight line was determined by linear regression. Slope (95% confidence intervals) was between 0.92–1.19 (b) and 0.49–0.99 (d).

E-6801 and E-6837 display similar efficacy as 5-HT when taking their pK_i/pEC_{50} values into account. Indeed, 5-HT illustrated a larger pK_i/pEC_{50} ratio (22 and 58 for wild-type and S267 K 5-HT6 receptor, respectively) as compared to 11 and 9 (E-6801) and 6 and 2 (E-6837), respectively. Agonism by E-6837 was also observed at the rat 5-HT6 receptor but principally in the copresence of forskolin. E-6801 was a potent partial agonist at rat 5-HT6 receptor in the absence of forskolin. Moreover, it could be converted in the copresence of forskolin into a highly efficacious agonist with similar efficacy to 5-HT. Therefore, we can postulate the agonist response by E-6801 at the rat 5-HT6 receptor resembles more closely that of 5-HT than E-6837. This also fits with corresponding pK_i/pEC_{50} ratios: 18, 13 and 0.5 for 5-HT, E-6801 and E-6837, respectively. Other examples of reported 5-HT6 receptor partial agonists are: SB-331711 (5-chloro-3-methyl-*N*-(4-(piperazin-1-yl)quinolin-6-yl)benzo[*b*]thiophene-2-sulfonamide) and related 4-piperazinyl quinolines (Bromidge *et al*., 2001b), and several similar substituted piperazine analogs (Bromidge *et al*., 2001b) producing some stimulation of basal adenylyl cyclase. It seems that each of the investigated 5-HT6 compounds actually possesses intrinsic activity. However, the 'observable' magnitude of this effect is variable and mainly dependent on the experimental model system. Non-observance of efficacy does not necessarily imply absence of efficacy. The experimental conditions must be appropriate for the effect to be monitored. The challenge is still to find a neutral, silent 5-HT6 receptor antagonist in order to learn more about the advantages/disadvantages under physiological and pathological CNS conditions of either a 5-HT6 neutral antagonist *versus* either a partial agonist or inverse agonist/antagonist. Interestingly, the predominance of inverse agonism agrees with theoretical predictions which indicate that neutral antagonists are the minority species in pharmacological space (Kenakin, 2004). The model systems described here will be useful to identify truly silent 5-HT6 receptor antagonists as they can exclude any ligand with efficacy, either positive or negative.

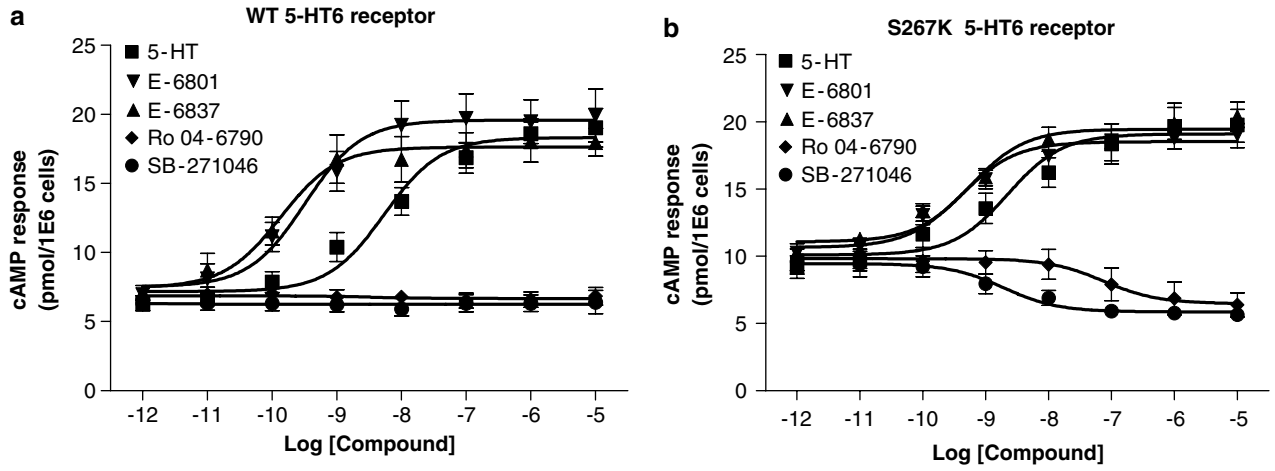

Figure 7 5-HT6 receptor ligand-mediated cAMP formation in Cos-7 cells transiently transfected with either human wild-type or S267K 5-HT6 receptor. Cos-7 cells were transiently transfected with either human wild-type 5-HT6 (a) or S267K 5-HT6 receptor (b) as described in Methods. cAMP formation was determined after a 24 h serum-free incubation in the presence of indicated ligands using HTRF. Mean dose–response curves ± s.e.m. are shown from five to ten independent experiments performed in duplicate. Mean E_{max} and pEC_{50} values ± s.e.m. are summarized in Table 3. Specific [^{3}H]LSD binding (2.5–10 nM) to membranes of the wild-type 5-HT6 and S267K 5-HT6 receptor cell lines indicated 1.08 ± 0.01 and 0.52 ± 0.01 pmol mg protein^{-1}, respectively.

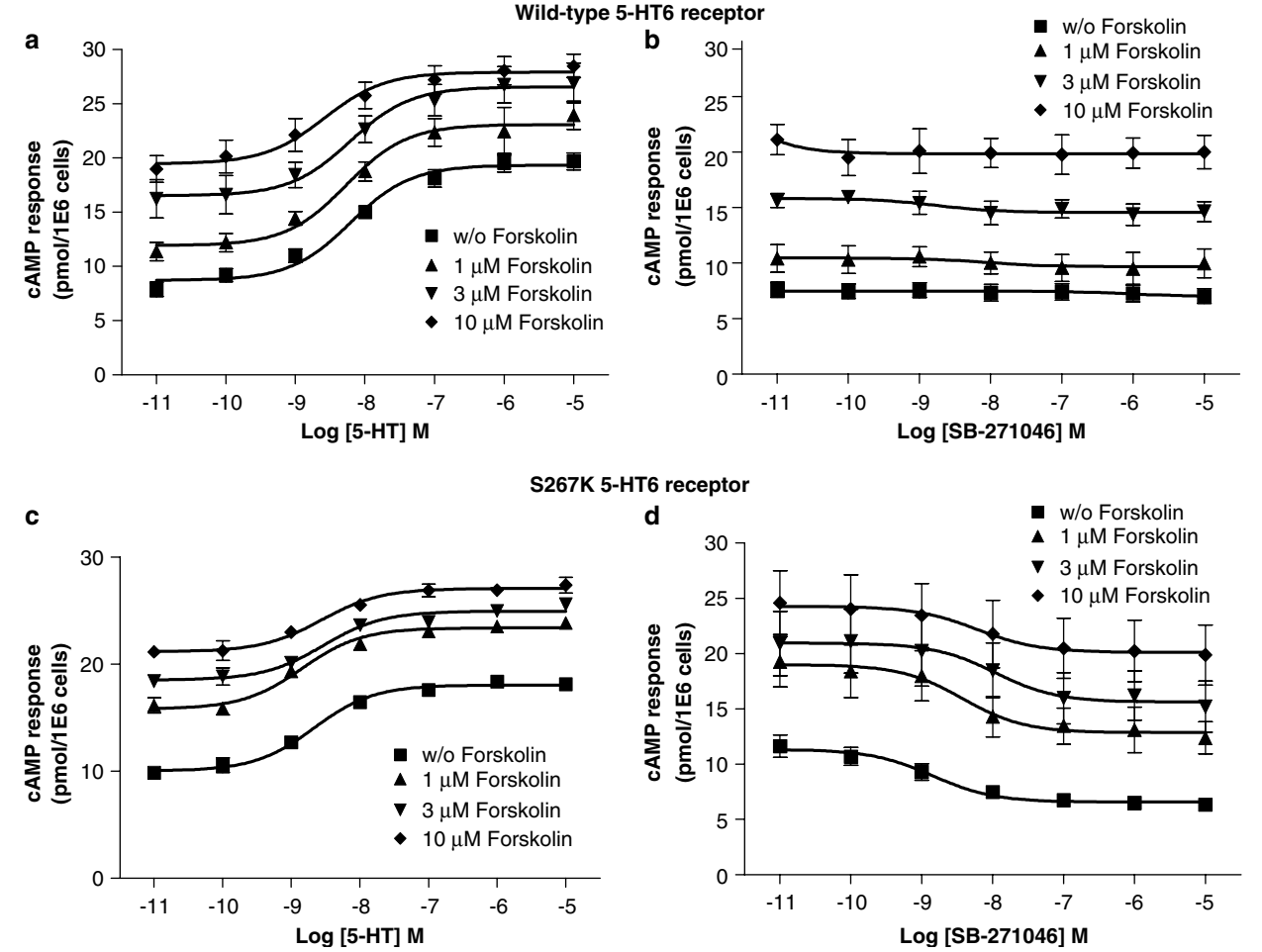

Figure 8 Effect of forskolin on 5-HT and SB-271046-mediated cAMP formation in Cos-7 cells transiently transfected with either human wild-type or S267K 5-HT6 receptor. Cos-7 cells were transiently transfected with either human wild-type 5-HT6 (a, b) or S267K 5-HT6 receptor (c, d) as described in Methods. cAMP formation was determined after a 24 h serum-free incubation in the presence of 5-HT (a, c) or SB-271046 (b, d) in either the absence or copresence of indicated concentrations of forskolin using HTRF. pEC_{50} and E_{max} values related to 5-HT are summarized in Table 4. pEC_{50} values of SB-271046 at S267K 5-HT6 receptor: 8.85 ± 0.26, 8.42 ± 0.64, 7.97 ± 0.88 and 8.22 ± 1.29 in, respectively, the absence and presence of 1, 3 and 10 μM forskolin. Data points correspond to mean values ± s.e.m. from four to eight independent experiments performed in duplicate.

Table 3 E_{max} and pEC_{50} values of several 5-HT6 receptor ligands for inducing cAMP formation in transiently transfected COS-7 cells with either human wild-type or S267K mutant 5-HT6 receptor

	cAMP formation			
5-HT6 receptor	*Wild-type*		*S267K*	
Compound	E_{max} *(pmol 10^6 cells^{-1})*	p*EC*$_{50}$	E_{max} *(pmol 10^6 cells^{-1})*	p*EC*$_{50}$
5-HT	18.3±0.6	8.31±0.16	19.1±0.3	8.73±0.25
E-6801	19.6±0.6	9.50±0.21	18.5±0.3	9.40±0.14
E-6837	17.6±0.5	9.89±0.21	19.4±0.4	9.31±0.18
SB-271046	6.3±0.2	<5	5.9±0.4[a]	8.69±0.36
Ro 04-6790	6.7±0.3	<5	6.5±0.7[b]	7.13±0.78
Basal	6.8±0.2	—	9.7±0.4[c]	—

E_{max} and pEC_{50} values were derived from cAMP-mediated agonist/inverse agonist curves as illustrated in Figure 7. Data correspond to mean±s.e.m. values of five to ten independent experiments performed in duplicate. a: $P<0.001$ *versus* basal value of mutant 5-HT6 receptor; b $P<0.01$ *versus* basal value of mutant 5-HT6 receptor; c: $P<0.001$ *versus* basal value of wild-type 5-HT6 receptor.

Table 4 Effect of forskolin on basal, E_{max} and pEC_{50} values of 5-HT-induced cAMP formation in transiently transfected COS-7 cells with either human wild-type or S267K mutant 5-HT6 receptor

	cAMP formation					
5-HT6 receptor	*Wild-type*			*S267K*		
	Basal (pmol 10^6 cells^{-1})	*5-HT* E_{max} *(pmol 10^6 cells^{-1})*	p*EC*$_{50}$	*Basal (pmol 10^6 cells^{-1})*	*5-HT* E_{max} *(pmol 10^6 cells^{-1})*	p*EC*$_{50}$
w/o Forskolin	7.8±0.3	19.5±0.8	8.21±0.13	10.4±0.5[a]	18.1±0.2	8.60±0.07
1 μM Forskolin	11.6±0.4[b]	23.1±1.5	8.25±0.17	18.8±0.7[c]	23.6±0.4[d]	8.86±0.04[e]
3 μM Forskolin	16.1±0.9	26.6±0.9[f]	8.21±0.10	21.5±2.2	24.9±0.1	8.62±0.04
10 μM Forskolin	20.9±0.9	27.9±0.7	8.57±0.11	24.4±2.0	27.1±0.6	8.58±0.17

E_{max} and pEC_{50} values were derived from cAMP-mediated 5-HT response curves as illustrated in Figure 8. Data correspond to mean±s.e.m. values of four to eight independent experiments performed in duplicate. a: $P<0.05$ *versus* basal value wild-type 5-HT6 receptor; b: $P<0.001$ *versus* basal w/o forskolin wild type 5-HT6 receptor; c: $P<0.01$ *versus* basal value w/o forskolin mutant 5-HT6 receptor; d: $P<0.001$ *versus* E_{max} 5-HT w/o forskolin mutant 5-HT6 receptor; e: $P<0.01$ *versus* pEC_{50} wild-type 5-HT6 receptor; f: $P<0.01$ *versus* E_{max} 5-HT w/o forskolin wild type 5-HT6 receptor.

In conclusion, the use of either forskolin or a constitutively active S267K 5-HT6 receptor enhances the resolution to analyze the efficacy of 5-HT6 compounds. SB-271046 and Ro 04-6790 exhibit an inverse agonist/antagonist profile, whereas the novel 5-HT6 receptor ligands E-6801 and E-6837 are potent partial agonists at the 5-HT6 receptor *in vitro*.

In honor of the memory of our dear friend and colleague Dr Gonzalo Romero (26th of September 1964, Sevilla), who had a great sense of humour and love of life, who died much too soon on the 9th of July 2006. We sincerely thank Professor P. Strange for critical reading of the manuscript. We acknowledge Dr J. Giraldo for support with the statistical analysis. We also thank A. Dordal for providing pK_i values and X. Monroy for sequence analysis of rat 5-HT6 receptor.

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