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Pharmacology of 5-HT6 Receptors-Part 1

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PREFACE

The field of 5-HT_6 receptors is quite recent story. The number of patents (Ruiz and Oranias, this book) and scientific publications is increasing every year. If one considers the very first indication of the probable presence of 5-HT_6 receptors in 1979 (MacDermont *et al.*, 1979), until 1998, only 33 scientific communications were published in this 19-year period, mainly focused on distribution, structure, and gross function of the receptor. Pharmaceutical companies found potentially interesting the interference with the function of this receptor and started to synthesize various 5-HT_6 receptor ligands (Liu and Robichaud, this book). Between 1999 and 2004, in 5 years, the number of publications raised to 79. After the first period, with the advent of several 5-HT_6 ligands, the number of publications increased even more and, between 2005 and mid-2010, reached 154.

With the rise of the number of researchers working in this field and then with the increase of different experimental settings, the information on 5-HT_6 receptors started to appear inconsistent. In fact, the definition of 5-HT_6 receptor agonist or antagonist may be test dependent (Codony *et al.*, this book), and this may explain why both agonists and antagonists may exert similar pharmacological effects (part two of this book). Also the first enthusiasm in going to clinical phase with 5-HT_6 ligands (part two of this book), as antiobese or anti-amnesic agents, was attenuated by the unsatisfactory clinical results. Also the first idea to use 5-HT_6 ligands against cognitive impairment associated with schizophrenia seems destined to be unsuccesful (part two of this book). However, so far, no genetic modification has clearly been associated with any pathology (Gennarelli and Cattaneo, this book).

In the field of 5-HT_6 receptors, neurochemical (part two of this book) and electrophysiological (Tassone *et al.*, this book) properties of the receptor are also far to be clearly understood. Therefore, it is difficult to ascertain 5-HT_6 receptor pharmacological effects, since many pieces of information are unsatisfactory, such as pharmacokinetics/metabolism of the 5-HT_6 ligands (Mancinelli, this book), or how radioligands bind to the receptor (Riccioni, this book) (Figure 1).

This book was thought to summarize all the data so far obtained in order to put forward some hypothesis for the inconsistencies in the 5-HT_6 field. One is that 5-HT_6 receptors might exist in different conformational states, according to the G protein with which the 5-HT_6 receptor interacts. Since the coupling with the various G proteins may depend on cellular environment, which is differently

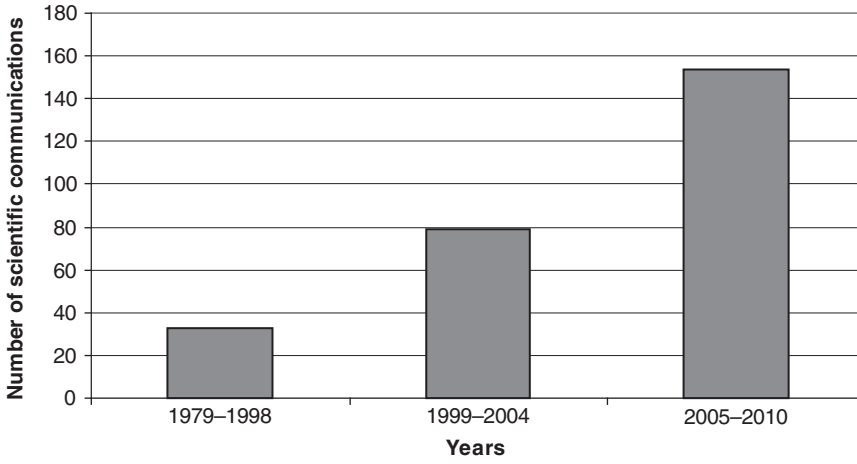

FIG. 1. Number of scientific publications in the field of 5-HT_6 receptors since the first possible publication.

sensitive to various stressors, the final output (agonist versus antagonist) might depend on cellular state itself. If one assumes that the various 5-HT_6 receptor ligands might bind to different receptor sites, it might become clearer why similar pharmacological properties are shared by alleged agonists and antagonists. If this hypothesis is confirmed, 5-HT_6 receptor might be a type of receptor that is cell-state sensitive. However, there is no clear explanation for the inconsistencies in the 5-HT_6 field.

This book wants to evidence the state of the art in the field of 5-HT_6 receptor physiology and pharmacology. We hope that this book may represent a reference information for all researchers who work in the field of 5-HT_6 receptors. The book on "Pharmacology of 5-HT_6 receptors" is published in two parts. The first part deals with in-vitro results and pharmakinetics of 5-HT_6 receptor ligands, and the second part with in-vivo findings.

Reference

MacDermont, J., Higashida, H., Wilson, S.P., Matsuzawa, H., Minna, J., and Nirenberg, M. (1979). Adenylate cyclase and acetylcholine release regulated by separate serotonin receptors of somatic cell hybrids. *Proc. Natl. Acad. Sci. U. S. A.* **76**, 1135–1139.

FRANCO BORSINI

5-HT_6 MEDICINAL CHEMISTRY

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I. Introduction

First reported as a vasoconstrictor more than 60 years ago, serotonin (**1**, 5-hydroxytryptamine, 5-HT) has been appreciated since that time as an important neurotransmitter in the brain (Rapport *et al.*, 1948). The receptors through which serotonin acts represent a diverse family of receptors, which had initially been classified pharmacologically into multiple subtypes. The advent of molecular cloning provided additional discrimination within the serotonin receptor family ultimately revealing the existence of seven subfamilies containing a total of 14 distinct receptors based on primary sequence, pharmacology, and signal transduction pathways (Hoyer and Martin, 1996; Hoyer *et al.*, 1994). The various subtypes are located both centrally and peripherally, influence a number of physiological functions, and are implicated in many disease states (Murphy *et al.*, 1998). With the exception of the 5-HT_3 receptor, which is a ligand-gated ion channel, these receptors belong to the superfamily of G-protein-coupled receptors (GPCRs).

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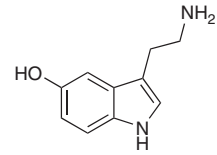


1 5-HT, 5-HT$_6$ K_i = 75 nM

The 5-hydroxytryptamine-6 (5-HT$_6$) receptor is the most recent addition to this receptor family and was identified by molecular biology in early 1990s (Kohen *et al.*, 1996, 2001; Monsma *et al.*, 1993; Ruat *et al.*, 1993). This GPCR is positively coupled to adenylate cyclase and is localized primarily in the central nervous system (Sleight *et al.*, 1998a). Extensive investigation has shown that these receptors are expressed in brain regions known to be associated with learning and memory (Hamon *et al.*, 1999). In addition, studies have shown that blockade of 5-HT$_6$ receptor function increases neurotransmission, specifically cholinergic and glutamatergic (Bourson *et al.*, 1995; Sleight *et al.*, 1996), and this leads to improvement in cognition in a number of rodent behavioral models (King *et al.*, 2004; Rogers and Hagan, 2001).

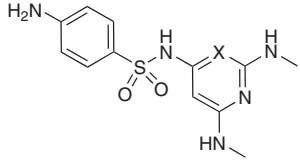
In addition to its relatively low level of sequence homology (<50%) as compared to other 5-HT receptors, the 5-HT$_6$ receptor exhibits a unique pharmacological profile. Known 5-HT ligands including 5-HT **1**, 5-methoxytryptamine and LSD bind to the 5-HT$_6$ subtype receptor with high affinity, as do a number of typical and atypical antipsychotic agents including clozapine, loxapine, and ritanserin, and tricyclic antidepressants such as mianserin and amitriptyline (Boess *et al.*, 1998; Monsma *et al.*, 1993; Roth *et al.*, 1994). The unique pharmacology and nearly exclusive central nervous system (CNS) expression spurred significant interest in the functional role of the 5-HT$_6$ receptor. Before selective 5-HT$_6$ tool molecules were available, investigation of the functional role of the receptor was attempted using antisense oligonucleotides (AO) targeting 5-HT receptor mRNA to block translation to the receptor protein (Bourson *et al.*, 1995; Sleight *et al.*, 1996).

Since the first selective 5-HT$_6$ ligand was reported over a decade ago, a plethora of potent and selective ligands for the 5-HT$_6$ receptor have been identified by an army of researchers in both academic and industrial laboratories (Glennon *et al.*, 2010; Johnson *et al.*, 2008; Liu and Robichaud, 2009). This array of structurally diverse compounds has served as excellent tools to investigate the functional role of the 5-HT$_6$ receptor in greater detail. In addition, a number of compounds have been advanced into development, undergoing Phase I and II clinical trials for cognitive impairment in Alzheimer's disease (AD) and schizophrenia (Maher-Edwards *et al.*, 2010; Robichaud, 2010). It is this work, specifically, that is the subject of this chapter.

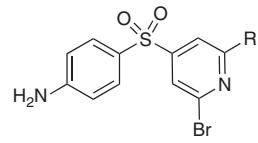
II. 5-HT$_6$ Antagonists

A. Bisaryl Sulfonamides and Sulfones

The first selective 5-HT$_6$ ligands, **2** (Ro 04-6790) and **3** (Ro 63-0563), were reported by Sleight and coworkers over a decade ago (Sleight *et al.*, 1998b). These biaryl sulfonamides bearing basic amino groups were identified by optimization of leads found by screening the Roche compound library (Bos *et al.*, 2001). Both compounds displayed high affinity with $K_i = 55$ and 12 nM, respectively, and showed full antagonist efficacy in an adenylyl cyclase assay ($IC_{50} = 178$ and 94 nM, respectively). In addition, they had over 100-fold selectivity against 23 other receptors including various dopamine and 5-HT receptor subtypes. Although both compounds had very poor blood–brain barrier (BBB) penetration (brain/plasma < 0.01), the concentration of **2** in cerebrospinal fluid (CSF) following intraperitoneal (i.p.) administration of 30 mg/kg in rats was sufficient to occupy more than 70% of the 5-HT$_6$ receptors. Similar to earlier antisense oligonucleotide studies to probe 5-HT$_6$ receptor function in the absence of selective 5-HT$_6$ ligands, **2** (i.p. dose) produced a dose-dependent increase in the number of stretches and yawning in rats, which could be dose dependently blocked by the brain-penetrant muscarinic antagonists atropine and scopolamine (Bentley *et al.*, 1999).



2 X = N, Ro 04–6790, $K_i = 55$ nM
3 X=CH, Ro 63–0563, $K_i = 12$ nM



4 R = 1-pyrrolidinyl, $K_i = 1.0$ nM
5 R = 1-piperazinyl, $K_i = 0.11$ nM

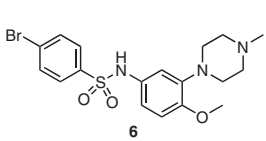
A structure–activity relationship (SAR) was subsequently carried out in order to improve the brain penetration and potency of this chemical series (Bos *et al.*, 2001). It was shown that small groups such as CH_3NH, EtNH, azetidine, and pyrrolidine at the 2- and 6-positions of the pyrimidine ring were tolerated and provided compounds with similar affinity to the lead compound **2**. It was also concluded that compounds with log D values between 2 and 3.5 were most favorably suited for brain penetration based on the minimal effective dose in a behavior study. To further optimize this class of compounds and to extend the structural scope of these 5-HT$_6$ ligands, a limited number of sulfones were synthesized (Bos *et al.*, 2001; Riemer *et al.*, 2003). The most potent compounds identified in the pyridyl sulfone series were compounds with a cyclic amine replacing the aniline NMe (**4** and **5**).

Piperazinyl derivative **5**, a picomolar 5-HT_6 antagonist, also displayed significant affinity for the 5-HT_{2c} receptor subtype and was quickly deemphasized. Further profiling of pyrrolidine **4**, lacking this cross-reactivity, revealed it to be a full 5-HT_6 antagonist with $IC_{50} = 3.2$ nM in the cyclase assay and selectivity against more than 50 protein targets screened. Compound **4** had an adequate pharmacokinetic profile with 50% bioavailability and low to intermediate plasma clearance (20 mL/min/kg) following an oral dose of 10 mg/kg in rats and was significantly more brain penetrant (brain/plasma ratio = 0.24) than the sulfonamide analogs such as Ro 04-6790 (**2**) and Ro 63-0563 (**3**). Oral administration of **4** at 30 mg/kg produced a clear twofold increase of the extracellular level of acetylcholine (ACh) in the rat frontal cortex, as measured by microdialysis, providing evidence for the role of the 5-HT_6 receptor in the cholinergic system. Compound **4** was also shown to reverse a scopolamine-induced passive avoidance retention deficit in a behavior assay at doses of 10–100 mg/kg p.o. acutely.

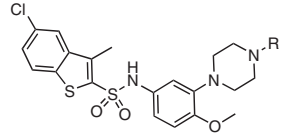
B. Piperazinylbenzenesulfonamides

Shortly after the disclosure of 5-HT_6 antagonists **2** and **3**, Bromidge and coworkers reported a series of sulfonamides as potent and selective ligands (Bromidge *et al.*, 1999, 2001a). The potent initial lead compound **6** (SB-214111), bearing a basic piperazine, was also reportedly identified from the high-throughput screening campaign. Pharmacokinetic studies in rats (i.v. infusion) demonstrated that **6** was moderately brain penetrant (brain/plasma = 0.25), but was subject to rapid clearance (~60 mL/min/kg) resulting in low oral bioavailability ($F = 12\%$). The SAR around the lead compound **6** was rapidly investigated through parallel synthesis and it was shown that lipophilic sulfonyl moieties, in particular halogen-substituted aromatics, were beneficial to 5-HT_6 affinity, whereas polar groups were detrimental. The 5-chloro-3-methylbenzothiophenesulfonyl group in particular was found to be optimal which provided the advanced analog **7** (SB-258510) with sub-nanomolar 5-HT_6 affinity in the binding assay and an $IC_{50} = 3.2$ nM in the functional assay with greater than 300-fold selectivity against a range of other receptors. Although **7** was slightly less brain penetrant (brain/plasma = 0.18) than the lead compound **6**, it benefited from a much improved plasma clearance property (12.5 mL/min/kg). However, in the Pharmacokinetic (PK) studies of **7**, significant levels of the desmethyl derivative were found in the plasma, indicating the metabolic instability of the parent **7**. The putative metabolite **8** was then prepared and shown to have excellent potency and selectivity and was a full antagonist with $IC_{50} = 2.0$ nM in the functional assay. Furthermore, **8** demonstrated no significant cross-reactivity (>200-fold selectivity) against more than 69 receptors, enzymes, and ion channels (Routledge *et al.*, 2000; Stean *et al.*, 2002).

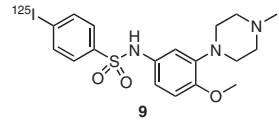
Although **8** was even more poorly brain penetrant (brain/plasma = 0.10), it had a low blood clearance (7.7 mL/min/kg), a moderate half-life ($t_{1/2}$ = 4.8 h), and excellent oral bioavailability (F > 80%) in rats, thus making it suitable for advanced development. As one of the early potent and selective 5-HT$_6$ antagonists, SB-271046 has become one of the most widely used tool molecules to probe 5-HT$_6$ receptor function. Shortly thereafter, radiolabeled **9** ([^{125}I]-SB-258585), an antagonist and a structurally related analog of **8**, was reported (Hirst *et al.*, 2000). Due to its high affinity (K_i = 1.8 nM), selectivity (>100-fold over other 5-HT receptor subtypes), and high level of specific binding (68% to native human receptor), this radioligand together with [^{3}H]-Ro 63-0563 (Boess *et al.*, 1998) provided significantly better tools than the initial non-selective radioligands such as [^{3}H]-LSD, [^{125}I]-LSD, [^{3}H]-5-HT, and [^{3}H]-clozapine for studies of the 5-HT$_6$ receptor.



6
SB-214111
K_i = 5.0 nM

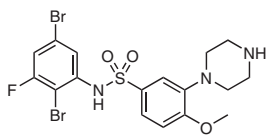


7 R = Me, SB-258510, K_i = 0.63 nM
8 R = H, SB-271046, K_i = 1.3 nM

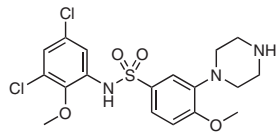


9
SB-258585
K_i = 1.8 nM

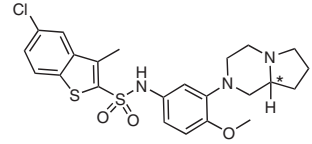
Continued research on these active ligand classes led to further advances. Conformational constraint of the piperazinylbenzenesulfonamide by tethering the sulfonamide nitrogen (Witty *et al.*, 2009) or the methoxy group (Bromidge, 2001) to the phenyl ring resulted in indolines and dihydrobenzofurans with similar potency but with further improved selectivity. Subsequent SAR studies around the sulfonamide linkage demonstrated that reverse sulfonamides were equally potent and a wide range of substituted phenyl groups on the left-hand side of the molecule were then explored which led to identification of **10** and **11** as potent, selective, brain-penetrant, and orally active 5-HT$_6$ antagonists (Bromidge *et al.*, 2001a). In a further effort to block the potential *N*-dealkylation metabolism of analogs such as **7**, constrained bicyclic piperazines such as **12** and **13** were synthesized (Bromidge *et al.*, 2002). The two enantiomers had identical 5-HT$_6$ affinity indicating that there is no preference of 5-HT$_6$ receptor for either of the chiral configurations of the piperazine moiety, in agreement with previous studies (Bromidge *et al.*, 2001b).



10
SB-257134
K_i = 2.6 nM



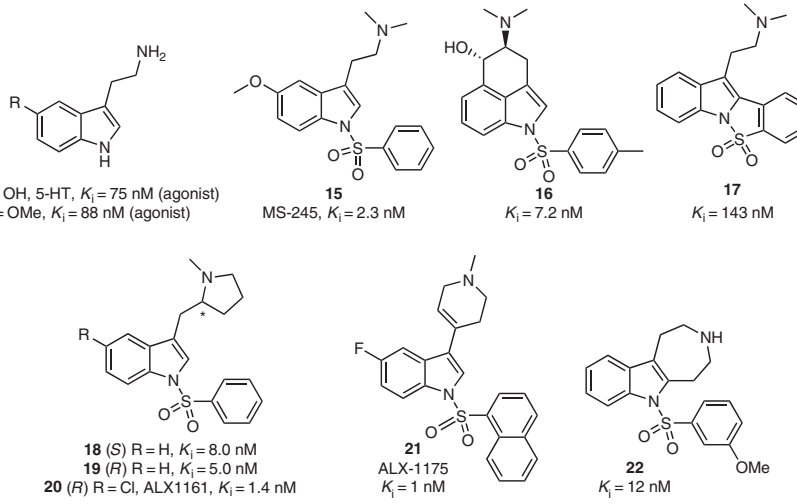
11
SB-399885
K_i = 0.7 nM



12 (*S*) K_i = 0.8 nM
13 (*R*) K_i = 0.8 nM

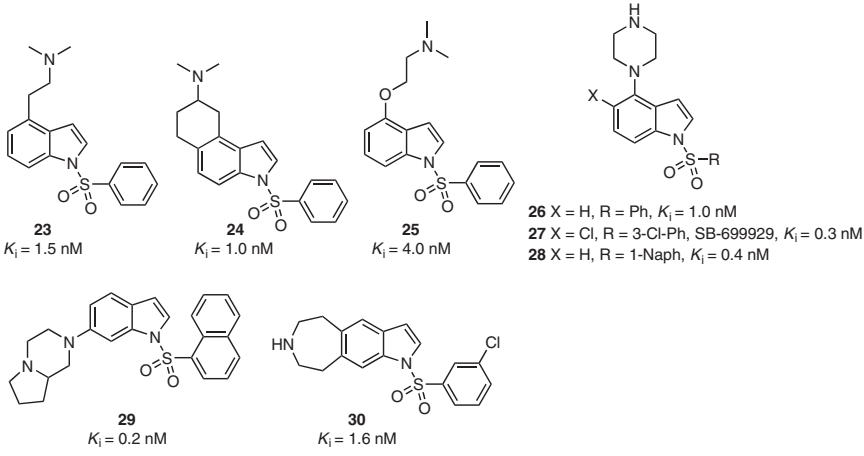
C. Indoles

In an attempt to identify potent and selective 5-HT_6 ligands, Glennon took a different but obvious approach starting with the natural ligand 5-HT (Abate *et al.*, 2005; Chang-Fong *et al.*, 2004; Glennon, 2003; Glennon *et al.*, 1999, 2000; Kolanos *et al.*, 2006; Lee *et al.*, 2000, 2005; Pullagurla M. *et al.*, 2004, 2005; Pullagurla M. R. *et al.*, 2003; Pullagurla M. R. *et al.*, 2004; Sikazwe *et al.*, 2006; Siripurapu *et al.*, 2006; Tsai *et al.*, 2000). Initial efforts were aimed at identification of agonists based on the rationale that 5-HT and the related natural ligand 5-methoxytryptamine (**14**) act as agonists at the 5-HT_6 receptor subtype. While substitution at the 2-position of the indole core or dimethylation on the primary amine resulted in agonists with similar potency to 5-HT, *N*1-arylsulfonylation provided full 5-HT_6 antagonists with significantly improved potency (**15**, $K_i = 2.3$ nM). Russell and coworkers independently reported **15** and its analogs as potent and selective 5-HT_6 antagonists (Russell *et al.*, 2001). While conformational constraint of the basic amine on the phenyl ring is tolerated (**16**) (Russell *et al.*, 2001), constraining the molecule by tethering the sulfonyl group to the indole core (**17**) resulted in a dramatic reduction in affinity (Kolanos *et al.*, 2006).



Pyrrolidine derivatives such as **18–20** have been independently reported by Cole, Abate, and Slassi as potent 5-HT_6 ligands (Abate *et al.*, 2005; Cole *et al.*, 2005c; Slassi *et al.*, 1999, 2002). Among the various analogs explored in these SAR studies, **20** (ALX-1161) exhibited high affinity in both 5-HT_6 binding ($K_i = 1.4$ nM) and functional ($IC_{50} = 8.5$ nM) assays and greater than 100-fold

selectivity over a panel of 40 other receptors and binding sites. This compound was shown to possess excellent brain exposure (brain/plasma = 23.4, i.p.) and a moderate oral bioavailability in rats (F = 17%) (Demchyshyn, 2001; Slassi, 2002). Again, there was no preference of 5-HT$_6$ receptor to the stereochemistry at the basic amine (**18** and **19**). Subsequently, additional classes of pyrrolidine and piperidine derivatives were reported by Cole *et al.* (2005a, 2005c) and others (Demchyshyn, 2001; Jasti *et al.*, 2005; Slassi *et al.*, 2000, 2002; Zhou *et al.*, 2002b). Among those reported, **21** (ALX-1175) was a potent (K_i = 1 nM and IC_{50} = 24 nM), selective (>100-fold), orally bioavailable (F = 19%, rats), and brain-penetrant (brain/plasma = 43) 5-HT$_6$ antagonist. In addition, Liu and Robichaud reported a novel class of azepinoindoles (**22**) as potent 5-HT$_6$ antagonists (Liu *et al.*, 2008). This class of compounds was designed by rigidifying the 1-sulfonylindole derivatives through tethering the amino side chain to the 2-position of the core with the hope to improve the selectivity against other 5-HT subtypes, a reoccurring issue for the 1-sulfonyltryptamine compounds.

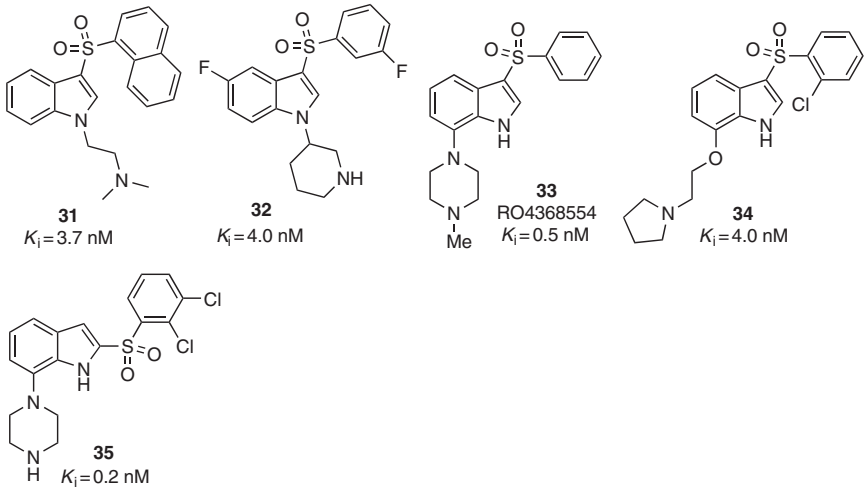


Related work in this chemical space was reported by several independent groups. Russell and coworkers demonstrated that migration of the amine side chain from the 3-position of 1-arylsulfonyl indole derivatives to the 4-position afforded compounds (**23**) that retained excellent 5-HT$_6$ affinity (Russell *et al.*, 2001). The conformationally constrained analogs (**24**) were shown to be equally potent compounds as reported by Cole (Cole and Asselin, 2005). Zhou reported a class of 1-sulfonylindole derivatives with an aminoalkoxy side chain at the 4-position (**25**) (Li and Zhou, 2002; Zhou *et al.*, 2002a, 2005). Indazole derivatives (*vide infra*) had similar affinity as their indole counterparts, showing the diverse tolerance to the core heterocycle. Cole showed that 4-piperazinyl-

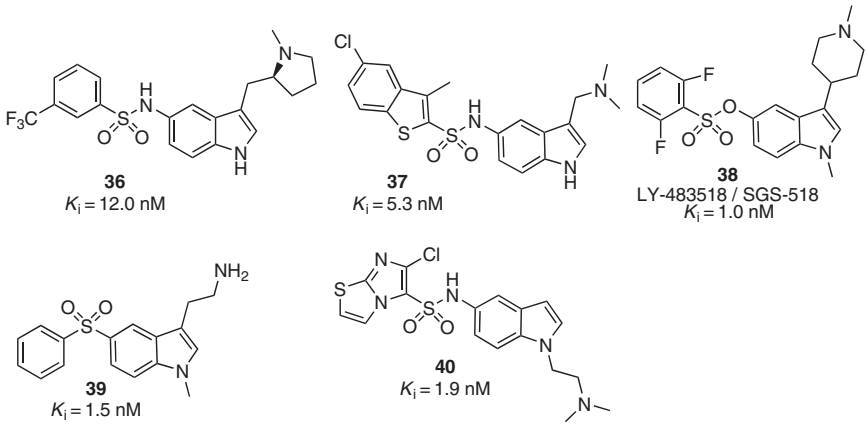
1-sulfonylindoles (**26**) were potent 5-HT_6 antagonists, further demonstrating the promiscuous nature of the receptor site (Kelly and Cole, 2004, 2006). This was supported by additional 4-piperazinyl-1-sulfonylindole 5-HT_6 antagonists independently discovered by researchers at GlaxoSmithKline (**27**) (Ahmed *et al.*, 2005a; Bromidge, 2002), Roche (**28**) (Briggs *et al.*, 2002), and Biovitrum (Caldirola *et al.*, 2002; Johansson *et al.*, 2003). In addition to the excellent affinity, **27** (SB-699929) was also shown to have high selectivity (>100-fold against a range of 50 receptors), good BBB exposure (brain/plasma = 3), and a suitable PK profile (oral $F = 49\%$ and CL = 44 mL/min/kg in rats) to warrant additional focus. Although most of the compounds in this group have a basic amine at the 4-position, similar substituents at the 6-position have also afforded potent compounds (**29**) (Isaac *et al.*, 2000; Xin *et al.*, 2001). Trani and Witty reported a class of indolylazepines (**30**) as potent 5-HT_6 antagonists. This class of compounds was designed by conformationally constraining azepine derivatives (**86**, vide infra) in an attempt to improve the poor brain penetration (Trani *et al.*, 2008). Though this was achieved, the compounds displayed poor microsomal stability.

Bernotas and coworkers took an interesting approach to the development of novel 5-HT_6 ligands by "flipping" the basic amine side chain and the arylsulfonyl group in 1-sulfonyltryptamine derivatives (Bernotas *et al.*, 2004a, 2010). This approach had been successfully utilized in the past to develop other 5-HT ligands in the 5-HT_{2C} and 5-HT_{1D} areas (Boes *et al.*, 1997). In general, the "flipped" analogs had somewhat weaker 5-HT_6 affinity as compared to their non-flipped analogs (Cole *et al.*, 2005a, 2005c); however, they were still potent 5-HT_6 antagonists, in particular analogs with the 1-naphthalenesulfonyl group (**31**) (Bernotas *et al.*, 2004a). Cyclic amino group analogs of **31**, such as piperidine or pyrrolidine, also provide potent compounds (**32**) with selected examples demonstrating agonist efficacy (*vide infra*). Potent 5-HT_6 antagonists were also obtained when the 3-sulfonylindole core was substituted with a basic amino side chain at the 4- to 7-positions of the indole nucleus rather than the 1-position. Researchers at Roche have claimed 3- as well as 2-sulfonylindole derivatives with a basic amine such as piperazine or piperidine at positions 4 through 7 on an indole template (**33–35**) as potent 5-HT_6 antagonists in several patent applications (Beard *et al.*, 2002; Madera and Weikert, 2004a, 2004b; Zhao, 2004). In the 3-sulfonylindole series (**33** and **34**), basic amines at the 5- and 7-positions were reported to be optimal, whereas in the corresponding 2-sulfonylindoles (**35**), the 4- and 7-positions were best. In addition to its potency, **33** (RO4368554) was also shown to be selective (>50-fold over 35 other targets) and brain penetrant (brain/plasma = 0.8–1.1). In scopolamine-impaired rats, **33** was shown to be efficacious in reversal of the effects of scopolamine in several behavioral models including novel object recognition, social recognition, social discrimination, and passive avoidance tasks (Schreiber *et al.*, 2007). In unimpaired rats, **33** enhanced object recognition and autoshaping learning, but was inactive in a water maze task

(1–10 mg/kg, i.p.). Researchers at GlaxoSmithKline also claimed the 7-piperazinyl-3-sulfonylindole derivatives in a patent application (Bromidge *et al.*, 2003a).



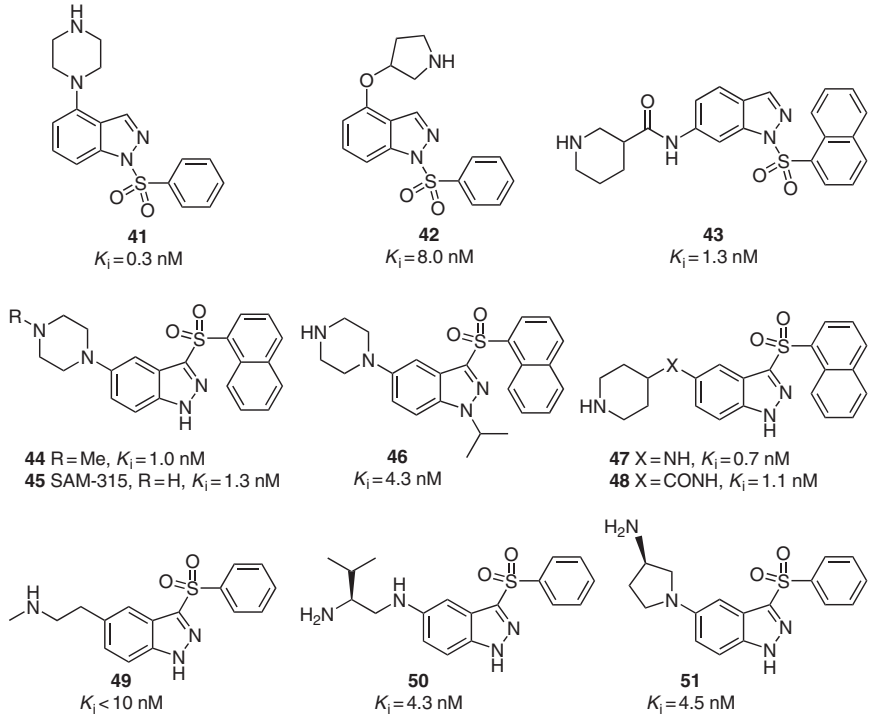
While working on tryptamine derivatives as potential 5-HT$_6$ ligands, Cole and coworkers found that lipophilic moieties such as a benzyloxy group in the 5-position of the tryptamine nucleus afforded compounds with high affinity for the 5-HT$_6$ receptor. Based on this observation, it was hypothesized that moving the sulfonyl group from the 1-position to 5-position may also lead to potent 5-HT$_6$ ligands (Cole *et al.*, 2005b). These 5-sulfonamidoindole derivatives (**36**) were indeed potent compounds, and even more interestingly, both potent 5-HT$_6$ agonists and antagonists were identified within this single chemical series (*vide infra*). Holenz and coworkers independently discovered a similar class of 5-sulfonamidoindole derivatives (**37**) with the guide of a hypothetical model based on several known 5-HT$_6$ ligands (Holenz *et al.*, 2005). Not surprisingly, potent 5-HT$_6$ agonists and antagonists were identified within the same chemical series (*vide infra*). In this area, researchers at Lilly reported a class of arylsulfonic esters (**38**) (LY-483518) as potent 5-HT$_6$ antagonists (Filla *et al.*, 2002; Pineiro-Nunez *et al.*, 2005). Pharmacia & Upjohn (now Pfizer) claimed 5-arylsulfonylindole derivatives (**39**) as potent 5-HT$_6$ antagonists in two patent applications (Fu, 2003a, 2003b). Holenz reported a series of indole derivatives with a basic amine at the 1-position of an indole template and with a sulfonamido group at the 4-, 5-, 6-, or 7-position (Holenz, 2005). In this series, the 5-position was studied in greatest detail and compound **40**, with the imidazothiazole sulfonyl group found in the Wyeth reported clinical compound SAX-187 (WAY-181187), proved to be the most potent analog.



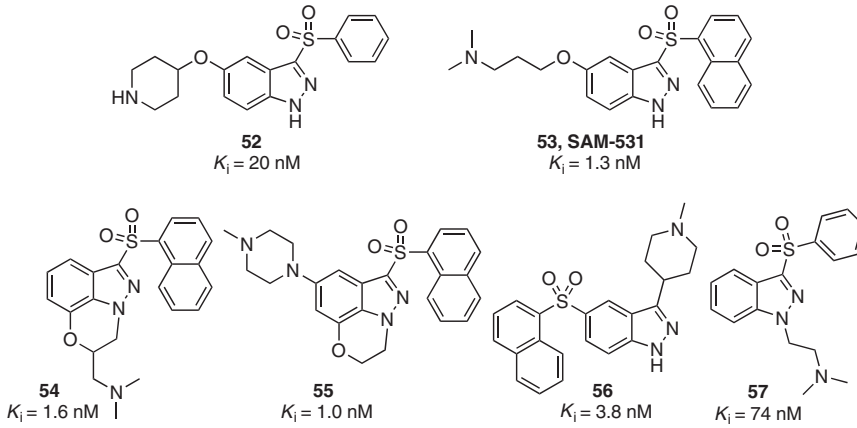
D. Indazoles

Indazoles have been shown to be excellent replacements for indoles as potent and selective 5-HT_6 antagonist cores. Cole (Kelly and Cole, 2004) and Zhou (Li and Zhou, 2002; Zhou *et al.*, 2002a, 2005) reported 1-sulfonylindazoles with a basic amine such as a piperazine (**41**) or an aminoalkoxy side chain (**42**) at the 4-position as potent 5-HT_6 antagonists. The amino side chain can be replaced by other amino groups (e.g., **43**) and the 6-position was found to be the optimal position for some of the chemical series as demonstrated by Liu and Robichaud (Liu *et al.*, 2009a). Liu, Robichaud, and Bernotas further demonstrated that the sulfonyl group can be migrated from the 1-position to the 3-position of the indazole core to provide compounds with great potency and improved physical properties (i.e., water solubility) (Bernotas *et al.*, 2004b; Liu *et al.*, 2010a). From this "1 to 3" migration approach, one of the most potent compounds in both binding and functional assays identified was 1-naphthalenesulfonyl derivative **44**. It should be noted that this particular sulfonyl group has been shown to be one of the optimal sulfonyl groups for a number of classes of 5-HT_6 ligands (Liu *et al.*, 2008, 2009a, 2009b, 2009c). Pharmacokinetic studies in rats demonstrated that **44** was subject to rapid metabolism to generate the demethylated analog **45**. Compound **45** (SAM-315, WAY-255315) was then prepared and showed an improved PK profile and maintained potency, $K_i = 1.3$ nM (Liu *et al.*, 2010a). Though it still had relatively modest brain exposure properties (brain/plasma < 0.20), SAM-315 had excellent water solubility (>100 μg/mL) and low brain and plasma protein binding (80–85%) and displayed over 200-fold selectivity over more than 80 other receptors, enzymes, and ion channels. The potency and attractive pharmacokinetic profile of this antagonist warranted further interest and the compound was quickly advanced to development. In the novel object

recognition (NOR) assay (Comery and Schechter, 2007) paradigms in rats, SAM-315 significantly blocked scopolamine-induced memory deficit with a MED of 0.03 mg/kg and enhanced retention after 48-h delay with a MED of 3 mg/kg in a dose-dependent manner. In the *in vivo* microdialysis studies in rats, SAM-315 significantly increased ACh and glutamate release in hippocampus of the brain in a time-course study (Liu *et al.*, 2010a). As part of the efforts to expand the SAR around the SAM-315 series, it was found that substitution at the *N*1 position of the indazole core (e.g., **46**) resulted in loss of potency, especially with large groups, but with a much improved PK profile. These *N*1-substituted compounds displayed significantly improved oral bioavailability and brain penetration worthy of additional interest (Liu *et al.*, 2010a). A great number of diverse amino side chains were explored to provide compounds (**47–51**) with similar potency (Elokdah *et al.*, 2007a, 2007b; Haydar *et al.*, 2010b; Liu *et al.*, 2010b). The extensive studies on various cores and substitution patterns underscore the hypothesis that the relative positions of the basic amine and the arylsulfonyl moieties, but not necessarily the nature of the template, are important for effective interaction, and thus affinity, with the 5-HT$_6$ receptor (Liu *et al.*, 2009a, 2009c).



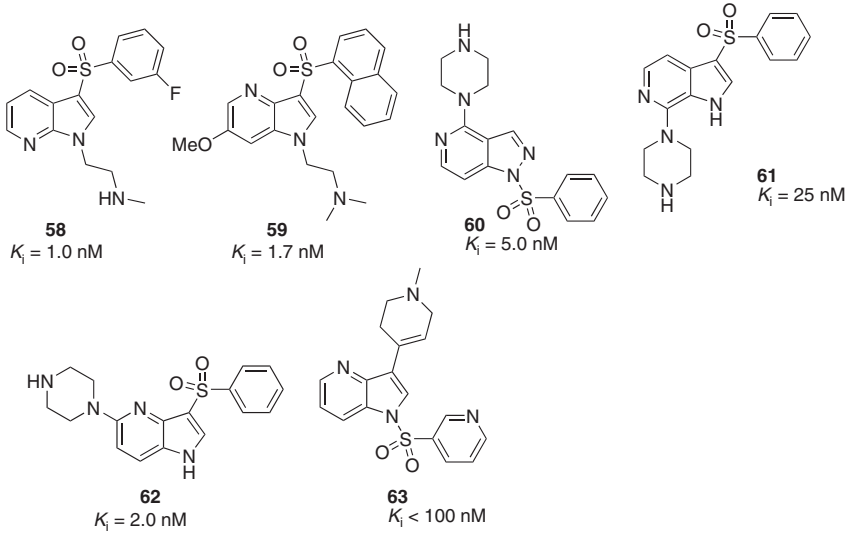
The "1 to 3" sulfonyl group migration approach, in order to identify novel 5-HT_6 ligands, also worked very well for 1-sulfonylindazoles with an aminoalkoxy side chain. Utilizing this approach, a new series (**52**) was identified by Robichaud, Liu, and Elokdah (Elokdah *et al.*, 2007a, 2007b; Robichaud, 2010). Extensive optimization of this chemical series led to identification of the clinical development candidate SAM-531 (WAY-262531), **53**. SAM-531 displayed excellent potency at the 5-HT_6 receptor, an optimized pharmacokinetic profile and >200-fold selectivity over more than 80 other targets. This orally available compound showed *in vivo* efficacy in a variety of preclinical cognition models in rodents and is reported to be currently in Phase II clinical development for the treatment of cognitive deficits in AD (Robichaud, 2010). Greenfield and Robichaud later reported tricyclic derivatives (**54** and **55**) designed by tethering the 1- and 7-positions of SAM-531 derivatives. Based on this expanding database of chemical derivatives, Liu and Robichaud designed another novel series (**56**) as potent 5-HT_6 antagonists by "flipping" the basic amine and arylsulfonyl pharmacophores (Liu *et al.*, 2009b). Finally, 3-sulfonylindazoles with a basic amino side chain at the 1-position (**57**) as 5-HT_6 antagonists have been reported by Bernotas (Bernotas *et al.*, 2004a).



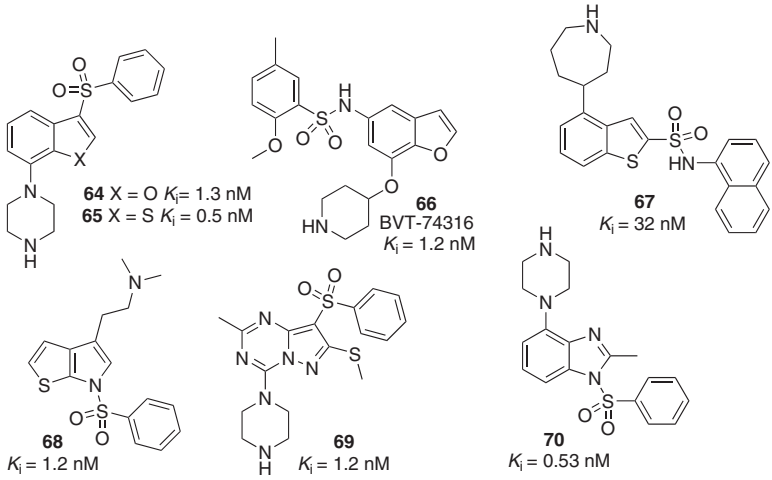
E. Azaindoles and Azaindazoles

Based on the success of the discovery of 5-HT_6 ligands (e.g., **31**) by "flipping" the amino side chain and the arylsulfonyl group on the indole

core, Bernotas further modified the indole core leading to the identification of azaindole derivatives **58** and **59** as potent 5-HT$_6$ antagonists (Bernotas *et al.*, 2009a, 2009b). 5- and 6-azaindoles were also synthesized, but were shown to be much less potent than their 4- and 7-counterparts. One possible explanation for this may be that differences in the basicity of the 5- and 6-azaindoles relative to the 4- or 7-azaindoles were responsible for the reduced affinity. Alternatively, the pyridine nitrogens of the 5- or 6-azaindoles may have found an unfavorable interaction with the 5-HT$_6$ receptor backbone. Interestingly, this chemical series provided both antagonists and agonists, a heretofore scarce commodity, depending on the arylsulfonyl groups and substitution on the basic amine (*vide infra*). Researchers at Wyeth also reported piperazine-substituted azaindoles or azaindazoles (**60–62**) as potent 5-HT$_6$ antagonists in several subsequent patent applications (Bernotas and Yan, 2004; Johansson *et al.*, 2003). More recently, researchers at Memory Pharmaceuticals (now Roche) reported a number of classes of azaindole and azaindazoles (**63**) as potent 5-HT$_6$ ligands with structures closely related to some classes previously reported by Wyeth and other companies (Conticello *et al.*, 2010a, 2010b; Danca *et al.*, 2010; Dunn *et al.*, 2007, 2009; Schumacher, 2010). Though the function of the compounds was not specified, most of them are likely to be antagonists due to the bulky cyclic basic amine in the molecule and the previous knowledge developed in this area.



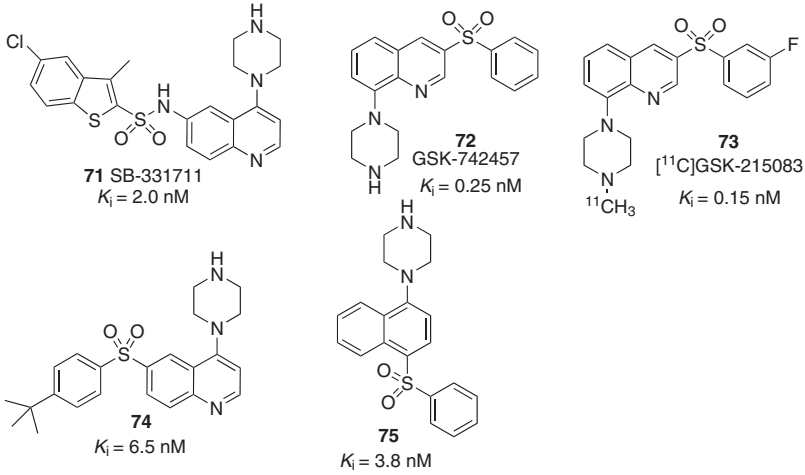
F. Benzofuran, Benzothiaphenes, Benzimidazoles, Thienopyrroles, and Pyrazolotriazines



Benzofurans and benzothiophenes (**64** and **65**) have been reported to be potent 5-HT_6 ligands, comparable to their corresponding indole derivatives (Ahmed, 2005a). Researchers at Biovitrum claimed a range of these benzofuran- and benzothiophene-based derivatives (**66** and **67**) as 5-HT_6 antagonists in several patent applications (Caldirola *et al.*, 2006; Dykes, 2006; Johansson *et al.*, 2003, 2005). The key benzofuran analog **66** (BVT-74316) was recently reported to be in Phase I development. Wyeth claimed a class of thienopyrrole derivatives, such as **68**, as potent 5-HT_6 ligands (Cole, 2005). Furthermore, scientists at Roche have demonstrated that the pyrazolotriazine (**69**) template was a good replacement for indole in terms of 5-HT_6 activity (Boes *et al.*, 1999). However, one could surmise that introduction of too many hydrogen bond acceptors or donors will dramatically increase the hydrophilicity of the molecule and may result in poor brain exposure, as was seen in some other early Roche compounds such as **1** (Ro 04-6790) and **2** (Ro 63-0563). Haydar and Robichaud recently disclosed a class of benzimidazoles (**70**) as potent 5-HT_6 antagonists, extending the work of the Wyeth group even further (Haydar *et al.*, 2010a).

G. Quinolines, Isoquinolines, and Naphthalenes

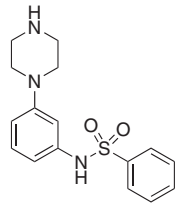
Bromidge first reported a series of quinoline-based sulfonamides as potent 5-HT_6 antagonists, represented by **71**, SB-331711 (Bromidge *et al.*, 2001b). Subsequent



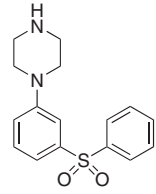
work showed that migration of the piperazine moiety from the 4- to the 3-position of the quinoline template resulted in a significant loss in affinity, further indicating the importance of the relative positions of the pharmacophoric elements for 5-HT$_6$ binding. It was later found that a sulfone was a good replacement for the sulfonamide moiety (Ahmed *et al.*, 2003, 2005b, 2007; Johnson and Witty, 2005; Johnson *et al.*, 2005b), which ultimately led to the identification of the potent 5-HT$_6$ antagonist **72** (GSK-742457). GSK-742457 is reportedly in Phase II clinical trials and has been shown to be efficacious in two trials conducted in patients with mild to moderate AD (Johnson *et al.*, 2008). A PET ligand **73** was later identified and developed from this sulfonylquinoline class to allow a direct measurement of 5-HT$_6$ receptor occupancy in patients, an important tool for the development of the clinical moiety (Gee *et al.*, 2006). Biovitrum claimed another class of quinoline- or isoquinoline-based derivatives with the sulfonyl group at the 5- to 8-position and with piperazine (**74**) or other basic amines on the quinoline core as potent 5-HT$_6$ antagonists (Johansson *et al.*, 2003). The naphthalene-based derivatives (**75**) were broadly claimed by Pharmacia & Upjohn, but no specific 5-HT$_6$ activity was reported (Tenbrink and Kortum, 2003). Glennon also investigated naphthalene-based analogs for their utility as 5-HT$_6$ ligands (Lee *et al.*, 2005; Sikazwe *et al.*, 2006).

H. Benzene and Its Fused Carbocyclic Derivatives

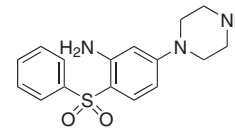
Based on the SAR studies of the indole-based 5-HT$_6$ ligands from his own and other works, Glennon envisioned that the structurally simpler benzene



76
K_i = 62 nM

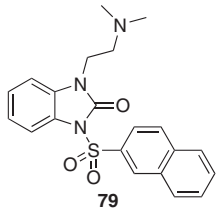


77
K_i = 1.2 nM

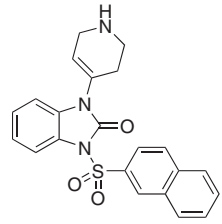


78
K_i = 2.6 nM

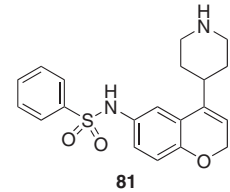
template may retain the 5-HT_6 binding properties of the indole derivatives provided that other pertinent substituents are present and in the right positions (Sikazwe *et al.*, 2006). Compound **76**, which can be viewed as a truncated version of indole **26**, has modest 5-HT_6 receptor affinity. Abbreviation of the sulfonamide to sulfone results in a 50-fold enhanced affinity improvement (**77**). Adding to activity in this class, Pharmacia & Upjohn (now Pfizer) claimed *bis*-arylsulfone derivatives (**78**) as potent ligands (Jacobsen and King, 2004). In this series, a small or unsubstituted amine substituent is reportedly necessary for good affinity. Epix Pharmaceuticals also claimed a number of derivatives related to this class as potent 5-HT_6 and D_2/D_3 ligands (Becker *et al.*, 2006).



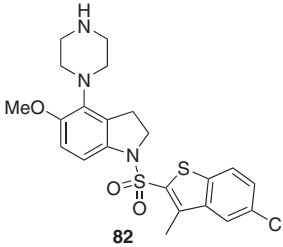
79
K_i = 17 nM



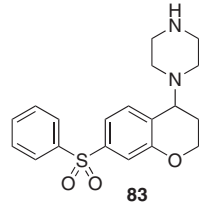
80
K_i = 5.7 nM



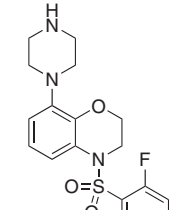
81
K_i = 1.0 nM



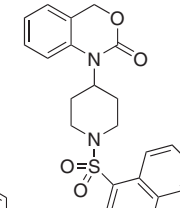
82
K_i = 7.9 nM



83
K_i = 0.4 nM



84 R1485
K_i = 1.3 nM

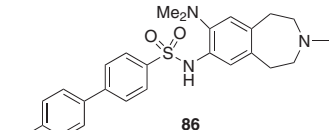


85
K_i = 52 nM

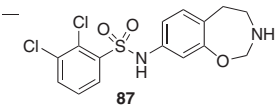
A number of partially reduced aromatic systems have also been reported to provide potent 5-HT$_6$ ligand cores. Researchers at Wyeth have claimed a series of dihydrobenzimidazolones (**79** and **80**) as potent 5-HT$_6$ antagonists (Cole and Bernotas, 2005). However, compounds with a primary amine side chain showed partial or full agonist activity, a result that challenges a plausible explanation (*vide infra*). Researchers at Wyeth also claimed a series of 4-piperidinyl chromens/chromans (**81**) thus expanding the scope of this class (Greenblatt, 2005; Greenblatt and Kelly, 2003; Kelly *et al.*, 2003). Witty demonstrated that conformational constraint of the previously mentioned piperazinylbenzenesulfonamides (**7**, *vide supra*) by tethering the sulfonamide nitrogen (Witty *et al.*, 2009) or the methoxy group (Bromidge, 2001) to the phenyl ring resulted in indolines (**82**) or dihydrobenzofurans with similar potency but with further improved selectivity. Nonetheless, these indolines and dihydrobenzofurans were quite susceptible to metabolic oxidation, thus limiting their utility. AstraZeneca claimed a series of chroman and tetralin sulfonamides and from the limited data reported, it appeared that both series had similar affinity indicating that the oxygen in the chromans did not afford additional affinity for the receptor (Chu *et al.*, 2006; Nordvall *et al.*, 2006). Adding to the flurry of activity in this area, Roche claimed several series of chromans (**83**), tetralins, benzodioxins, benzoxazines (**84**), and tetrahydroisoquinolines in a number of applications (Berger *et al.*, 2003a, 2003b; Greenhouse *et al.*, 2006; Harris *et al.*, 2006; Krauss and Zhao, 2006; Putman, 2004). In particular, compound **84** (R1485) was reported to be a potent and selective 5-HT$_6$ antagonist with excellent brain penetration (B/P = 1.8) and low hERG activity (10% at 10 μM) (Zhao *et al.*, 2007). Investigators at Akzo Nobel claimed a series of indolines together with their indole counterparts with no specific data reported (Spinks *et al.*, 2003). Finally, several benzoxazine derivatives (**85**) were reported by Holenz as potent ligands for the 5-HT$_6$ receptor (Holenz *et al.*, 2006).

Garzya and coworkers reported attempts to identify a new generation of atypical antipsychotic agents by targeting multiple receptors including D$_2$, D$_3$, 5-HT$_{2A}$, 5-HT$_{2C}$, and 5-HT$_6$ with a single molecular entity (Garzya *et al.*, 2007). Based on the history of atypical and typical antipsychotics, this may be a reasonable approach for treatment of complex diseases like schizophrenia, although quite complex from a ligand design standpoint. Through various studies and pharmacological exploration it could be hypothesized that the D$_2$/D$_3$ component may address the positive symptoms, while the 5-HT$_{2A/2C}$ component may reduce extra pyramidal side effects (EPS), and 5-HT$_6$ may be beneficial for cognitive effects. Several analogs with different ring sizes were synthesized and the seven-membered ring benzazepine (**86**) was found to have the most desirable profile against the five-receptor panel. In particular, compound **86** showed a moderate clearance (39 mL/min/kg), good oral bioavailability (F = 69%), and excellent brain exposure properties (brain/plasma = 3.4). In rats, **86** was shown to reverse amphetamine-induced hyperactivity at 20.6 mg/kg (p.o.) and no

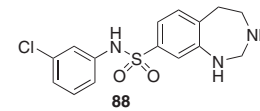
propensity to induce catalepsy at up to 100 mg/kg (p.o.). A number of applications by GSK covering the benzazepine derivatives have been published, noting the importance of these findings (Bromidge and Moss 2002; Bromidge *et al.*, 2003b; Castagnoli *et al.*, 2005; Cooper *et al.*, 2005a, 2005b; Forbes *et al.*, 2004, 2005; Gribble *et al.*, 2003a, 2003b). Adding further to this class, AstraZeneca claimed similar structures (**87** and **88**) in two separate applications (Nordvall and Sehgelmeble, 2007; Nordvall *et al.*, 2007).



86
K_i = 7.9 nM



87
K_i = 31 nM

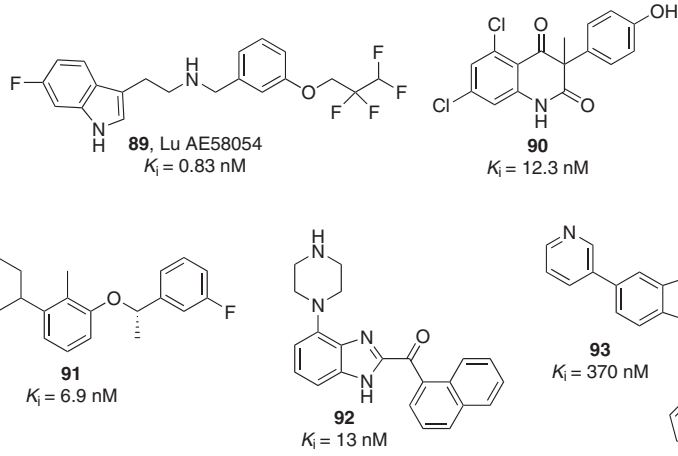


88
K_i = 82 nM

I. Miscellaneous Derivatives Lacking a Sulfonyl Group

For most 5-HT_6 antagonists and 5-HT_6 ligands in general, it has been shown that a sulfonyl group is generally required in order to achieve adequate affinity. However, there are reports of several compound classes that do not contain this ubiquitous sulfonyl group have been shown to be potent 5-HT_6 ligands. Arnt and coworkers at Lundbeck Research disclosed Lu AE58054 (**89**) which is reportedly in Phase II clinical trials for schizophrenia (Arnt *et al.*, 2010). Lu AE58054, a potent ($K_i = 0.83$ nM) antagonist with modest affinity for adrenergic α1A and α1B, demonstrated greater than 50-fold selectivity for more than 70 other targets examined. Oral administration of the compound in a dose range of 5–20 mg/kg displayed 65% striatal 5-HT_6 receptor binding and reversed cognitive impairment in a rat behavioral NOR task induced after subchronic treatment for 7 days with phencyclidine (PCP). Seong reported a class of quinoline derivatives (**90**) without a basic amine as 5-HT_6 antagonists (Seong *et al.*, 2008). Albeit most of the compounds in this class are not potent, it is still quite surprising because it is duly noted that a basic amine is generally required for 5-HT_6 or any monoamine receptor-targeted affinity. Schwarz reported a class of tolylamines (**91**) where it was observed that the (*S*)-configuration is preferred for 5-HT_6 potency (Singer *et al.*, 2009). Though it was noted that compound **91** is highly protein bound, it is selectively partitioned into rat brain. Consequently the brain free drug concentration was sufficient to provide efficacy in a rat NOR behavioral

paradigm. Lopez–Rodriguez reported a class of benzimidazoles (**92**) identified based on a ligand pharmacophore model (de la Fuente *et al.*, 2010). Derivatives of Dimebon (e.g., **93**), which belongs to a class of compounds called γ-carbolines, with broad spectrum of biological activities, have also been reported to have 5-HT$_6$ antagonist activity (Ivachtchenko *et al.*, 2009). Additional non-sulfonyl containing derivatives have also been reported as 5-HT$_6$ antagonists, including benzimidazolones (Bonhaus and Martin, 2006; Owens *et al.*, 2007), quinolinones/quinazolinones (Bonhaus and Martin, 2006; Harris *et al.*, 2004; Sui and Zhao, 2005), benzoxazinones (Bonhaus and Martin, 2006; Maag *et al.*, 2004), quinolines (Harris *et al.*, 2007; Johnson *et al.*, 2005a), carbazoles (Tenbrink, 2001a, 2001b), chromans (Kelly *et al.*, 2003), and carboximidamides (Cole *et al.*, 2003).

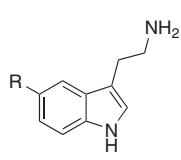


III. 5-HT$_6$ Agonists

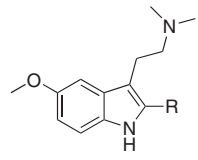
While many classes of potent and selective 5-HT$_6$ antagonists have been discovered, identification of potent and selective agonists has proven to be considerably more challenging. 5-HT(**1**), and 5-methoxytryptamine (**14**) were the first compounds demonstrated to act as 5-HT$_6$ agonists and produce a potent dose-dependent increase in cAMP levels (Monsma *et al.*, 1993). However and not unexpectedly, these endogenous ligands are not selective and bind to other 5-HT receptor subtypes as well. In an attempt to identify potent and selective 5-HT$_6$ agonists, Glennon studied the SAR of tryptamine derivatives at human

5-HT_6 receptors. With minimal molecular modification, it was shown that simple related tryptamine derivatives can be developed to display enhanced selectivity for different populations of 5-HT receptors (Glennon *et al.*, 2000). One interesting finding was that methylation at the 2-position of the indole core (**94**) was tolerated and surprisingly provided compounds with good selectivity over 5-HT_3. This is noteworthy because until this work, 2-methyl 5-HT (**94**) had been considered a 5-HT_3 selective agonist. Analogs with bulkier groups at the 2-position were then synthesized to provide compounds such as **95** (EMDT) with improved affinity at 5-HT_6. EMDT displayed 10-fold selectivity over 5-HT_{1A} and greater than 20-fold selectivity against other 5-HT receptor subtypes and behaved as a full agonist relative to the natural agonist 5-HT. Interestingly, while being even more selective, 2-phenyl 5-HT is a full antagonist.

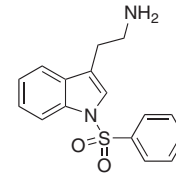
Cole and coworkers attempted to identify 5-HT_6 agonists through focused screening of the biogenic amine-type compounds in the Wyeth compound collection. After several rounds of SAR optimization of the initial lead, an indolyl aminoguanidine derivative **96**, representative of a potent class of 5-HT_6 agonists, was identified (Cole *et al.*, 2007). It was soon found that the benzenesulfonyl group could be replaced by a variety of other arylsulfonyl groups to retain potency and full agonist function, with a few notable exceptions in which antagonists were obtained. The optimized compound identified in this series, **97** (WAY-181187, SAX-187), displayed high binding affinity, potent full agonism ($EC_{50} = 6.6$ nM, $E_{max} = 96\%$), and greater than 50-fold selectivity in a panel of 31 other receptors and ion channels and was quickly advanced to development (Cole *et al.*, 2007; Schechter *et al.*, 2008). In rats, SAX-187 displayed high volume of distribution ($V_{ss} = 24$ L/Kg) and high systemic clearance (7.4 L/h/kg) following a 1 mg/kg i.v. dose and an elimination half-life ($t_{1/2}$) of 3.1 h and an oral bioavailability (F) of 23% following 10 mg/kg oral dose. The compound had moderate brain penetration in rats with brain to plasma ratios of 0.1–0.7 depending on the dose. A similar pharmacokinetic profile was observed in dogs. Acute administration of SAX-187 (3–30 mg/kg, sc) in rats significantly increased extracellular GABA concentrations in the frontal cortex without altering the levels of glutamate or norepinephrine as measured by microdialysis. The neurochemical effects could be blocked by pretreatment with the known selective 5-HT_6 antagonist SB-271046 (**7**) directly implicating 5-HT_6 receptor mechanisms in mediating these responses. Furthermore, it was reported that in the rat schedule-induced polydipsia (SIP) model, acute administration of SAX-187 (56–178 mg/kg, po) decreased adjunctive drinking behavior in a dose-dependent manner suggesting a potential role of 5-HT_6 agonists in treating obsessive-compulsive disorder (OCD), considered to be a type of anxiety. SAX-187 was advanced to Phase I clinical development based on its robust preclinical profile.



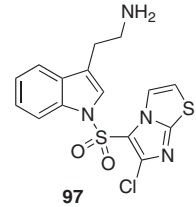
13 R = OH, 5-HT, K_i = 75 nM
14 R = OMe, K_i = 88 nM



94 R = Me, K_i = 60 nM
95 R = Et, EMDT, K_i = 16 nM

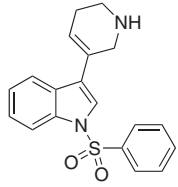


96
K_i = 10 nM

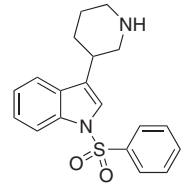


97
WAY-181187, SAX-187
K_i = 2.2 nM

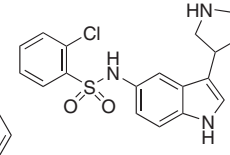
To build on the success of *N*1-ayrlsulfonyl-tryptamines, such as **96** and **97**, Cole and coworkers examined cyclic amino side chains where **98** was quickly identified to be a partial agonist (Cole *et al.*, 2005c). Saturation of the double bond of **98** led to **99** with significantly improved potency and full agonist function. The two enantiomers of **99** were resolved and it was determined that they were equally potent in binding, however, all the agonist activity came from a single enantiomer. In an effort to further expand the scope of the indole-based 5-HT$_6$ ligands, Cole moved the sulfonamido group from the 1- to the 5-position on the indole core leading to a class of potent 5-HT$_6$ agonists as represented by **100**. Interestingly, for this class of compounds, while (*R*)-enantiomers were shown to be full agonists, the (*S*)-enantiomers were silent antagonists.



98
K_i = 159 nM (partial)



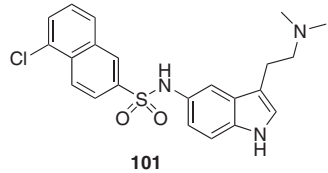
99
K_i = 2.0 nM



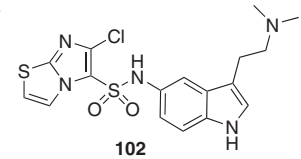
100
K_i = 1.1 nM

Adding to the confusion around functional efficacy, Holenz and coworkers independently discovered a similar class of 5-sulfonamidoindole derivatives resulting in both antagonists (**37**, *vide supra*) and agonists (**101**) (Holenz *et al.*,

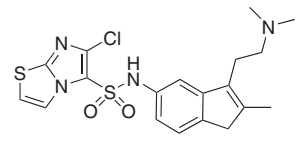
2005). Compound **101** (E-6837) displayed partial agonism in 5-HT_6 rat receptors ($EC_{50} = 0.6$ nM, $E_{max} = 67\%$) but full agonism ($EC_{50} = 0.3$ nM, $E_{max} = 96\%$) in human receptors and greater than 150-fold selectivity over more than 60 targets (Fisas *et al.*, 2006). Oral administration of E-6837 at 30 mg/kg (twice a day) in diet-induced obesity (DIO) rats during a 4-week period resulted in a sustained body weight loss and a decreased cumulative food intake during a 4-week treatment indicating that 5-HT_6 agonists may provide a promising approach to treat obesity (Fisas *et al.*, 2006). In this series, an analog (**102**, E-6801) with the SAX-187 6-Cl-imidazothiazolesulfonyl group was also shown to be potent full agonist (Alcalde *et al.*, 2009; Holenz *et al.*, 2005). Alcalde expanded this 5-sulfonamidoindole series by replacing the indole core with indene leading to the identification of a number of potent full agonists including **103** (E-15136) (Alcalde *et al.*, 2009). It should be noted that indene as a template has previously been examined by Glennon and has been found to be a good replacement to indole for ligands focused on 5-HT_6 affinity (Kolanos *et al.*, 2005).



101
E-6837
$K_i = 0.7$ nM



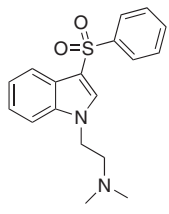
102
E-6801
$K_i = 2.2$ nM



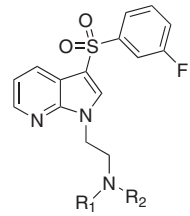
103
E-15136
$K_i = 4.5$ nM

As before, Bernotas "flipped" the basic amine side chain and the arylsulfonyl group in 1-sulfonyltryptamine derivatives in order to expand the scope of novel 5-HT_6 ligands (*vide supra*) (Bernotas *et al.*, 2004a, 2009a, 2009b). From this approach, indole derivative **104** was identified as a weak partial agonist (EC_{50} 366 nM, $E_{max} = 63\%$). While most of the compounds in the indole series were shown to be antagonists (e.g., **31**), introduction of an additional nitrogen to the indole core provided azaindoles predominately as potent full agonists. Among these compounds, **105** (WAY-208466) was a standout with an $EC_{50} = 7.3$ nM and $E_{max} = 100\%$. Remarkably, the secondary amine **106** was a potent full antagonist ($IC_{50} = 6.2$ nM, $I_{max} = 93\%$), and the primary amine **107** reverted

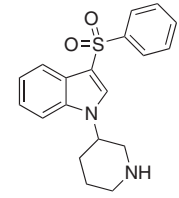
back to a full agonist ($EC_{50} = 24$ nM, $E_{max} = 100\%$). Needless to say, one is challenged to provide a plausible explanation for the changing functional efficacy in a series of derivatives differing only by a methyl group. Nevertheless, WAY-208466 was shown to be selective against a panel of other receptors and ion channels and produced a similar neurochemical effects to that observed with SAX-187 and was designated as the backup to the clinical development compound (Schechter *et al.*, 2008). Elokdah and Bernotas also explored cyclic amino side chains such as piperidine and pyrrolidine in both the indole and the azaindole series (Bernotas *et al.*, 2010; Elokdah *et al.*, 2005). While **108** was identified to be a potent full agonist, most of the compounds in these classes were found to be partial weak agonists or antagonists (e.g., **32**) adding to the mystery around the functional efficacy of this class of compounds.



104
$K_i = 20$ nM



105 $R_1 = R_2 =$ Me, WAY-208466, $K_i = 4.8$ nM
106 $R_1 = R_2 =$ H, $K_i = 4.7$ nM
107 $R_1 =$ H, $R_2 =$ Me, $K_i = 4.7$ nM



108
$K_i = 13$ nM

IV. Summary

Since the identification and cloning of the 5-HT$_6$ receptor almost two decades ago, tremendous progress has been made in elucidating 5-HT$_6$ receptor function using modern biology techniques. The pharmacological potential of the modulation of this receptor was demonstrated by the many potent and selective 5-HT$_6$ ligands identified subsequently. The advancement of a number of 5-HT$_6$ antagonists into clinical development for cognitive enhancement in Alzheimer's disease and schizophrenia shows the translational potential of this receptor for the treatment of significant diseases. Alzheimer's disease represents one of the greatest human health challenges in this century and current palliative treatments (Robichaud, 2006) reportedly exert their activity via the cholinergic or glutamatergic systems. Extensive neurobiology and *in vivo* pharmacology studies in rodents have shown that 5-HT$_6$ antagonists can improve memory and cognition through involvement in multiple neurotransmission systems, in particular cholinergic and glutamatergic systems. Therefore, central antagonism of the 5-HT$_6$ receptor may provide a mechanistically distinct palliative treatment for Alzheimer's disease that

works through both of these neuronal systems. The reader is directed to the plethora of publications by the key groups mentioned in this review, as well as several academic labs, in further researching the utility of 5-HT_6 antagonists for various therapeutic indications. The potential for this approach for cognitive enhancement, in particular, will be validated by the ongoing clinical trials with the various 5-HT_6 antagonists currently in development. The medical and scientific communities eagerly await the results of these and future trials to demonstrate the potential utility of these various classes of molecules.

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PATENTS

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A. Glossary

1. *Patent Application*

Document containing the description of the invention, including the state of the art (background), the experimental part giving examples, and the claims determining the scope of protection.

2. *Patent Application Publication*

Document published 18 months after the filing date, or after the date of the filing of the priority application.

3. *Filing Date*

Date attributed by the patent office in which the patent document is filed.

4. *Priority*

Designate the first patent application filed for an invention (Basic Patent Application). During 12 months after the filing date of this priority, the applicant may file further patent application(s) claiming the same invention. In such case, the state of the art used for evaluating the patentability will be determined on the filing date of this first patent application filed.

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5. *Patent (Granted)*

At the end of the examination, the patent office grants a patent which is published and will determine the final protection given to the applicant. The patent is most of the time different compared with the patent application filed.

6. *Patent Family*

All the patents and/or patent applications claiming the same priority(ies) are grouped in a patent family.

I. Introduction

Intellectual property (IP) and more specifically patents can be an important source of information in relation to the pharmacology of 5-HT6 receptors. This is true not only for the patents and patent application documents published but also for the existing databases whose main objective is to gather and compile all the available information from different points of view. Following the expansion and generalization of the use of the patent system, the number of documents generated has grown enormously. One has to remember that, although the main objective of patents is to protect the inventions and thereby provide the inventor an exclusivity on the exploitation of his own invention, the counterpart required by the legislators has been to make all the information contained in the patents available to the public after a period of 18 months from the filing date. Nevertheless, for the public not specialized in patents, the format may be somewhat complex to understand and the information may be difficult amidst the documents that not only contain scientific information but also have to comply with formal legal requirements. This renders even more attractive the use of databases for a first screening of the huge and complex documentation before going deeply into the more interesting documents to find the scientific information.

In this regard, information related to the pharmacology of 5-HT6 receptor will be approached in the following discussion from two different perspectives. On the one hand, a general overview of the patent situation for the 5-HT6 receptor will focus on the evolution of the filing and publication of patent applications over time since the mentioned receptor was identified as a target of interest. On the other hand, a more specific and detailed approach will focus on the information available from a chemical, pharmacological, and therapeutical point of view. The data and information discussed are obtained from commercially available databases. When searching for such information, one has to bear in mind important parameters, for example the database coverage, the different raw sources of information compiled in the database, or the main

focus or interest of some databases. Consequently, the same query, using the same keywords or key parameters, will give different results when executed in a scientific-oriented database or in a business-oriented database. Having said that, the source of information used for the present discussion is Thomson Reuters Integrity (http://integrity.prous.com/integrity/xmlxsl/).

II. Patent Applications Filed: Evolution over Time

The interest for 5-HT6 receptors compared to other serotonin receptors can be drawn from Fig. 1, showing the number of patent application families filed for different receptor subtypes (when dopaminergic receptors appear, it means one of the serotonin receptors is mentioned as a secondary target).

Searching the existing patent information related to "drugs acting on 5-hydroxytryptamine receptors" as "mechanism of action," more than 3000 patent families are retrieved. Grouping the results according to the different subtypes of serotonin receptors, 5-HT6 represents 9.47% of the total answers retrieved, meaning the fourth target of interest after 5-HT1A, 5-HT2A, and 5-HT1D. More specifically and focusing on "5-HT6 receptor ligands" as mechanism of action, 271 different patent families are retrieved.

The 5-HT_6 receptor is the latest serotonin receptor subtype identified by molecular cloning both in rats (Monsma *et al.*, 1992; Ruat *et al.*, 1993) and in humans (Kohen *et al.*, 1996).

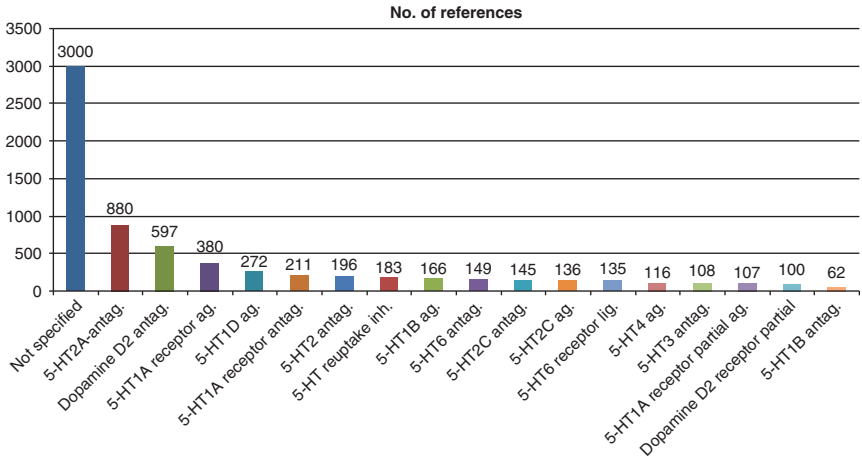


FIG. 1. Number of patent applications filed for each serotonin receptor subtype.

Figure 2 shows the number of patent applications filed sorted by priority years. From 1998, 5 years after the publication of the first cloning, the number of filing increased significantly. Specifically, 2001 meant the start of a huge activity, and two other periods, 2001–2003 (33.95%) and 2006–2008 (34.69%), concentrated on the maximum numbers of patent filing, with 33 and 34, respectively.

Figure 3, focusing on the basic patent publication year for the different patent families, confirms the same tendency. The major range of publication took place between 2002 and 2009, the four years 2003 (13.28%), 2005 (11.81%), 2007 (12.55%), and 2009 (12.92%) being the most important periods. It also shows the decrease observed in 2008 for the filing of applications, confirmed in 2010 with much less publications.

Four companies are recorded to be titular of over 25 patent families filed: GSK (United Kingdom), Wyeth (United States), Roche (Switzerland), and Esteve (Spain) (Fig. 4).

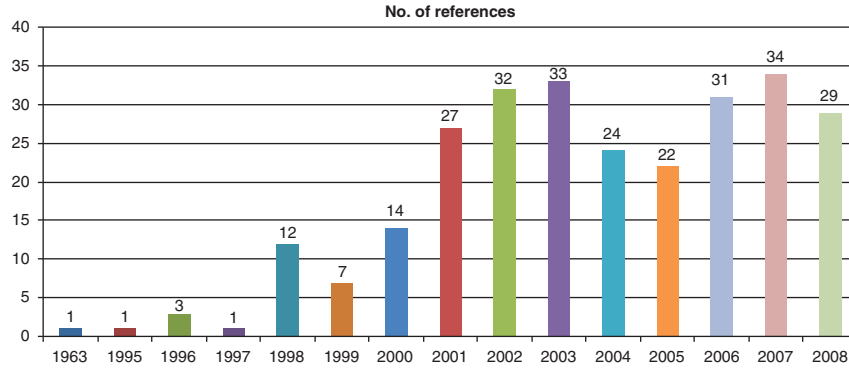


FIG. 2. Number of patent applications sorted by *priority* year.

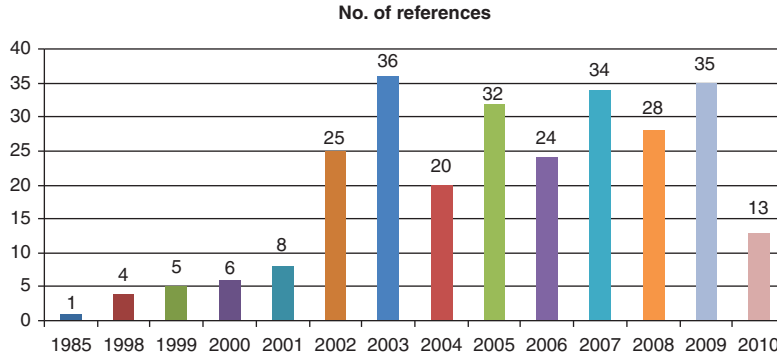


FIG. 3. Number of patent applications sorted by *publication* year.

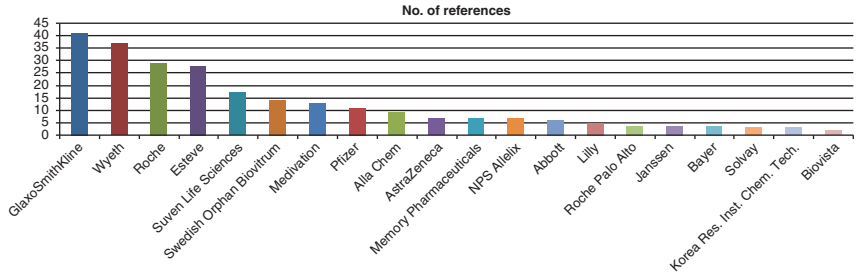


FIG. 4. Number of patent families sorted by *applicant*.

From the data related to the rest of companies, it can be noticed that the so-called Big Pharma are mixed with other smaller companies, which means that the 5-HT6 receptor is a target of importance independently of the company size.

On reviewing the countries from the view, either of patent families filed or of the origin of the companies, United States appears as predominant (Fig. 5). On the other hand, there are some countries such as Spain or Korea where only one company has generated IP related to this target.

Figure 6 attends to the main subject matter claimed in the different patent families.

For a big majority, the patent applications filed are related to new chemical entities (NCE, 79.8%) and also contain claims related to process for manufacturing, therapeutical indications (uses), and sometimes combinations. The other important matters covered by the claims are therapeutical indications, also referenced as method of use/treatment (12.1%) and combinations (4.8%).

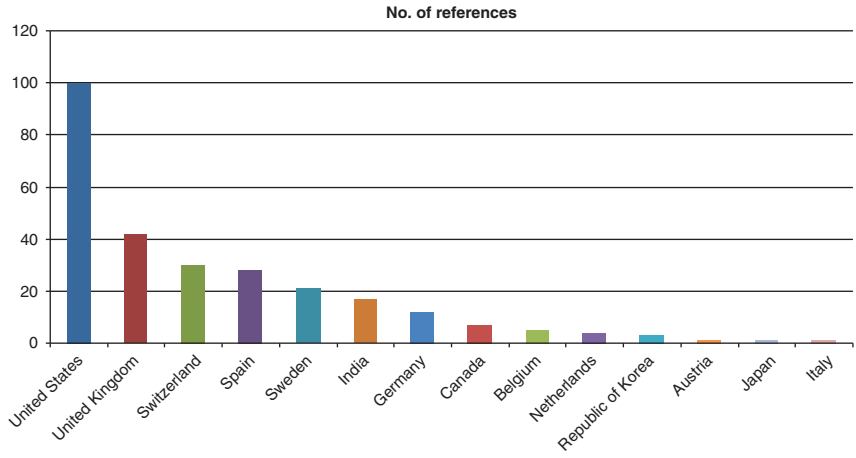


FIG. 5. Number of patent families sorted by *countries*.

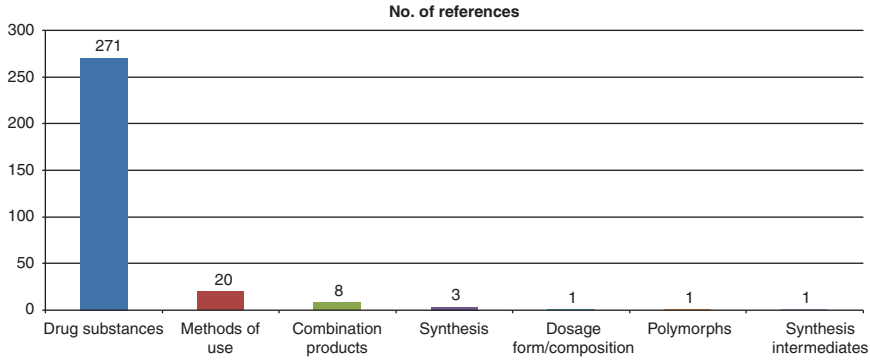


FIG. 6. Number of patent applications sorted by *subject matter* of the main claim.

As shown in Fig. 6, the occurrence of other subject matter, as formulations, polymorphs, or process for manufacturing, is sensibly low compared with the NCE patent applications.

With regard to the subject matter claimed in the different patent applications commented upon in relation to Fig. 6, some important aspects are to be highlighted, before going deeply into the results described in the documents.

In a patent application whose main claims relate to NCEs, usually the information contained in the description, besides a general approach to the current state of the art, is centered in a general formula also called "Markush formula." It will be complemented, in the experimental part, with specific examples according to a general synthetic process described and/or also claimed and also with specific binding data for the receptor, which also implies a complete description of the protocol involved. From this point of view, patents can also be a powerful information tool for knowing how to obtain chemical and pharmacological data complementing the information available in scientific publications, sometimes more focused in the discussion of the results obtained or in the explanation of the mechanism involved.

This kind of patent applications on becoming granted patents gives the strongest protection to the applicant/owner.

Totally different information will be found in patent applications whose main claims relate to *new therapeutical indications/uses*. In this case the named "experimental data" are more focused on pharmacological tests, either *in vivo* or *in vitro*, that support the new indication(s) claimed. In this kind of patent applications, it is often usual to include even data coming from human clinical trials. These patent applications on becoming granted patents usually form part of a product life-cycle management and expire later than the related patents covering the NCEs. Obviously, this is not true for patents covering new therapeutical indications of known compounds. This may be the case for compounds

already described in the literature, but for which a special new and unexpected interest has been found in relation with an activity related to 5-HT6 receptors.

Finally, the third important group shown in Fig. 6 is that of the patent applications whose main claims are related to *combinations*. Again in this case the named "experimental data" are more focused on pharmacological results, usually *in vitro*, that support the synergistic effect of the new combination for a specific indication(s) claimed. In this kind of application, it is often usual to claim the combination itself as a "new product." As the "use" patent applications, these patent applications on becoming granted patents usually form part of a product life-cycle management and expire later than the related compound patents.

Another important issue is to evaluate how many patent applications become granted patents. Patent applications are examined separately in each country, and after several years of this process, the examiners of the corresponding patent offices grant the patent. A document corresponding to these granted patents is then made available to the public in order for third parties to be aware of the final protection obtained by the applicant. Additionally, it is a way to measure the value of the invention, meaning that when the object of the claim has passed the examination process successfully, it complies with the patentability criteria and has a greater value. Focusing on United States (US) and/or Europe (EP) granted patents, the data show that from the 324 filed and published patent application families, 21.3% (69 families) become EP-granted patents, 30.9% (100 families) become a US-granted patent, and 13.27% (43 families) have been granted in both countries (data from DERWENT World Patents Index, covering 1963 to date).

III. Results Described and Claimed in Patent Related to 5-HT6 Receptors: Main Focus, Therapeutical Areas, and Chemical Diversity

As mentioned in the previous section, the patents and patent applications related to 5-HT6 receptors mainly focus on NCEs. This is usual for any pharmacological target of interest. Nevertheless, in almost all of these documents, as well as in the documents specifically related to pharmacological applications, an important part is dedicated to the conditions that may be treated or prevented through the action of the claimed compounds on 5-HT6 receptors.

Examining the different diseases mentioned in the patent literature in depth, Fig. 7 reveals that five indications are the most claimed: cognitive disorders with almost 16% are the most relevant followed by psychosis, depression, obesity, anxiety, and dementia/Alzheimer's disease.

As far as chemical diversity is concerned, in order to have a picture of the different scaffolds published in the patent literature, one has to focus on patent applications directed to cover NCEs. Following this approach, 233 patent

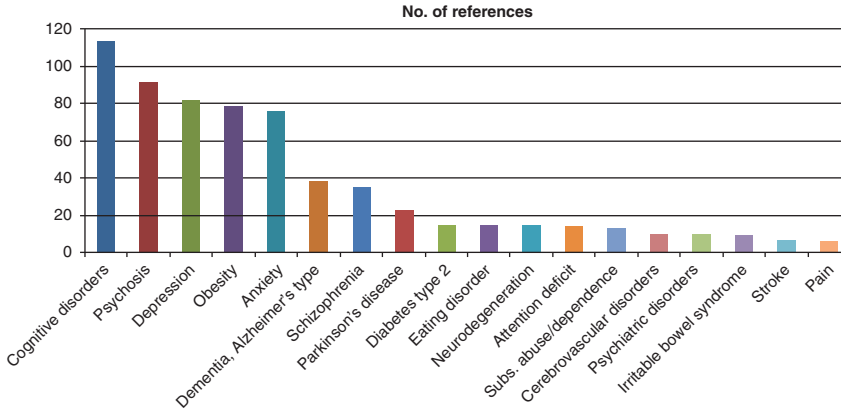


FIG. 7. Number of patent applications sorted by *therapeutical area.*

families may be found, among which 27 will not be considered, because the main subject matter relates to another pharmacological target, namely 5-HT6 receptors only appearing as a secondary mechanism of action. Among the 206 remaining patent families, the chemical diversity is quite important.

The most relevant scaffold appears to be that of indole. A total of 83 patent families have been published, describing chemical formulae containing an indole ring or a close analog. A big majority of these compounds present an aryl sulfonyl substitution, which has been thought on any position of the basic indole structure described in 80% of the documents (the more recent examples are WO-2010/032257, WO-2009/053997,WO-2009/034581, WO-2008/136017, WO-2008/084492, WO-2008/084491, WO-2007/138611, WO-2007/046112, WO-2007/020653, and WO-2005/066157 all from Suven Life Sciences; WO-2009/016225 and WO-2006/038006 from Glaxo Group Limited; WO-2007/053352 and WO2007/084841 from Wyeth; WO-2008/003703 from Biovitrium AB; WO-2008/055808 from F. Hoffman-La Roche AG; WO-2009/094668 from Medivation Technologies Inc.), or a sulfonamide moiety also linked to any position (WO-2006/024535, WO-2006/015867, WO-2005/013979, WO-2005/013978, WO-2005/013977, and WO-2005/013976 all from Esteve; WO-2009/073118 from Merck & Co. Inc.). Azaindole has also been investigated and represents 12% of the documents (the more recent examples are WO-02/051837 from American Home Product Corporation; WO-2010/024980, WO-2010/021797, and WO-2010/002802 from Memory Pharmaceutical Corporation) as well as indazoles representing 8% (for example, WO-2009/155399 and WO-2007/142905 from Wyeth).

The second important group of compounds patented for a 5-HT6 modulation activity is represented by quinoline and its analogs (23 patent families). Quinoline ring has attracted a considerable interest, again associated to a sulfonyl

substitution, and is the object of 65% of these documents (the more recent ones are WO-2007/039219, WO-2005/095346, WO-2005/040124, and WO-2005/030724 from Glaxo Group Limited; WO-2008/116831 from Abbot GmbH & Co.; WO-2007/025798 from F. Hoffmann-La Roche AG). Closely related structural analogs such as quinolinediones (for example, WO-2008/004716 and WO-2007/032572 from Korea Research Institute of Chemical Technology), tricyclic isoquinolines (WO-2008/110598 from Biovitrium AB), quinazoline (WO-2007/108744 and WO-2007/108743 from AstraZeneca AB), quinazolinone (WO-2005/067933 from F. Hoffmann-La Roche AG), and quinoxaline (WO-2006/037481 from F. Hoffmann-La Roche AG) have also been reported to have a potential interesting affinity for the 5-HT6 receptors and may be mentioned among others.

A third group of compounds that has drawn attention is constituted by chroman (for example, WO-2006/066756 and WO-2006/066746 from F. Hoffmann-La Roche AG; WO-2006/126939 from AstraZeneca AB), chromen (including WO-2005/037830 from Wyeth), benzodioxane (for example, WO-2005/105776 from F. Hoffmann-La Roche AG), benzoxazine (WO-2005/058847 from F. Hoffmann-La Roche AG), benzoxazinone (for example, WO-2005/014589 from Esteve), and benzofuran (for example, WO-2006/062481 from Biovitrum AB) rings. Once again a majority of these compounds bear a sulfonyl or sulfonamide substitution on various positions of the basic structure and represent a total of 19 patent families.

Mono- and bicyclic rings lacking any heteroatoms have also been considered in nine patent families published. Thus results involving tetraline (for example, WO-2007/147771, WO-2007/147762, WO-2006/066748, and WO-2006/066745 from F. Hoffmann-La Roche AG; WO-2007/108742 from AstraZeneca AB) and indene (WO-2007/054257 from Esteve) were objects of various claims. As for the preceding families of compounds, they all bear a sulfonyl or sulfonamide substituent, and more specifically an aryl sulfonyl or aryl sulfonamide.

Benzazepine (for example, in WO-2005/051397, WO-2005/025576, and WO-2005/016891 from Glaxo Group Limited; WO-2007/004960 from AstraZeneca AB) and benzoxazepinone (for example, WO-2006/061126 from F. Hoffmann-La Roche AG) have also been found to have an interesting activity in the modulation of the 5-HT6 receptors. In contrast to the preceding scaffold, they are not associated to the sulfonyl or sulfonamide substitution. These structures appear in 11 patent families.

The last group of compound of importance that has been published in seven patent families contains benzimidazoles (for example, US-2006/0116384 from Wyeth) and benzimidazolones (for example, WO-2005/010003 and WO-2005/009996 from Wyeth; WO-2007/006677 from F. Hoffmann-La Roche AG), all bearing a sulfonyl or sulfonamide substituent.

Apart from the above-mentioned scaffolds, several patent applications have been filed to cover various chemical formulae whose common characteristic relies

on the presence of a piperazine or piperidine ring and/or a sulfonyl or sulfonamide group. A total of 54 patent families may be found, representing 20% of the documents retrieved. By way of example, the list of these structures includes piperazinyl or pyridazine derivatives (WO-2010/000456 from Janssen Pharmaceutica NV), sulfonyl heterocyclopyrrolylalkylamine (WO-2005/012311 from Wyeth), pyrrolo[3,2-*b*]pyridine (WO-2005/037834 from Biovitrum AB), sulfonylpyrazoles and sulfonylpyrazoline (WO-2009/115515 and WO-2008/034863 from Solvay Pharmaceuticals BV), aryl sulfonamides (WO-2010/032258 and WO-2007/020652 from Suven Life Sciences Limited; WO-2007/118900 and WO-2007/118899 from Abbott GmbH & Co.), aminopyrimidine (WO-2010/053825 from Janssen Pharmaceutica NV), phenylpiperazines (WO-2006/069808 and WO-2006/069807 from Esteve), pyridinylpiperazine (WO-2009/098576 from Pfizer Inc.), carbazoles and derivatives (WO-2007/046111 and WO-2005/066184 from Suven Life Sciences; WO-2009/038764 from D2E, LLC; WO-2007/028460 from Esteve), pyrazolo[1,5-*a*]pyrimidine (WO-2009/093210, WO-2009/093209, WO-2009/093208, and WO-2009/093206 from Alla Chem LLC), azetidines substituted by indole, indazole, or benzimidazole (WO-2008/116833 from Abbott GmbH & Co.), pyrimidines (WO-2008/147812 and WO-2007/098418 from Memory Pharmaceuticals Corporation), thiazones (WO-02/083863 from Sepracor Inc.), and others (WO-2006/091703 from Psychenomics Inc.).

The full list of documents will be found in the reference section, as well as the one regarding therapeutical uses, processes of synthesis, and combinations.

IV. Preclinical and Clinical Candidates: Status of the Studies Reported

A number of compounds are now under active development in different phases. Fourteen of these compounds are studied for their 5-HT6 antagonist properties, whereas only three of them are reported to be agonists. Two of these agonists are compounds of Avineuro (AVN-492 and AVN-457), currently in preclinical phase. There are no major differences with regard to the diseases treated associated with the functionality of the ligand on the receptor. Thus both antagonists and agonists are studied and developed for the management of cognitive disorders. Nevertheless, one agonist compound from Sigma-Tau (ST-1936) is reported to be unique at a predevelopment stage for depression. Central nervous system appears to be the major target since only one compound is in preclinical development for obesity (SUVN-504 from Suven Life Sciences).

Among the compounds referred to for their antagonist properties, cognitive disorders appear to be the most relevant therapeutic focus for compounds.

Two of them are currently at preclinical stage, SUVN-507 (Suven Life Sciences) and AVN-458 (Avineuro). In Phase I, two compounds are reported, SYN-114 and SYN-120, both from Roche/Synosia Therapeutics. Lu-AE-58054 (SGS-518), in a co-development between Lundbeck and Eli Lilly, reached Phase II as did also AVN-211 of Avineuro. Besides these studies, others are more specifically dedicated to schizophrenia, dementia, and Alzheimer's: SUV-52 of Suven Life Sciences is in preclinical development, SB-742457 (or GSK-742457) of GlaxoSmithKline is reported undergoing a Phase II trial, and PF-5212365 of Pfizer is also in Phase II.

Two other compounds undergoing preclinical or clinical studies are reported for a dual activity. SUVN-501 of Suven Life Sciences is at preclinical stage for the same indication of schizophrenia, dementia, and Alzheimer with a mechanism of action including enhancement of the release of acetylcholine in addition to a 5-HT6 antagonism. PF-01913539 from Pfizer and Russian Academy of Sciences, under registration process in both United States and Europe for dementia and Alzheimer's disease, has the same 5-HT6 antagonist activity associated to an *N*-methyl-D-aspartate (NMDA) antagonist effect. A Phase II trial is also reported for Huntington disease. This same product has also been previously approved only for Russia in 1983 for treating allergy or rhinitis.

GlaxoSmithKline is performing a Phase I study for a diagnostic compound radiolabeled [11C]-GSK-215083 in the field of dementia and Alzheimer's.

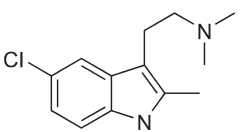
All these data are reported in Table I.

V. Conclusion

The great interest of the scientific community for the pharmacology of the 5-HT6 receptors is also reflected in the important number of patent literature published, meaning most of the important companies have considered this target to possibly have a great clinical and commercial value. New compounds, methods of synthesis, and therapeutical indications are objects of claims published in patent applications as well as in granted patents. Despite all promising results covered and published in patents, to date only one compound has reached the registration phase for entering the market, and 16 other molecules are under active preclinical or clinical development. This reflects the complexity of the target as highlighted in the numerous scientific publications dealing with the potential application of 5-HT6 receptors and their corresponding ligands.

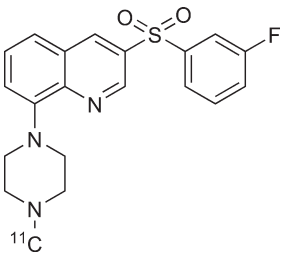
Tables II–V show as reference information the 271 families retrieved initially grouped from the subject matter point of view above described. It has to be pointed out that the tables do not show the complete family of patents.

Table I
Compounds under Active Preclinical or Clinical Development

Drug name Chemical name and structure	Mechanism Condition	Originator	Status
SUVN-504 Unknown	5-HT6 antagonist Obesity	Suven Life Sciences	Preclinical
SUVN-501 Unknown	5-HT6 antagonist; acetylcholine release enhancer Schizophrenia, dementia, Alzheimer's	Suven Life Sciences	Preclinical
SUVN-507 Unknown	5-HT6 antagonist Cognitive disorders	Suven Life Sciences	Preclinical
ST-1936  2-(5-Chloro-2-methyl-1*H*-indol-3-yl)-*N*,*N*-dimethylethylamine; 5-chloro-2-methyl-*N*,*N*-dimethyltryptamine	5-HT6 agonist Depression	Sigma-Tau	Preclinical
AVN-492 Unknown	5-HT6 agonist Cognitive disorders	Avineuro	Preclinical
AVN-457 Unknown	5-HT6 agonist Cognitive disorders	Avineuro	Preclinical
AVN-458 Unknown	5-HT6 antagonist Cognitive disorders	Avineuro	Preclinical
SUVN-502 Unknown	5-HT6 antagonist Schizophrenia, dementia, Alzheimer's	Suven Life Sciences	Phase I

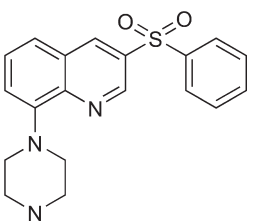
(*continued*)

[11*C*]-GSK-215083	5-HT6 antagonists Diagnostics: dementia, Alzheimer's	GlaxoSmithKline	Phase I



3-(3-Fluorophenylsulfonyl)-8-(4[11*C*]-methylpiperazin-1-yl)quinoline

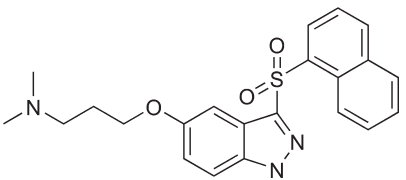
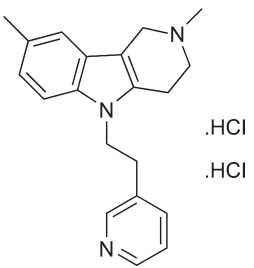
SYN-114 Unknown	5-HT6 antagonist Cognitive disorders	Roche Synosia Therapeutics	Phase I
AVN-322 Unknown	5-HT6 antagonist Anxiety, dementia, Alzheimer's	Avineuro	Phase I
SYN-120 Unknown	5-HT6 antagonist Cognitive disorders	Roche Synosia Therapeutics	Phase I
Lu-AE-58054; SGS-518 Unknown	5-HT6 antagonist Cognitive disorders, schizophrenia, dementia, Alzheimer's	Lilly; Lundbeck	Phase II
SB-742457; GSK-742457 Unknown	5-HT6 antagonist Schizophrenia, dementia, Alzheimer's	GlaxoSmithKline	Phase II



-(Phenylsulfonyl)-8-(1-piperazinyl) quinoline

(*continued*)

Table I (*continued*)

Drug name Chemical name and structure	Mechanism Condition	Originator	Status
SAM-531; PF-5212365; WAY-262531  *N,N*-Dimethyl-3-[3-(naphthalen-1-ylsulfonyl)-1*H*-indazol-5-yloxy]propan-1-amine	5-HT6 antagonist Schizophrenia, dementia, Alzheimer's	Pfizer	Phase II
AVN-211Unknown	5-HT6 antagonist Cognitive disorders, schizophrenia; dementia, Alzheimer's	Avineuro	Phase II
PF-01913539 Latrepirdine hydrochloride; Dimebolin hydrochloride; Dimebon  2,8-Dimethyl-5-[2-(6-methylpyridin-3-yl)	5-HT6 antagonist; NMDA antagonist Dementia, Alzheimer's type, Huntington's disease, rhinitis, allergy	Russian Academy of Sciences Pfizer	Phase III Launched (RU)

(*continued*)

Table II
Patent Applications Published Covering New Compounds

Title	Applicant	Patent Number	Publication Date
2-Aminopyrimidine compounds as serotonin receptor modulators	Janssen Pharmaceutica NV	WO 2010053825	May 14, 2010
Azepinof[4,5-b]indoles and methods of use	Medivation Technologies, Inc.	WO 2010051503	May 6, 2010
Aryl sulfonamide amine compounds and their use as 5-HT6 ligands	Suven Life Sciences Ltd.	WO 2010032258	March 25, 2010
Aryl indolyl sulfonamide compounds and their use as 5-HT6 ligands	Suven Life Sciences Ltd.	WO 2010032257	March 25, 2010
4'-Amino cyclic compounds having 5-HT6 receptor affinity	Memory Pharmaceuticals Corp.	WO 2010024980	March 4, 2010
Condensed heterocyclic compounds having 5-HT6 receptor affinity	Memory Pharmaceuticals Corp.	WO 2010021797	February 25, 2010
Piperazin-1-yl-trifluoromethyl-substituted-pyridines as fast dissociating dopamine 2 receptor antagonists	Janssen Pharmaceutica NV	WO 2010012758	February 4, 2010
5-HT6 receptor and modulators thereof for the treatment of insulin-related disorders	Arena Pharmaceuticals, Inc.	WO 2010011305	January 28, 2010
Pyrrolidine-substituted azaindole compounds having 5-HT6 receptor affinity	Memory Pharmaceuticals Corp.	WO 2010002802	January 7, 2010
Substituted 6-(1-piperazinyl)-pyridazines as 5-HT6 receptor antagonists	Janssen Pharmaceutica NV	WO 2010000456	January 7, 2010
1-Substituted-3-(naphthalen-1 -ylsulfonyl)-5-(piperazin-1 -yl)-1 H indazole compounds as 5-hydroxytryptamine-6 ligands	Wyeth	WO 2009155399	December 23, 2009
Tetrahydro-pyrazolo[1,5-a]pyrido-pyrimidines as antagonists of serotonin 5-HT6 receptors, methods for the production and use thereof	Alla Chem, LLC	WO 2009136814	Novembers.12, 2009
2-Amino-3-sulphonyl-tetrahydro-pyrazolo[1,5-a]pyrido-pyrimidi antagonists of serotonin 5-HT6 receptors, methods for the production and use thereof	Alla Chem, LLC	WO 2009136813	November 12, 2009

(*continued*)

Table II (*continued*)

Title	Applicant	Patent Number	Publication Date
Substituted N-phenyl-2,3-dihydroimidazo[2,1-b]thiazole-5-sulfonamide derivatives as 5-HT6 ligands	Laboratories del Dr. Esteve, SA	WO 2009135927	November 12, 2009
Substituted N-imidazo[2,1-b]thiazole-5-sulfonamide derivatives as 5-HT6 ligands	Laboratories del Dr. Esteve, SA	WO 2009135925	Nowmber 12, 2009
Pyrido[3,4-b]indoles and methods of use	Medivation Technologies, Inc.	WO 2009120717	October 1, 2009
Arylsulfonyl pyrazoline carboxamidine derivatives as 5-HT6 antagonists	Solvay Pharmaceuticals BV	WO 2009115515	September 24, 2009
Pyridinyl amides for the treatment of CNS and metabolic disorders	Pfizer Inc.	WO 2009098576	August 13, 2009
New 2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole compounds and methods of use thereof	Medivation Technologies, Inc.	WO 2009094668	July 30, 2009
Substituted 3-sulphonyl-[1,2,3]triazolo[1,5-a]pyrimidines-antagonists of serotonin 5-HT6 receptors and methods for the production thereof	Alla Chem, LLC	WO 2009093934	July 30, 2009
Substituted cycloalcano[e and d]pyrazolo[1,5-a]pyrimidines/ antagonists of serotonin 5-HT6 receptors and methods for production and the use thereof	Alla Chem, LLC	WO 2009093210	July 30, 2009
2-Alkylamino-3-arylsulfonyl-cycloalcano[e or d]pyrazolo[1,5-a] pyrimidines/antagonists of serotonin 5-HT6 receptors, methods for the production and the use thereof	Alla Chem, LLC is	WO 2009093209	July 30, 2009
Substituted 2-amino-3-sulfonyl-pyrazolo[1,5-a]pyrimidines/ antagonists of serotonin 5-HT6 receptors, methods for the production and the use thereof	Alla Chem, LLC	WO 2009093208	July 30, 2009
3-Sulfonyl-pyrazolo(1,5-a]pyrimidines/antagonists of serotonin HT6 receptors, methods for the production and the use thereof	Alla Chem, LLC	WO 2009093206	July 30, 2009
Tryptamine sulfonamides as 5-HT6 antagonists	Merck & Co., Inc.	WO 2009073118	June 11, 2009

(*continued*)

Benzenesulfonanilide compounds suitable for treating disorder; that respond to modulation of the serotonin 5-HT6 receptor	Abbott GmbH & Co. KG	WO 2009056632	May 7, 2009
Amino arylsulfonamide compounds and their use as 5-HT6	Suven Life Sciences Ltd.	WO 2009053997	Apri 30, 2009
Sulfonyl-3-heterocyclylindazde derivatives as 5-hydroxytryptamine-6 ligands	Wyeth	US 2009105303	Aprl 23, 2009
Fluoro-containing derivatives of hydrogented pyrido[4,3-b] indoles with neuroprotective and cognition enhancing properties, process for preparinq, and 'use	D2E, LLC	WO 2009038764	March 26. 2009
Naphthyl-substituted sulfonamides	Laboratories del Dr. Esteve, SA	WO 2009036955	March 26, 2009
Substituted indolyl compounds and their use as 5-HT6 ligands	Suven Life Sciences Ltd.	WO 2009034581	March 19, 2009
3' Substituted compounds having 5-HT6 receptor affinity	Memory Pharmaceuticals Corp.	WO 2009023844	February 19, 2009
Quinoline compounds suitable for treating disorders that respond to modulation of the serotonin 5-HT6 receptor	Abbott GmbH & Co. KG	WO 2009019286	February 12, 2009
Novel compounds	GlaxoSmithKline plc	WO 2009016227	February 5, 2009
1-Piperidinyl-6-piperidinylsulfonylindoles as 5-HT (2B) receptor antagonists	GlaxoSmithKline plc	WO 2009016225	February 5, 2009
Substituted tetrahydro-quinoline-sulfonamide compounds, their preparation and use as medicaments	Laboratories del Dr. Esteve, SA	EP 2016943	January 21, 2009
4' Substituted compounds having 5-HT6 receptor affinity	Memory Pharmaceuticals Corp.	WO 2008147812	December 4,2008
Aminoazacyclyl-3-sulfonylindazoles as 5-hydroxytryptamine-6 liqands	Wyeth	WO 2008144299	November 27, 2008
Aminoalkoxy aryl sulfonamide compounds and their use as 5-HT6 liqands	Suven Life Sciences Ltd.	WO 2008136017	November 13, 2008
Azetidin compounds suitable for treating disorders that respond to modulation of the serotonin 5-HT6 receptor	Abbott GmbH & Co. KG	WO 2008116833	October 2, 2008
Quinoline compounds suitable for treating disorders that responds to modulation of the serotonin 5-HT6 receptor	Abbott GmbH & Co. KG	WO 2008116831	October 2, 2008

(*continued*)

Table II (*continued*)

Title	Applicant	Patent Number	Publication Date
Tricyclic isoquinoline derivatives for treatment of obesity	Swedish Orphan Biovitrum AB (publ)	WO 2008110598	September 18, 2008
6' Substituted compounds having 5-HT6 receptor affinity	Memory Pharmaceuticals Corp.	WO 2008101247	August 21,2008
Fast dissociating dopamine 2 receptor antagonists	Janssen Pharmaceutica NV	WO 2008098892	August 21,2008
Substituted indole sulfonamide compounds, their preparation and use as medicaments	Laboratories del Dr. Esteve, SA	EP 1947085	July 23, 2008
5-(Heterocyclyl)alkyl-N-(arylsulfonyl)indole compounds and their use as 5-HT6 ligands	Suven Life Sciences Ltd.	WO 2008084492	July 17, 2008
4-(Heterocyclyl)alkyl-N-(arylsulfonyl)indcle compounds and their use as 5-HT6 ligands	Suven Life Sciences Ltd.	WO 2008084491	July 17, 2008
Arylsulfonyl pyrrolidines as 5-HT6 inhibitors	F. Hoffmann-La Roche AG	WO 2008055847	May 15, 2008
Indole and benzofuran 2-carboxamide derivatives	F. Hoffmann-La Roche AG	WO 2008055808	May 15,.2008
8-Sulfonyl-1,3 4,8-tetrahydro-2H-[1,4]oxazepino[6,7-e]indole derivatives and their use as 5-HT6 receptor ligands	Swedish Orphan Biovitrum AB (publ)	WO 2008054288	May 8, 2008
Sulfonylpyrazoline carboxamidine derivatives as 5-HT6 antagonists	Solvay Pharmaceuticals BV	WO 2008034863	March 27, 2008
Novel substituted-1H-quinazoline-2,4-dione derivatives, preparation method thereof and pharmaceutical composition containing the same	Korea Research Institute of Chemical Technology	WO 2008004716	January 10, 2008
Indoles as 5-HT6 modulators	Swedish Orphan Biovitrum AB (publ)	WO 2008003703	January 10, 2008
Tetralin and indane derivatives and uses thereof	F. Hoffmann-La Roche AG	WO 2007147771	December 27, 2007
Arylsulfonamidyl tetralin derivatives and uses thereof	F. Hoffmann-La Roche AG	WO 2007147762	December 27, 2007
1-Sulfonylindazolylamine and -amide derivatives as 5-hydroxytryptamine-6 ligands	Wyeth	WO 2007142905	December 13, 2007
Benzoxazole and benzothiazole derivatives as 5-hydroxytrytamine-6 ligands	Wyeth	WO 2007142904	December 13, 2007

(*continued*)

3-(Heterocyclyl)-N-(arylsulfonyl)indole derivatives as functional 5-HT6 ligands	Suven Life Sciences Ltd.	WO 2007138611	December 6, 2007
Heterocyclic compounds suitable for treating disorders that respond to modulation of the serotonin 5HT6 receptor	Abbott GmbH & Co. KG	WO 2007118900	October 25, 2007
Heterocyclic arylsulphones suitable for treating disorders that respond to modulation of the serotonin 5HT6 receptor	Abbott GmbH & Co. KG	WO 2007118899	October 25, 2007
Sulfonyl-3-heterocyclylindazole derivatives as 5-hydroxytryptamine-6 ligands	Wyeth	WO 2007117413	October 18, 2007
Novel quinazolines as 5-HT6 modulators	AstraZeneca AB	WO 2007108744	September 27, 2007
Novel quinazolines as 5-HT6 modulators II	AstraZeneca AB	WO 2007108743	September 27, 2007
Novel tetralins as 5-HT6 modulators	AstraZeneca AB	WO 2007108742	September 27,2007
Novel substituted-1,1-dioxo-benzo[1,2,4]thiadizin-3-ones, preparation method thereof, and pharmaceutical composition containing the same	Korea Research Institute of Chemical Technology	WO 2007108569	September 27, 2007
Compounds having 5-HT6 receptor affinity	Memory Pharmaceuticals Corp.	WO 2007098418	August 30, 2007
Sulfonyl substituted 1H-indoles as ligands for the 5-hydroxytryptamine receptors	Wyeth	WO 2007084841	July 26, 2007
Indene derivatives, their preparation and use as medicaments	Laboratories del Dr. Esteve, SA	WO 2007054257	May 18, 2007
Pyrroloquinolinone derivatives as 5-hydroxytryptamine-6 ligands	Wyeth	WO 2007053352	May 10, 2007
Haloperidol analogs	Florida Agricultural and Mechanical University (FAMU)	WO 2007053145	May 10, 2007
Arylthioether tryptamine derivatives as functional 5-HT6 ligands	Suven Life Sciences Ltd.	WO 2007046112	April 26, 2007
Carbazole derivatives as functional 5-HT6 ligands	Suven Life Sciences Ltd.	WO 2007046111	April 26, 2007
Quinoline compounds capable of binding the CB2 and/or the 5-HT6 receptor	GlaxoSmithKline plc	WO 2007039219	April 12, 2007
N-Substituted-1H-quinoline-2,4-diones, preparation method thereof, and pharmaceutical composition containing the same	Korea Research Institute of Chemical Technology	WO 2007032572	March 22, 2007

(*continued*)

Table II (*continued*)

Title	Applicant	Patent Number	Publication Date
Tetrahydro-beta-carbolin-sulfonamide derivatives as 5-HT6 ligands	Laboratories del Dr. Esteve, SA	WO 2007028460	March 15, 2007
Aryloxy quinolines and uses thereof	F. Hoffmann-La Roche AG	WO 2007025798	March 8, 2007
Substituted-3-sulfonylindazole derivatives as 5-hydroxytryptamine-6 ligands	Wyeth	WO 20070217 11	February 22, 2007
Thioether derivatives as functional 5-HT6 ligands	Suven Life Sciences Ltd.	WO 2007020653	February 22, 2007
Aminoaryl sulphonamide derivatives as functional 5-HT6 ligands	Suven Life Sciences Ltd.	WO 2007020652	February 22, 2007
Benzimidazole derivatives as 5-HT6, 5-HT2A	F. Hoffmann-La Roche AG	WO 2007006677	January 18, 2007
New compounds, process for their preparation, intermediates, pharmaceutical compositions and their use in the treatment of 5-HT6 mediated disorders such as Alzheimer's disease, cognitive disorders, cognitive impairment associated with schizophrenia, obesity, and Parkinson's disease	AstraZeneca AB	WO 2007004960	January 11, 2007
New compounds, process for their preparation, intermediates, pharmaceutical compositions and their use in the treatment of 5-HT6 mediated disorders such as Alzheimer's disease, cognitive disorders, cognitive impairment associated with schizophrenia, obesity, and Parkinson's disease	AstraZeneca AB	WO 2007004959	January 11, 2007
Benzofuranyl derivatives as 5-HT6-receptor inhibitors	Swedish Orphan Biovitrum AB (publ)	WO 2006134150	December 21, 2006
Novel 8-sulfonylamino-3 aminosubstituted chroman or tetrahydronaphtalene derivatives modulating the 5-HT6 receptor	AstraZeneca AB	WO 2006126939	November 30, 2006
Novel 8-sulfonyl-3 aminosubstituted chroman or tetrahydronaphtalene derivatives modulating the 5-HT6 receptor	AstraZeneca AB	WO 2006126938	November 30, 2006

(*continued*)

Multimediator 5-HT6 receptor antagonists, and uses related thereto	Novasite Pharmaceuticals, Inc.	WO 2006091703	August 31,2006
Substituted arylamine compounds and their use as 5-HT6 modulators	Epix Pharmaceuticals, Inc.	WO 2006081332	August 3, 2006
Substituted indazolyl sulfonamide and 2,3-dihydro-indolyl sulfonamide compounds, their prepartion and use in medicaments	Laboratorios del Dr. Esteve, SA	WO 2006069809	July 6, 2006
Nitro-substituted phenyl-piperazine compounds, their preparation and use in medicaments	Laboratorios del Dr. Esteve, SA	WO 2006069808	July 6, 2006
Substituted phenyl-piperazine compounds, their preparation and use in medicaments	Laboratories del Dr. Esteve, SA	WO 2006069807	July 6, 2006
Tetralin and indane derivatives and uses thereof	F. Hoffmann-La Roche AG	WO 2006066790	June 29, 2006
Chroman derivatives and uses thereof in the treatment of CNS disorders	F. Hoffmann-La Roche AG	WO 2006066756	June 29, 2006
Tetralin and indane derivatives and uses thereof as 5-HT antagonists	F. Hoffmann-La Roche AG	WO 2006066748	June 29, 2006
Chroman derivatives and their use as 5-HT receptor ligands	F. Hoffmann-La Roche AG	WO 2006066746	June 29, 2006
Tetralin and indane derivatives and uses thereof	F. Hoffmann-La Roche AG	WO 2006066745	June 29, 2006
New benzofuran derivatives and their use in the treatment of obesity, type II diabetes and CNS disorders	Swedish Orphan Biovitrum AB (publ)	WO 2006062481	June 15, 2006
Dibenzoxazepinone derivatives	F. Hoffmann-La Roche AG	WO 2006061126	June 15, 2006
1-Aryl- or 1-alkylsulfonyl-heterocyclylbenzazoles as 5-hydroxytryptamine-6 ligands	Wyeth	US 2006116384	June 1, 2006
Radiolabelled quinoline-based ligands for the 5-HT6 receptor functionality	GlaxoSmithKline plc	WO 2006053785	May 26, 2006
3-Aryl-3-methyl-quinoline-2,4-diones, preparation method thereof and pharmaceutical composition containing same	Korea Research Institute of Chemical Technology	EP 1650190	April 26, 2006
5-Sulfonyl-1-piperidinyl substituted indole derivatives as 5-HT6 receptor antagonists for the treatment of CNS disorders	GlaxoSmithKline plc	WO 2006038006	April 13, 2006
Benzoxazine and quinoxaline derivatives and uses	F. Hoffmann-La Roche AG	WO 2006037481	April 13,2006

(*continued*)

Table II (*continued*)

Title	Applicant	Patent Number	Publication Date
Substituted indole compounds and their use as 5-HT6 receptor modulators	Laboratorios del Dr. Esteve, SA	WO 2006024535	March 9, 2006
Substituted indole compounds, their preparation and use in medicaments	Laboratories del Dr. Esteve, SA	WO 2006015867	February 16, 2006
Indolylalkylamine metabolites as 5-hydroxytrytamine-6 ligands	Wyeth	WO 2006002125	January 5, 2006
3-Arylsulfonyl-quinolines as 5HT6 receptor antagonists for the treatment of CNS disorders	GlaxoSmithKline plc	WO 2005113539	December 1, 2005
Arylsulfonyl benzodioxanes useful for modulation the 5-HT6 receptor, the 5-HT2A receptor or both	F. Hoffmann-La Roche AG	WO 2005105776	November 10, 2005
3-[(Hetero)arylsulfonyl]-8-[(aminoalkyl)oxy]quinolines as 5-HT6 receptor antagonists for the treatment of CNS disorders	GlaxoSmithKline plc	WO 2005095346	October 13, 2005
1-Benzyl-5-piperazin-1-yl-3,4-dihydro-1H-quinazolin-2-one derivatives and the respective 1H-benzo(1,2,6)thiadiazine-2,2-dioxide and 1,4-dihydro-benzo(d)(1,3)oxazin-2-one derivatives as modulators of the 5-hydroxytryptamine receptor (5-HT) for the treatment of diseases of the central nervous system	F. Hoffmann-La Roche AG	WO 2005067933	July 28, 2005
Novel indeno[2,1a]indenes and isoindol[2,1-a]indoles	Suven Life Sciences Ltd.	WO 2005066184	July 21, 2005
3-(Pyrolidin-3-yl) indoles as 5-HT6 receptor modulators	Suven Life Sciences Ltd.	WO 2005066157	July 21, 2005
Novel benzofuran derivatives, which can be used in prophylaxis or treatment of 5-HT6 receptor-related disorder	Swedish Orphan Biovitrum AB (publ)	WO 2005058858	June 30, 2005
Benzoxazine derivatives and uses thereof	F. Hoffmann-La Roche AG	WO 2005058847	June 30, 2005
7-Phenylsulfonyl-tetrahydro-3-benzazepine derivatives as antipsychotic agents	GlaxoSmithKline plc	WO 2005051397	June 9, 2005
Sulfonyltetrahydro-3H-benzo(e)indole-8-amine compounds as 5-hydroxytryptamine-6 ligands	Wyeth	WO 2005047252	May 26, 2005
A polymorphic form of 3-phenylsulfonyl-8-piperazin-1-yl-quinoline	GlaxoSmithKline plc	WO 2005040124	May 6, 2005

(*continued*)

Novel tetrahydrospiro(piperidine-2,7'-pyrrolo[3,2-b]pyridine) derivatives and novel indole derivatives useful in the treatment of 5-HT6 receptor-related disorders	Swedish Orphan Biovitrum AB (publ)	WO 2005037834	April 28, 2005
Piperidinylchromen-6-ylsulfonamide compounds as 5-hydroxytryptamine-6 ligands	Wyeth	WO 2005037830	April 28, 2005
Piperazinyl-quinoline derivatives useful for the treatment of CNS disorders	GlaxoSmithKline plc	WO 2005030724	April 7, 2005
Substituted piperazines of azepines, oxazepines, and thiazepines	Eli Lilly and Company	WO 2005026177	March 24, 2005
Quinoline compounds and pharmaceutical compositions containing them	GlaxoSmithKline plc	WO 2005026125	March 24, 2005
7-Heteroarylsulfonyl-tetrahydro-3-benzazepine derivatives as antipsychotic agents	GlaxoSmithKline plc	WO 2005025576	March 24, 2005
8-(1-Piperazinyl)-quinoline derivatives and their use in the treatment of CNS disorders	GlaxoSmithKline plc	WO 2005021530	March 10, 2005
7-Phenylsulfonyl-tetrahydro-3-benzazepine derivatives as antipsychotic agents	GlaxoSmithKline plc	WO 2005016891	February 24, 2005
Benzoxazinone-derived sulfonamide compounds, their preparation and use as medicaments	Laboratories del Dr. Esteve, SA	WO 2005014589	February 17, 2005
Phenylsulfonyl compounds as antipsychotic agents	GlaxoSmithKline plc	WO 2005014578	February 17, 2005
Indol-4 sulfonamide derivatives, their preparation and their use 5-HT-6 as modulators	Laboratorios del Dr. Esteve, SA	WO 2005013978	February 17, 2005
lndol-5-yl sulfonamide derivatives, their preparation and their use 5-HT-6 as modulators	Laboratorios del Dr. Esteve, SA	WO 2005013977	February 17, 2005
lndol-6 sulfonamide derivatives, their preparation and their use 5-HT-6 as modulators	Laboratorios del Dr. Esteve, SA	WO 2005013976	February 17, 2005
1-Sulfonylindole derivatives, their preparation and their use as 5-HT6 ligands	Laboratorios del Dr. Esteve, SA	WO 2005013974	February 17, 2005
N-Sulfonylheterocyclopyrrolylalkylamine compounds as 5-hydroxytryptamine-6 ligands	Wyeth	WO 2005012311	February 10, 2005
Sulfbnyldihydroimidazopyridinone compounds as 5-hydroxytryptamine-6 ligands	Wyeth	WO 2005010003	February 3, 2005
Sulfonyldihydrobenzimidazolone compounds as 5-hydroxytryptamine-6 ligands	Wyeth	WO 2005009996	February 3, 2005

(*continued*)

Table II (*continued*)

Title	Applicant	Patent Number	Publication Date
Quinolinone/benzoxazinone derivatives and uses thereof	F. Hoffmann-La Roche AG	WO 2004080969	September 23, 2004
2,5- and 2,6-substituted tetrahydroisoquinolines for use as 5-HT6 modulators	F. Hoffmann-La Roche AG	WO 2004078176	September 16, 2004
Heterocyclyl-3-sulfonylazaindole or-azaindazole derivatives as 5 hydroxytryptamine-6 ligands	Wyeth	WO 2004074286	September 2, 2004
Heterocyclyl-3-sulfonylindazoles as 5-hydroxytryptamine-6 ligands	Wyeth	WO 2004074243	September 2, 2004
Aminoalkoxyindoles as 5-HT6-receptor ligands for the treatment of CNS-disorders	F. Hoffmann-La Roche AG	WO 2004050085	June 17, 2004
N-Arylsulfonyl-3-substituted indoles having serotonin receptor affinity, process for their preparation and pharmaceutical composition containing them	Suven Life Sciences Ltd.	WO 2004048330	June 10, 2004
N-Arylsulfonyl-3-aminoalkoxyindoles	Suven Life Sciences Ltd.	WO 2004048328	June 10, 2004
Substituted benzoxazinones and uses thereof	Roche Palo Alto LLC	WO 2004041792	May 21, 2004
Preparation of 3-aminoalkyl-substituted indole derivatives from phenylhydrazines and aminoketones	Suven Life Sciences Ltd.	WO 2004041781	May 21, 2004
4-Piperazinyl benzenes ulfonyl indoles with 5-HT6 receptor affinity	F. Hoffmann-La Roche AG	WO 2004035047	April 29, 2004
2.4-Substituted indoles and their use as 5-HT6 modulators	F. Hoffmann-La Roche AG	WO 2004026831	April 1, 2004
2,7-Substituted indoles	Roche Palo Alto LLC	WO 2004026830	April 1, 2004
Piperazine substituted aryl benzodiazepines	Eli Lilly and Company	WO 2004014895	February 19, 2004
Pyridopyrimidine derivatives as 5-HT6 antagonists	Bristol-Myers Squibb Co.	US 2004019064	January 29, 2004
1 -Heterocyclylalkyl-3-sulfonylazaindole or -azaindazole derivatives as 5-hydroxytryptamine-6 ligands	Wyeth	WO 2004009600	January 29, 2004
1-Heterocyclylalkyl-3-sulfonylindole or-indazole derivatives as 5-hydroxytryptamine-6 ligands	Wyeth	WO 2004009548	January 29, 2004
Indolylalkylidenehydrazine-carboximidamide derivatives as 5-hydroxytryptamine-6 ligands	Wyeth	US 2004002527	January 1, 2004

(*continued*)

New compounds useful for the treatment of obesity, type II diabetes and CNS disorders	Swedish Orphan Biovitrum AB (publ)	WO 2004000828	December 31, 2003
1-Sulfonyl-4-aminoalkoxy indole derivatives and uses thereof	Roche Palo Alto LLC	WO 2003104193	December 18, 2003
(1-Substituted-indol-3-yl)alkylidenehydrazinecarboximidamide derivatives as 5-hydroxytryptamine-6 ligands	Wyeth	US 2003232843	December 18, 2003
1-(Aminoalkyl)-3-sulfonylazaindoles as 5-hydroxytryptamine-6 ligands	Wyeth	WO 2003101990	December 11, 2003
1-(Aminoalkyl)-3-sulfonylindole and -indazole derivatives as 5-hydroxytryptamine-6 ligands	Wyeth	WO 2003101962	December 11, 2003
Salts of bis-arylsulfones for the treatment of CNS disorders	Pfizer Inc.	WO 2003099797	December 4, 2003
Benzoxazine derivatives as 5-HT6 modulators and uses thereof	F. Hoffmann-La Roche AG	WO 2003095434	November 20, 2003
Piperazine substituted aryl benzodiazepines and their use as dopamine receptor antagonists for the treatment of psychotic disorders	Eli Lilly and Company	WO 2003082877	October 9, 2003
Novel compounds	GlaxoSmithKline plc	WO 2003080608	October 2, 2003
Novel compounds	GlaxoSmithKline plc	WO 2003080580	October 2, 2003
Arylsulfone derivatives	Pfizer Inc.	WO 2003072558	September 4, 2003
Pyridyl sulfone derivatives as 5-HT receptor antagonists	Pfizer Inc.	WO 2003072548	September 4, 2003
Benzenesulfonamide derivatives as antipsychotic agents	GlaxoSmithKline plc	WO 2003068752	August 21, 2003
7-Arylsulfonamido-2,3,4,5-tetrahydro-1H-benzo[d]azepine derivatives with 5-HT6 receptor affinity for the treatment of CNS disorders	GlaxoSmithKline plc	WO 2003068751	August 21, 2003
Pyrrolylalkylidene-hydrazinecarboximidamide derivatives as 5-HT6	Wyeth	WO 2003068740	August 21, 2003
1-Arylsulfonyl-3-substituted indole and indoline derivatives useful in the treatment of central nervous system disorders	Akzo Nobel NV	WO 2003068220	August 21, 2003
Sulphonyl compounds with 5-HT6 receptor activity	GlaxoSmithKline plc	WO 2003066632	August 14, 2003
7-Sulfonyl-3-benzazepine derivatives as modulators of the dopamine receptor and their use for the treatment of CNS disorders	GlaxoSmithKline plc	WO 2003062205	July 31, 2003

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Table II (*continued*)

Title	Applicant	Patent Number	Publication Date
Azaindolylalkylamine derivatives as 5-hydroxytryptamine-6 ligands	Wyeth	WO 2003053970	July 3, 2003
Indolylalkylamine derivatives as 5-hydroxytryptamine-6 ligands	Wyeth	WO 2003053433	July 3, 2003
Sulphonamides derivatives, the preparation thereof and application of same as medicaments	Laboratories del Dr. Esteve, SA	WO 2003042175	May 22, 2003
Arylsulphonyl-substituted tetrahydro- and hexahydro-carbazoles as 5-HT-6 receptor ligands	Pfizer Inc.	WO 2003030901	April 17, 2003
Chroman and benzofuran derivatives as 5-HT6 ligands	Wyeth	WO 2003029239	April 10, 2003
Chroman derivatives as 5-HT6 ligands	Wyeth	WO 2003029238	April 10, 2003
Optical isomers of an iloperidone metabolite	Novartis AG	WO 2003020707	March 13, 2003
Arylsulfonyl derivatives with 5-HT6 receptor affinity	F. Hoffmann-La Roche AG	WO 2003014097	February 20, 2003
3-Arylsulfonyl-7-piperazinyl-indoles, -benzofurans and -benzothiophenes with 5-HT6 receptor affinity for treating CNS disorders	GlaxoSmithKline plc	WO 2003013510	February 20, 2003
5-Arylsulfonyl indoles having 5-HT6 receptor affinity	Pfizer Inc.	WO 2003011284	February 13, 2003
5-Halo-tryptamine derivatives used as ligands of the 5-HT6 and/or 5-HT7 serotonin receptors	Sigma-Tau Industrie Farmaceutiche Riunite SpA	WO 2003000252	January 3, 2003
4-Piperazinylindole derivatives with 5-HT6 receptor affinity	F. Hoffmann-La Roche AG	WO 2002102774	December 27, 2002
Sulfonyloxazolamines and their use as 5-HT6 ligands	Merck Patent GmbH	WO 2002100842	December 19, 2002
Substituted sulfonamide compounds, process for their use as medicament for the treatment of CNS disorders, obesity, and type II diabetes	Swedish Orphan Biovitrum AB (publ)	WO 2002100822	December 19, 2002
New indole derivatives with 5-HT6 receptor affinity	F. Hoffmann-La Roche AG	WO 2002098857	December 12, 2002
Novel, arylsulfonamide compounds for the treatment of obesity, type II diabetes and CNS-disorders	Swedish Orphan Biovitrum AB (publ)	WO 2002092585	November 21, 2002
Benzo[d]azepine derivatives as 5-HT6 receptor antagonists	GlaxoSmithKline Plc	WO 2002089811	November 14, 2002

(*continued*)

Heterocyclyloxy-, thioxy-, and -aminobenzazole derivatives as 5-HT6 ligands	Wyeth	WO 2002085892	October 31, 2002
Heterocyclylalkoxy-, alkylthio- and -alkylaminobenzazole derivatives as 5-HT6 ligands	Wyeth	WO 2002085853	October 31, 2002
Thiazole and other heterocyclic ligands and use thereof	Sepracor, Inc.	WO 2002083863	October 24, 2002
N-(2-Arylethyl)benzylamines as antagonists of the 5-HT6 receptor	Eli Lilly and Company	WO 2002078693	October 10, 2002
Substituted indolines as 5-HT receptor ligands	Pfizer Inc.	WO 2002060903	Augusts 8, 2002
Benzenesulfonic acid indol-5-yl esters as antagonists of the 5-HT6 receptor	Eli Lilly and Company	WO 2002060871	August 8, 2002
1-Aryl- or 1-alkylsulfonylbenzazole derivatives as 5-hydroxytryptamine-6 ligands	Wyeth	WO 2002059088	August 1, 2002
Heterocyclindazole and azaindazole compounds as 5-hydroxytryptamine-6 ligands	Wyeth	WO 2002051837	July 4, 2002
Heterocyclylalkylindole or -azaindole compounds as 5-hydroxytryptamine-6 ligands	Wyeth	WO 2002051832	July 4, 2002
Isoquinoline derivatives useful in the treatment of CNS disorders	GlaxoSmithKline plc	WO 2002042293	May 30, 2002
Compounds useful in the treatment of CNS disorders	GlaxoSmithKline plc	WO 2002041889	May 30, 2002
1-Aryl- or 1-alkylsulfonyl-heterocyclylbenzazoles as 5-hydroxytryptamine-6 ligands	Wyeth	WO 2002036562	May 10, 2002
Sulphonamides for the treatment of central nervous system diseases	Bayer Healthcare AG	WO 2002036115	May 10, 2002
2-, 3-, 4-, or 5-substituted-N1-(benzensulfonyl)indoles and their use in therapy	Swedish Orphan Biovitrum AB (publ)	WO 2002032863	April 25. 2002
Tricyclic indole compounds having affinity for serotonin receptor	Shionogi & Co. Ltd.	WO 2002024641	March 28, 2002
N-(3, 5-Dichloro-2-methoxyphenyl)-4-methoxy-3-piperazin-1-yl-benzenesulfonamide	GlaxoSmithKline plc	WO 2002018358	March 7, 2002
Aryl sulfonamides as serotonin antagonist for the treatment of obesity	Swedish Orphan Biovitrum AB (publ)	WO 2002008179	January 31, 2002

(*continued*)

Table II (*continued*)

Title	Applicant	Patent Number	Publication Date
Bis-arylsulfones	Pfizer Inc.	WO 2001098279	December 27, 2001
Compounds having 5-HT6 receptor antagonist activity	NPS Allelix Corp.	WO 2001032660	May 10, 2001
Novel compounds	GlaxoSmithKline plc	WO 2001032646	May 10, 2001
Aminoalkoxy carbazoles for the treatment of CNS diseases	Pfizer Inc.	WO 2001017963	March 15, 2001
Pyrazolopyrimidines and pyrazolotriazines with 5-HT6 receptor affinity	F. Hoffmann-La Roche AG	US 6194410	February 27, 2001
Azaindoles having serotonin receptor affinity	NPS Allelix Corp.	WO 2001012629	February 22, 2001
Azaindoles having serotonin receptor affinity	NPS Allelix Corp.	US 6191141	February 20, 2001
Indole and indoline derivatives as 5-HT6 selective ligands	Merck Sharp & Dohme Ltd.	US 6187805	February 13. 2001
Oxazinocarbazoles for the treatment of CNS diseases	Pfizer Inc.	WO 2001009142	February 8, 2001
Piperidine-indole compounds having 5-HT6 affinity	NPS Allelix Corp.	WO 2000063203	October 26, 2000
Piperidine-indole compounds having 5-HT6 affinity	NPS Allelix Corp.	US 6133287	October 17, 2000
Sulphonyloxazolamines as therapeutic active ingredients	Merck Patent GmbH	WO 2000037452	June 29, 2000
Selective 5-HT6 receptor ligands	Virginia Commonwealth University	WO 2000034242	June 15, 2000
Bicyclic piperidine and piperazine compounds having 5-HT6 receptor affinity	NPS Allelix Corp.	WO 1999065906	December 23, 1999
Benzosulfone derivatives	F. Hoffmann-La Roche AG	US 5990105	November 23, 1999
Pyrrolidine-indole compounds having 5-HT6 affinity	NPS Allelix Corp.	WO 1999047516	September 23, 1999
Novel compounds	GlaxoSmithKline plc	WO 1999042465	August 26, 1999
Sulphonamides and their use	F. Hoffmann-La Roche AG	US 5939451	August 17, 1999
Compounds	GlaxoSmithKline plc	WO 1999037623	July 29, 1999
Novel compounds	GlaxoSmithKline plc	WO 1999002502	January 21, 1999
Sulphonamide derivatives, process for their preparation, and their use as medicaments	GlaxoSmithKline plc	WO 1998027081	June 25, 1998
Novel compounds	GlaxoSmithKline plc	WO 1998027058	June 25, 1998
Antihistaminic agent “Dimebon”		SU 1138164	February 7, 1985

Table III
PATENT APPLICATIONS PUBLISHED COVERING PROCESSES FOR MANUFACTURING

Title	Applicant	Patent Number	Publication Date
Synthesis of 9-(arylalkyl)-1,2,3,4-tetrahydro-gamma-carboline and analogues and intermediates	Wista Laboratories Ltd. (SG)	WO 2010067085	June 17, 2010
Process for the preparation of N-(phenylethyl) anilines salts and solvates thereof useful as serotonin 5-HT6 antagonists	Laboratories del Dr. Esteve, SA	WO 2009115393	September 24, 2009
Process for the preparation of 6-substituted imidazo[2,1-b]thiazole-5-sulfonyl halide	Laboratories del Dr. Esteve, SA	WO 2009053378	April 30, 2009
Novel chemical process for the synthesis of quinoline compounds	GlaxoSmithKline plc	WO 2007039238	April 12, 2007

Table IV
PATENT APPLICATIONS PUBLISHED COVERING THERAPEUTICAL USES

Title	Applicant	Patent Number	Publication Date
Compositions and methods for treating multiple sclerosis	Biovista, Inc.	WO 20 10045265	April 22, 2010
Compositions and methods for treating epilepsy	Biovista, Inc.	WO 20 1003 1054	March 18, 2010
Use of hydrogenated pyridol[4,3-b] indoles for the treatment of oxidative stress	Edison Pharmaceuticals, Inc.	WO 2010014758	February 4, 2010
Ligands of alpha-adrenoceptors and of dopamine, histamine, imidazoline and serotonin receptors and the use thereof	Alla Chem, LLC	WO 2009082268	July 2, 2009
Methods and compositions for treating neuronal death mediated ocular diseases	Medivation Neurology, Inc.	WO 2009039420	March 26, 2009
Methods and compositions for treating schizophrenia using antipsychotic combination theraphy	Medivation Neurology, Inc.	WO 2009017836	February 5, 2009
5HT6-ligands such as sulfonamide derivatives in drug-induced weight-gain	Laboratories del Dr. Esteve, SA	WO 2009013010	January 29, 2009
A drug demonstrating anxiolytic effect based on hydrogenated pyrido(4,3-b) indoles, its pharmacological compound and application method	Medivation Neurology, Inc.	WO 2009005771	January 8, 2009

(*continued*)

Table IV (*continued*)

Title	Applicant	Patent Number	Publication Date
Methods and compositions for stimulating cells	Medivation Neurology, Inc.	WO 2008147551	December 4, 2008
Use of quinoline derivatives in the treatment of pain and irritable bowel syndrome	GlaxoSmithKline plc	WO 2008113818	September 25, 2008
2,8-Dimethyl-5-[2-(6-methylpyridin-3-yl)-ethyl]-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole dihydrochloride (Dimebon) for the treatment of chronic pain	Grey Fox LLC	WO 2008112238	September 18, 2008
Use of 5-HT6 antagonists to prevent relapse into addiction	Solvay Pharmaceuticals BV	WO 2002027123	July 24, 2008
Means of the treatment of acute and chronic disorders of cerebral circulation, including insult, based on hydrogenated pyrido(4,3-b)indoles (variants), pharmacological means based thereon and method are the use thereof	Medivation Neurology, Inc.	WO 2008073231	June 19, 2008
Means for improving cognitive functions and memory based on hydrogenated pyrido(4,3-b)indoles (variants), pharmacological means based thereon and method are the use thereof	Medivation Neurology, Inc.	WO 2008069963	June 12, 2008
Ligands of 5-HT6 receptors, a pharmaceutical composition, method for the production and use thereof	Alla Chem, LLC	WO 2008060190	May 22, 2008
Combinations therapies for treating Alzheimer's disease using i.a.dimebon and dolepezil	Medivation Neurology, Inc.	WO 2008051599	May 2, 2008
Methods and compositions for treating amyotrophic lateral Sclerosis (ALS)	Medivation, Inc.	WO 2008036410	March 27, 2008
Methods and compositions for treating schizophrenia	Medivation, Inc.	WO 2007087425	August 2, 2007
Method for the treatment of cognitive dysfunction	Wyeth	WO 2007087151	August 2, 2007
Hydrogenated pyrido-indole compounds for the treatment of Huntington's disease	Medivation, Inc.	WO 2007041697	April 12, 2007
Compositions and methods for treating cognitive disorders	F. Hoffman-La Roche AG	WO 2006037482	April 13, 2006
Use of sulfonamide derivatives for the manufacture of a medicament for the prophylaxis and/or treatment of disorders of food ingestion	Laboratories del Dr. Esteve, SA	WO 2004098588	November 18, 2004

(*continued*)

Table IV (*continued*)

Title	Applicant	Patent Number	Publication Date
The use of a benzenesulfonamide compound in the treatment of obesity	GlaxoSmithKline plc	WO 2003072198	September 4, 2003
Method of promoting neuronal growth	GlaxoSmithKline plc	WO 2003066056	August 14, 2003
Use of sulfonamide derivatives in the treatment of obesity or for	Swedish Orphan Biovitrum AB (publ)	WO 2003039547	May 15, 2003
Use of indole and indoline derivatives in the treatment of obesity or for the reduction of food intake	Swedish Orphan Biovitrum AB (publ)	WO 2003035061	May 1, 2003
New use of napthylsulfonamides	Bayer Healthcare AG	DE 10053795	May 16, 2002
Use of tetrahydroisoquinolinyl-sulfonamides	Bayer Healthcare AG	DE 10053799	May 8, 2002
New use for amino- and amidosulfonamides	Bayer Healthcare AG	DE 10053794	May 8, 2002
Use	GlaxoSmithKline plc	WO 2000012623	March 9, 2000
Use of 5HT-6 antagonists	GlaxoSmithKline plc	WO 2000012073	March 9, 2000
Agents for treating neurodegenerative disorders	Selena Pharmaceuticals, Inc.	WO 1997015225	May 1. 1997

Table V
Patent Applications Published Covering Combinations

Title	Applicant	Patent Number	Publication Date
Combinations comprising 3-phenylsulfonyl-8-piperazinyl-1yl-quinoline	GlaxoSmithKline plc	WO 2009074607	June 18, 2009
Combination of at least two 5-HT6-ligands	Laboratories del Dr. Esteve, SA	EP 2020230	February 4, 2009
Pharmaceutical carrier composition and pharmaceutical composition	Dafra Pharma NV	WO 2008098586	August 21, 2008
Combination of a NMDA-receptor ligand and a compound with 5-HT6 receptor affinity	Laboratories del Dr. Esteve, SA	WO 2008034815	March 27, 2008

(*continued*)

Table V (*continued*)

Title	Applicant	Patent Number	Publication Date
Combinations comprising 5HT6 modulators and cholinesterase inhibitors	Epix Pharmaceuticals, Inc.	WO 2008002539	January 3, 2008
Combination of a cholinesterase inhibitor and a compound with 5-HT6 receptor affinity	Laboratories del Dr. Esteve, SA	WO 2007147883	December 27, 2007
Active substance combination comprising a compound with NPY receptor affinity and a compound with 5-HT6 receptor affinity	Laboratories del Dr. Esteve, SA	WO 2005014045	February 17, 2005
Active substance combination comprising a compound with NPY receptor affinity and a compound with 5-HT6 receptor affinity	Laboratories del Dr. Esteve, SA	WO 2005014000	February 17, 2005

Some sources where patent information can be found:

- STN International (subscription-based access)
- ThomsonPharma databases (subscription-based access)
- Thomson Reuters Integrity (subscription-based access)
- Espacenet (EPO database, free access)
- Specific Patent Offices databases (free access), for instance PAIR in USPTO, USPTO itself, JAPIO, OEPM, etc.

References

Kohen, R., Metcalf, M. A., Khan, N., Druck, T., Huebner, K., Lachowicz, J. E., Meltzer, H. Y.; Sibley, D. R., Roth, B. L., and Hamblin, M. W. (1996). Cloning, characterization and chromosomal localization of a human 5-HT_6 serotonin receptor. *J. Neurochem.* **66**(1), 47–56.

Monsma, F. J. Jr., Shen, Y., Ward, R. P., Hambun, M. W., and Sibley, D. R. (1992). Cloning and expression of a novel serotonin receptor with high affinity for tricyclic psychotropic drugs. *Molecular Pharmacol.* **43**, 320–327.

Ruat, M., Traiffort, E., Arrang, J.-M., Tardivel-Lacombe, J., Díaz, J., Leurs, R., and Schwartz, J.-C. (1993). A novel rat serotonin (5-HT_6) receptor: molecular clonning, localization and stimulation of cAMP accumulation. *Biochemical Biophysical Research Communications* **193**(1), 268–276.

5-HT6 RECEPTOR CHARACTERTIZATION

Teresa Riccioni

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I. Evidence of Existence of 5-HT6 Receptor before Receptor Cloning

5-HT6 receptors were not formally described prior to cloning in the early 1990s, but evidence of their existence was published some years earlier.

MacDermot *et al.* (1979) described two species of serotonin receptors in NCB-20 neuroblastoma-brain hybrid cells, one coupled to the activation of adenylate cyclase and the other with cell depolarization and acetylcholine release. In these cells, serotonin and lysergic acid diethylamide (LSD) activated adenylate cyclase with activation constants (K_{act}) of 530 and 12 nM, respectively, and LSD partially inhibited the stimulation of the enzyme elicited by serotonin. In NCB-20 homogenates, binding sites for [^3H]LSD with an apparent dissociation constant (K_{dapp}) of 36 nM and a receptor concentration of 385 fmol/mg of protein were also found. In competition experiments, serotonin was able to displace [^3H]LSD with a K_i of 110–180 nM. When [^3H]serotonin was used to label NCB-20 homogenates, two binding sites were detected with a K_{dapp} of 200 and 3750 nM.

In 1983, Berry-Kravis and Dawson confirmed the presence in NCB-20 cell line of both high- ($K_d = 180$ nM) and low- ($K_d > 3000$ nM) affinity binding sites for [^3H]serotonin, the latter disappearing in the presence of 1 μM spiperone.

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0074-7742/10 $35.00

The order of drug potency for inhibition of high-affinity [^{3}H]5-HT binding, as well as the order of potency for agonist stimulation of cyclic AMP (cAMP) (EC_{50} of 360 ± 150 nM for 5-HT) and 5-HT antagonist-mediated reversal of agonist-stimulated cAMP levels, were interpreted as the consequence of interaction with the 5-HT1 receptor, being at that time the only well-characterized serotonin receptor reported to activate adenylate cyclase.

Conner and Mansour (1990), working on the same NCB-20 cell line, obtained similar results on cAMP stimulation (EC_{50} of 320 ± 120 nM for 5-HT and of 27 ± 21 nM for LSD, which behaved as partial agonist). They were the first to speak of a novel serotonin receptor present at a low density in these cells. In their paper the pharmacological characteristics of the serotonin-mediated activation of adenylate cyclase were analyzed in order to compare this serotonin receptor with other subtypes of the serotonin receptor family. Correlation analysis between the potencies of different agonists and antagonists at the cyclase in these cells and their reported relative potencies for different serotonin receptor subtypes allowed to exclude the presence in NCB-20 cell line of the 5-HT1A, 5-HT1B, 5-HT1D, 5-HT2, and 5-HT3 receptors as well as of 5-HT1C and 5-HT4 receptors, pointing out the existence of a novel serotonin receptor.

More or less the same conclusions were drawn by Cossery *et al.* (1990) in the same year. They suggested that in NCB-20 cells the 5-HT3 receptor was involved in 5-HT-mediated cell depolarization, while a still unknown receptor was responsible for the 5-HT-mediated cAMP increase (EC_{50} of 500 ± 100 nM).

In conclusion, all these four papers reported a 5-HT-mediated stimulation of cAMP formation in NCB-20 cells, which varied between two- and fourfold, with EC_{50} values ranging between 300 and 500 nM, and had the merit to have characterized many known compounds for affinity and activity to the, at that time putative, 5-HT6 receptor.

A confirmation of the presence of 5-HT6 receptors in NCB-20 cells came from a paper published in 1994 by Unsworth and Molinoff, in which the authors described the properties of a 5-HT receptor on the parental neuroblastoma N18TG2 cell line which resembled those of the 5-HT6 receptor cloned just the year before.

II. Cloning of 5-HT6 Receptor: Receptor Binding and Functional Characterization

In 1993, Monsma and colleagues, as part of the effort to identify and clone G protein receptors from rat brain, used polymerase chain reaction technique to selectively amplify cDNA sequences from mRNA purified from rat striatum. Some of the amplified cDNA fragments ranging from 300 to 500 bp in size

exhibited sequence homology to previously cloned G protein-coupled receptors (GPCRs) and one in particular, clone St-B17, selected for further characterization, exhibited high homology to various serotonin receptors. St-B17 presented a combination of a relatively short third cytoplasmic loop (≈57 residues) and a long carboxyl terminus (≈117 residues), which is common among receptors that couple to the stimulation of either the adenylyl cyclase or phospholipase C signal transduction systems. St-B17 also contained one potential N-linked glycosylation site at Asn-9 in the extracellular N-terminus, in addition to several potential sites for phosphorylation by cAMP-dependent protein kinase or protein kinase C in both the third cytoplasmic loop and the intracellular C-terminal tail.

Transient transfection of COS-7 cells with the receptor resulted in the appearance of high-affinity and saturable binding sites for the serotonergic ligand [^{125}I]LSD, which exhibited a K_d of 1.26 ± 0.17 nM and B_{max} values ranging from 2 to 5 pmol/mg of protein. Specific binding of [^{125}I]LSD represented about 95% of total binding at a concentration of 1 nM. Preliminary characterization of the St-B17 pharmacology indicated that, among several endogenous biogenic amines, including dopamine, melatonin, epinephrine, norepinephrine, and histamine, only 5-HT was capable of completely displacing [^{125}I]LSD binding, exhibiting a K_i of 150 nM. The Hill coefficient for the 5-HT competition curve was not significantly different from unity and the affinity of 5-HT was not influenced by addition of the guanine nucleotide analogue guanosine 5′-(fl,-y-imido)triphosphate, probably because of the high level of transient expression obtained, resulting in an excess of receptor relative to G protein, thus precluding detection of the high-affinity, G protein-coupled state of the receptor.

The binding of 5-HT to St-B17 was also investigated using [^{3}H]5-HT as a radioligand. Saturation analysis of [^{3}H]5-HT binding revealed the presence of a single class of high-affinity, saturable binding sites with a K_d of 37 ± 5.0 nM and B_{max} values of 1–3 pmol/mg of protein. The binding of [^{3}H]5-HT was similarly not affected by the addition of guanine nucleotides, suggesting recognition of the low-affinity, uncoupled state of the receptor.

Further characterization of the St-B17 pharmacology, utilizing a variety of drugs that exhibited specificity for various serotonergic receptor subtypes and other binding sites, revealed that the pharmacology of clone St-B17 did not correspond to that of any previously described serotonin receptor subtypes. A number of drugs selective for 5-HT3 and 5-HT4 receptors (i.e., MDL-72222, ICS-205,930, and DAU-6285) exhibited virtually no affinity for St-B17, whereas agents selective for other 5-HT receptor subtypes, such as 8-OH-DPAT (5-HT1A), CGS 12066B (5-HT1B), mesulergine (5-HT1C), and ketanserin (5-HT2), bound with relatively low affinity. Ergot alkaloids, especially ergoline derivatives (i.e., LSD, lisuride, or pergolide), displayed relatively high affinity for St-B17, as did the nonselective serotonergic antagonist methiothepin. Interestingly, the atypical and typical antipsychotics clozapine and loxapine, respectively,

also exhibited high affinity for St-B17, as did several tricyclic antidepressant drugs (i.e., amoxipine, clomiprimine, and amitriptyline), which all exhibited K_i values <100 nM. In general, the drugs that exhibited the greatest affinity for St-B17 (i.e., $K_i < 100$ nM) were tricyclic, ergoline, or tryptamine derivatives. Competition for [^3H]5-HT binding by a number of drugs revealed, with a few exceptions, the same rank order of potency as for the inhibition of [^{125}I]LSD binding. However, for some drugs the K_i values determined by competition with [^3H]5-HT were up to fivefold lower than those determined by competition with [^{125}I]LSD, with the exception of clozapine, metergoline, and amitriptyline, which exhibited somewhat greater potency in competition with [^{125}I]LSD.

Although the pharmacological profile of St-B17 did not correspond to that of previously defined 5-HT receptor subtypes, it did resemble the profile described by Conner and Mansour for 5-HT stimulation of adenylyl cyclase activity in the NCB-20 neuroblastoma cell line (see Section I).

The effect of 5-HT on adenylyl cyclase was examined in stably transfected HEK-293 cells, because, despite several attempts, no activity was found in transiently transfected COS-7 cells. HEK-293 cells were used because they do not endogenously express serotonin receptors. Serotonin caused a potent, dose-dependent increase in cAMP levels in transfected HEK-293 cells, with an average EC_{50} value of 145 ± 40 nM. Pharmacological analysis of the cAMP response indicated that the serotoninergic agonists 5-methoxytryptamine and 5-carboxamidotryptamine were also able to elicit an increase in cAMP levels. The ergot alkaloids lisuride and dihydroergocryptine also stimulated cAMP accumulation, although these drugs appeared to function as partial agonists at the St-B17 receptor. Amoxipine, methiothepin, and clozapine all appeared to act as antagonists of this receptor, because they had no significant effect on cAMP levels on their own but were able to substantially inhibit the response elicited by 5-HT. These data thus indicated that the St-B17 receptor was functionally linked to activation of the adenylyl cyclase signal transduction system (see Table I)

In the same year, the 5-HT6 receptor was cloned independently by Ruat *et al.* (1993). Starting from a nucleotide probe encoding putative transmembrane domains (TM1–TM5) of the rat histamine H_2 receptor to screen a cDNA library from rat striatum, they cloned a cDNA encoding a functional receptor comprising 436 aa (amino acids) with an estimated molecular weight of 46.9 kDa. The gene sequence showed significant (40–45%) homology, particularly with the putative transmembrane domains of some serotonin receptors.

Transient expression of the encoded protein in COS-7 cells led to two- to eightfold cAMP accumulation upon 5-HT stimulation. The novel 5-HT6 receptor was described as a receptor characterized by the presence of a short third cytoplasmic loop (50 aa) and a rather long C-terminal tail (about 120 aa), two features widely found in receptors positively coupled to adenylyl cyclase.

Table I

Affinity Values

Molecule	Rat 5-HT6R Monsma et al (1993)		Rat 5-HT6R Roth et al (1994)	Human 5-HT6R Kohen et al (1996)	Rat 5-HT6R Boess et al (1997)		Rat 5-HT6R Grimaldi et al (1998)	Boess et al (1998)		Human 5-HT6R Bymaster et al (2001)	Hirst et al (2000)		Hirst et al (2003)		rat 5-HT6R Dupais et al (2008)
								Rat 5-H-T6R	Human 5-HT6R		Human 5-HT6R	Rat 5-HT6R	Human 5-HT6R	rat 5-HT6R	
	[^{125}I]LSD	[^{3}H]5-HT	[^{3}H]5-HT	[^{3}H]LSD	[^{3}H] LSD	[^{3}H] 5-HT	K_i (nM) vs [^{3}H]5-HT	[^{3}H] Ro63-0563	[^{3}H] Ro63-0564	[^{3}H]LSD	[^{125}I] SB258585	[^{3}H] LSD	[^{3}H]LSD	[^{3}H] LSD	[^{125}I] SB258585
Serotoninergic agents															
5-HT	151 ± 13	56 ± 9		65	234	12.6	20.0 ± 9.2	32.4	34.7		162	107	132	79.4	141.3
LSD					1.9	16.6		6.3	4.4				2.3	2.6	1.1
1-(1-Naphthyl)piperazine	104 ± 14														
2-Br-LSD	17.1 ± 1														
2-Methyl-5-HT					560	52.5									
5-Benzyloxytryptamine	110 ± 18				295	61.7									
5-Carboxamidotryptamine	774 ± 84	253 ± 20		720	3,470	186	165.7 ± 110								
5-CT											1,230	1230			2,089
5-Hydroxy-N-methyltryptamine	58 ± 8														
5-Methoxytryptamine	38.9 ± 4	18 ± 2		43	129	3.7	18.9 ± 9.4				79.4	56.2	69.2	22.4	
8-OH-DPAT				>10,000			>1,000								
Amitryptiline	69 ± 7	82 ± 6		65	72.4	339	222 ± 103	83.2	132		141	117	141	182	
Amoxapine	30.4 ± 2		6.3	50											
Bromocryptine				33											
Citalopram							>10,000								
Clomiprimine	53.8 ± 3						33.1 ± 9.1								
Cyproheptadine	134 ± 6			150											
DHE (Dihydroergotamine)	13.1 ± 09	5.4 ± 5					9.9 ± 1								
Dihydroergocryptine	161 ± 13														
Doxepin	136 ± 7														
Ergotamine					2.3	107		22.4	10.5						
ICS 205-930							>10,000								
Imipramine	209 ± 23	190 ± 3					316.7 ± 92								
Ketanserin				2,800			>1,000								
Lisuride	8.19 ± 05	5.3 ± 1			7.2	30.2	1.3 ± 0.8	11.7	8.5						
Mesulergine	1,720 ± 250			3,800	1,740	7,940		3,550	2,190		1,260	776			
Metergoline	30 ± 0.4	61 ± 23		400	50	263	47.3 ± 8.6	89.1	36.3						
Methiothepin	1.84 ± 0.2	0.39 ± 0.06		0.42	1.5	29.5	1.5 ± 0.9	5.5	2		2.8	3.2	1.4	1	2.4

(*continued*)

Table I (*continued*)

Molecule	Rat 5-HT6R Monsma et al (1993)		Rat 5-HT6R Roth et al (1994)	Human 5-HT6R Kohen et al (1996)	Rat 5-HT6R Boess et al (1997)		Rat 5-HT6R Grimaldi et al (1998)	Boess et al (1998)		Human 5-HT6R Bymaster et al (2001)	Hirst et al (2000)		Hirst et al (2003)		rat 5-HT6R Dupais et al (2008)
								Rat 5-HT6R	Human 5-HT6R		Human 5-HT6R	Rat 5-HT6R	Human 5-HT6R	rat 5-HT6R	
	[^{125}I]LSD	[^{3}H]5-HT	[^{3}H]5-HT	[^{3}H]LSD	[^{3}H] LSD	[^{3}H] 5-HT	K_i (nM) vs [^{3}H]5-HT	[^{3}H] Ro63-0563	[^{3}H] Ro63-0564	[^{3}H]LSD	[^{125}I] SB258585	[^{3}H] LSD	[^{3}H]LSD	[^{3}H] LSD	[^{125}I] SB258585
Methysergide	372 ± 73			180	257	339		347	257						
Mianserin	45.7 ± 9	38 ± 7		55	60.3	123	38.3 ± 11.9	97.7	123		135	79.4	81.3	39.8	
N,N-Dimethyl-5-methoxytryptamine	79.8 ± 4														
N.N-Dimethyltryptamine				68	112	4.5									
N,∪-Methyltryptamine	342 ± 32				107	3									
Nortryptyline	148 ± 3														
Quipazine				3,600											
Ritanserin	44 1 ± 4	16 ± 2		53	33.9	316		110	102		129	138			
RU24969				570											
Sumatriptan				2,600									30,000	30,000	
TFMPP	482 ± 37			430											
Tryptamine	438 ± 15				1,480	158									1,148
"Antipsychotic" agents															
"Atypical" drugs															
(—)-Octoclothepin				1.8											
Amperozide			63.1	1,600											
Aripiprazole															218.8
Bifeprunox															10,000
Clorotepine			0.4												
Clozapine	12.9 ± 2	20 ± 3	4.0	9.5	11.2	30.9	21.9 ± 9.3	9.3	13.8	5.2 ± 0.8	16.2	13.5	14.5	11.0	9.1
Fluperlapine			15.8	29											
ICI169369				11											
Melperone			1,260												
NDMC															5.8
Olanzapine			2,510	10						5.0 ± 0.8					5.9
Perlapine			63.1	150											
Pimozide			63.1												
Quetiapine										2,241 ± 151					2,630
Rilapine			6.3	17											
Risperidone			398	2,400						2,586 ± 570					1,318

(*continued*)

Table I (*continued*)

Molecule	Rat 5-HT6R Monsma et al (1993)		Rat 5-HT6R Roth et al (1994)	Human 5-HT6R Kohen et al (1996)	Rat 5-HT6R Boess et al (1997)		Rat 5-HT6R Grimaldi et al (1998)	Boess et al (1998)		Human 5-HT6R Bymaster et al (2001)	Hirst et al (2000)		Hirst et al (2003)		rat 5-HT6R Dupais et al (2008)
								Rat 5-H-T6R	Human 5-HT6R		Human 5-HT6R	Rat 5-HT6R	Human 5-HT6R	rat 5-HT6R	
	[^{125}I]LSD	[^{3}H]5-HT	[^{3}H]5-HT	[^{3}H]LSD	[^{3}H] LSD	[^{3}H] 5-HT	K_i (nM) vs [^{3}H]5-HT	[^{3}H] Ro63-0563	[^{3}H] Ro63-0564	[^{3}H]LSD	[^{125}I] SB258585	[^{3}H] LSD	[^{3}H]LSD	[^{3}H] LSD	[^{125}I] SB258585
Seroquel				33											
Tenilapine			100	570											
Tiospyrone			25.1	950											
ziprasidone										30 ± 2					26.9
Zotepine			1												15.5
"Typical" drugs															
(+)-Octoclothepin				2.6											
Acetophenazine			79.4												
Chlorpromazine			4	19											
Chlorprothixene			3.2												
Clothiapine			15.8												
Fluoxetine	1,770 ± 290						3,645.0 ± 125								
Fluphenazine			15.8	38											
Haloperidol			5,000	6,600			>1,000			4,475 ± 570					3,890
Loxapine	64.8 ± 0.6	56 ± 3	15.8	27			95.7 ± 9.8								
Mesoridazine			158	380											
Perphenazine			15.8												
Prochlorperazine			158												
Thioridazine			6.3												41.7
Thiothixine			39.8	320											
Trifluperazine			63.1	170											
Dopaminergic agents															
Lergotrile	36.5 ± 1														
Pergolide	29.9 ± 7														

In 1996, Kohen *et al.* reported the cloning and characterization of a human 5-HT6 receptor with homology to the previously described 5-HT6 rat receptor. The two receptors differed mostly in their C-terminal region, with the human receptor being 2 aa longer than its rat homolog (440 vs. 438 aa). In the same paper a corrected sequence for the rat 5-HT6 receptor was also reported. In fact these authors, noticing an apparent frameshift between the nucleotide sequences of the human 5-HT6 receptor and the rat sequences previously described (Monsma *et al.*, 1993; Ruat *et al.*, 1993), resequenced both rat and human 5-HT6 clones in parallel, using the same rat receptor, St-B17, that was described by Monsma *et al.* (1993).

The human 5-HT6 receptor was positively coupled to adenylyl cyclase in transfected mammalian cells and 5-HT caused a 2.5–8-fold dose-dependent increase in cAMP levels that could be surmountably antagonized by clozapine. The observed 5-HT EC_{50} was lower than the K_i as determined in binding experiments.

In receptor binding studies, the human 5-HT6 showed drug affinities similar to its rat homolog, with the exception of two of the tested compounds: methiothepin (human 5-HT6 $K_i = 0.42$ nM vs. rat $K_i = 1.84$ nM, but there is less than fivefold difference) and metergoline (human 5-HT6 $K_i = 400$ nM vs. rat $K_i = 30$ nM). The receptor displayed the following rank order of potency: methiothepin > clozapine ≈ olanzapine > ritanserin ≈ mianserin >> risperidone ≈ ketanserin. Among putative agonists, the rank order was 5-methoxytryptamine ≈ 5-HT ≈ *N*,*N*-dimethyltryptamine > TFMPP ≈ RU24969 ≈ 5-carboxamidotryptamine > sumatriptan >> 8-OH-DPAT. Like the rat receptor, the human 5-HT6 receptor showed high affinity for the atypical antipsychotic clozapine.

The same group (Kohen *et al.*, 2001) cloned the mouse 5-HT6 receptor and showed it to be a 440 aa peptide of 47 kDa molecular weight having 97% and 89% identity with the rat and human receptor protein, respectively. The authors also examined structure–function relationship in the C-terminal end of the third cytoplasmic (CIII) loop, introducing point mutations by site-directed mutagenesis at positions 264–268. These authors also demonstrated the presence of a BBXXB protein motif (where B = basic and X = nonbasic protein residue) in the C-terminal end of the third cytoplasmic loop, indicative of a constitutively active receptor. The ability of 5-HT6 wild-type and receptor mutants to activate a cAMP responsive reporter gene when transiently expressed in JEG-3 or COS-7 cells was investigated. The wild-type 5-HT6 receptor showed strong constitutive activity even when expressed at very low levels and increased in proportion to the amount of receptor cDNA transfected. Three of the five mutants investigated (K264I, K267A, and A268R) showed reduction in constitutive activity compared with wild type. These data suggested that constitutive activity may be important to 5-HT6 receptor activity *in vivo* and that, unlike some other GPCRs, alteration in the BBXXB CIII loop motif reduces rather than further activates basal activity of the murine 5-HT6 receptor.

III. Pharmacological Characterization of 5-HT6 Receptor: Affinity and Functional Profile of Serotoninergic, Antipsychotic, and Antidepressant Agents, and Tool Molecules

At the time of receptor cloning, as already mentioned, characterization of 5-HT6 receptor in the absence of selective agonists or antagonists was obtained by establishing the order of affinity of a variety of serotoninergic, antipsychotic, and antidepressant compounds. Since then, many papers have described the pharmacological characterization of 5-HT6 receptor by means of different tools. The principal affinity and functional activity values, respectively, of known compounds belonging to different molecular classes are reported in Tables I and II. In synthesis, the pharmacological profile of 5-HT6 receptor is characterized by a high affinity for a large number of typical and atypical antipsychotic compounds, such as loxapine and clozapine, both displaying antagonistic activity. Several antidepressant compounds, such as mianserin and amitryptiline, also display high affinity and antagonistic activity on the 5-HT6 receptor. Among the nonselective serotoninergic compounds many have high affinity to 5-HT6 receptor: methiothepin which behaves as an antagonist, and ergotamine, lisuride, and LSD which behave as agonists or partial agonists. It is noteworthy that 5-HT has a relatively low affinity to the 5-HT6 receptor compared with other 5-HT receptors (Borsini *et al.*, 2002).

Affinity and activity values seem to correlate well and to be rather consistent, with few exceptions (see previous section), between human and rat receptors.

The unique pharmacological profile showed by the 5-HT6 receptor made it an attractive target for medicinal chemistry and for the identification of selective ligands, some of which were used as tools in binding and behavioral studies. The first potent and selective ligands, Ro046790 and Ro630563, were found in 1998 by Sleight *et al.*, with K_i of 44.7 and 14.8 nM to human 5-HT6 receptor and of 55 and 12.3 nM to rat 5-HT6 receptor and >100-fold selectivity over a wide range of receptors. Both behaved as antagonists with K_B of 177.8 and 79.4 nM, respectively, and their radiolabeled form was used as tool in binding studies. The group of Bromidge (1999, 2001) identified other two highly selective 5-HT6 receptor antagonists, SB357134 and SB271046, with 2.5 and 1.26 nM affinity to 5-HT6 receptor and K_B values of 23.4 and 2.0 nM, respectively (Routledge *et al.*, 2000). SB258585, a structural analogue of SB271046, is another selective antagonist ($K_i = 3.2$ nM), whose iodine-radiolabeled form was extensively used in binding studies (Garcia-Alloza *et al.*, 2004; Hirst *et al.*, 2000, 2003). A more recent antagonist, SB399885, identified by Hirst *et al.* (2006), was found with K_i of 0.8 and 0.9 nM on human recombinant and native receptors, respectively, and 200-fold selectivity versus all receptors, ion channel, and enzymes.

The research of selective 5-HT6 receptor agonists has led to the identification (Holenz *et al.*, 2005) of two selective compounds, E6801 and E6837, with affinities

Table II
cAMP Assays

Molecule	cAMP measurement				[^{3}H]GTP assay	
	Rat 5-HT6R Monsma et al (1993)	Human 5-HT6R Kohen et al (1996)	Rat 5HT6R Boess et al (1997)	Rat 5HT6R Grimaldi et al (1998)	Human 5-HT6R Bymaster et al (2001)	Rat 5-HT6R Dupuis et al (2008)
	EC_{50} and KB (∗) or IC_{50} (°) for antagonism [nM]					
Serotoninergic agents						
LSD			12.3			0.67 ± 0.3
5-HT	145 ± 40	3.2	115	34.4		20.4 ± 07
5-Methoxytryptamine			34.7	21.4		
-N-Methyl-5-HT			29.5			
-N,N-Dimethyl-s-HT			45.7			
-N,N,N-Trimethyl-5-HT			Not active			
5-Benzyloxytryptamine			178			
2-Methyl-5-HT			200			
Tryptamine			295			741 ± 0.8
5-Carboxtryptamine						112 ± 0.9
5-Carboxamidotryptamine			501	414.7		
DHE				104.6		
Imipramine				4,985°		
Lisuride			148	178.9		
Methiothepin			18.6∗	52.5°		7.4 ± 0.6∗
Mianserin			72.4∗			
Ritanserin			26.9∗			

(*continued*)

Table II (*continued*)

Molecule	cAMP measurement				[^{3}H]GTP assay	
	Rat 5-HT6R Monsma et al (1993)	Human 5-HT6R Kohen et al (1996)	Rat 5HT6R Boess et al (1997)	Rat 5HT6R Grimaldi et al (1998)	Human 5-HT6R Bymaster et al (2001)	Rat 5-HT6R Dupuis et al (2008)
	EC_{50} and KB (∗) or IC_{50} (°) for antagonism [nM]					
"Antipsychotic" agents						
"Atypical" drugs						
Amperozide						
Clozapine			21.9∗	43.8°	38 ± 12∗	10∗
Olanzapine					42 ± 15∗	15.8∗
Risperidone					> 10,000∗	
"Typical" drugs						
Loxapine				132.9°		
Haloperidol					>10,000∗	
Fluoxetine				8699°		
Quetiapine					>10,000∗	
Ziprasidone					114 ± 27∗	57.5∗
NDMC						14.1∗
Aripiprazole						>10,000∗
Thioridazine						74.1∗
Zotepine						22.8∗
Asenapine						1.0∗

lower than 0.5 nM. Glennon *et al.* (2000) identified 2-ethyl-5-methoxy-*N*,*N*-dimethyltryptamine (EMDT) as a full agonist of 5-HT6 receptor ($K_i = 15.8$ nM and $EC_{50} = 3.6$ nM) but with limited selectivity over some other 5-HT receptors (only 10–30-fold selective over 5-HT1A, 5-HT1D, and 5-HT7 receptors).

Another selective 5-HT6 agonist was more recently developed (Schechter *et al.*, 2008), WAY-181187, which has a K_i of 2.2 nM and acts as full agonist with EC_{50} of 6.6 nM and with 50-fold selectivity versus other serotonin and dopaminergic receptors.

IV. Binding and Functional Methods for Receptor Characterization and Screening of Putative Ligands

This section intends to describe the principal binding and functional methods used for 5-HT6 receptor characterization and screening of putative ligands with the aim to give an overall overview on methodological aspects.

A. Receptor Source

Many cell types have been traditionally used for either transient or stable transfection of the 5-HT6 receptor. At the time of receptor cloning, Monsma *et al.* (1993) expressed transiently the rat 5-HT6 receptor in COS-7 cells and used their membranes to test the affinity of several compounds, whereas to study the effects of compounds on adenylyl cyclase they stably transfected the receptor in HEK-293 cells. On the contrary, Ruat *et al.*, in the same year, were able to perform cAMP studies using transiently transfected COS-7 with the rat 5-HT6 receptor.

The recombinant human receptor (Kohen *et al.*, 1996) was transiently expressed in COS-7 cells, used for radioligand binding assays, and stably expressed in HeLa cell line for cAMP studies.

The mouse receptor was transiently expressed either in JEG-3 or in COS-7 cells (Kohen *et al.*, 2001).

Since then, the use of recombinant expression system expressing either the rat or, as in the majority of the studies, the human 5-HT6 receptor has been extensively reported for screening of selective ligands, as it represents the easiest way for receptor supplying and the most controlled way to differentiate between closely related compounds. However, it is known that variations in the system used (i.e., cell line, receptor density, G protein combinations) may lead to different affinity and functional outputs. For ligand screening, the majority of papers report the use of HEK-293 or HeLa cells stably or transiently transfected with the human receptor.

For purposes different from ligand screening (i.e., receptor characterization and distribution and ligand characterization to native receptors), different mouse, rat, and human cerebral tissues were also used as receptor source (Garcia-Alloza *et al.*, 2004; Hirst *et al.*, 2000, 2003).

B. Radioligands

The radioligands mostly used with recombinant systems expressing the 5-HT6 receptor are the unselective LSD, either iodinated ([^{125}I]LSD) or tritiated ([^{3}H]LSD), and the tritiated serotonin ([^{3}H]5-HT), for both saturation and competition experiments. However, the use of a specific radioligand is not indifferent, as it has been repeatedly reported that some compounds exhibit lower or higher affinity values to 5-HT6 receptors, and the rank order of different molecules often changes, when LSD rather than 5-HT is used as radioligand competitor (see Table I).

However, in general, LSD rather than 5-HT is preferred because of its higher affinity to 5-HT6 receptors (K_d in the range of 0.8–8.6 nM for radiolabeled LSD compared to K_d in the range of 12.6–35 nM for radiolabeled 5-HT).

The use of the selective radioligands [^{3}H]Ro630563 and [^{125}I]SB258585 has also been reported for both ligand screening and receptor characterization in cerebral tissue.

C. Saturation Analysis

Saturation analysis is a common radioligand-based assay used to characterize membrane-bound receptors (see Frey and Albin, 2001 for a review). The major parameters are K_d, a measure of radioligand affinity, and B_{max}, a measure of the number of receptors present in the system.

Many papers reported saturation analysis of membranes expressing either recombinant or native 5-HT6 receptors, using different isotope-labeled radioligands and different binding conditions such as buffer, volume, time, and temperature of incubation, compounds for nonspecific binding evaluation, and filtration modalities. In Table III are listed the conditions and the results reported in some of the principal publications on this subject.

The following major considerations can be made: (1) K_d of [^{125}I]LSD) and [^{3}H]LSD is in the range of 0.6–8.6 nM, in the range of 12.6–35 nM for [^{3}H]5-HT, in the range of 4.96–11.7 nM for [^{3}H]Ro630563, and in the range of 0.8–2.8 nM for [^{125}I]SB258585; (2) in some cases, the use of different

Table III

SATURATION BINDING ANALYSIS

Cell line or tissue	Radioligand	Nonspecific binding	Buffer	Incubation time and temperature	Incubation volume	Filters	K_d (nM)	B_{max} (pmol/mg protein)	Ref
COS-7 transiently transfected with r 5-HT6R	[^{125}I]LSD (13–3500 pM)	100 μM 5-HT	50 mM Tris–HCl, pH 7.4, 10 mM $MgSO_4$, 0.5 mM EDTA, 200 μM sodium metabilsulfate	60 min 37°C	100 μl	GF/C presoaked with 0.3% PEI	1.5	3.4	Mosma *et al.* (1993)
	[^{3}H]5-HT (3–60 nM)	10 μM 5-HT	50 mM Tris–HCl, pH 7.4, 10 mM $MgSO_4$, 0.5 mM EDTA, 1 mM ascorbic acid	20 min 37°C	1000 μl	GF/C presoaked with 0.1% PEI	27.8	3.7	
Rat brain treated with 5-HT6 antisense oligonucleotides	[^{3}H]LSD (0.06–10 nM) in the presence of 300 nM spiperone	10 μM methiothepin	Buffer containing 10 μM pargyline, 4 mM $CaCl_2$, 0.1% ascorbic acid	60 min 23°C	?	GF/B	5.38	0.025	Bourson *et al.* (1995)
COS-7 transiently transfected with h 5-HT6R	[^{3}H]5-HT (1–60 nM)	5 μM methiothepin	50 mM Tris–HCl, pH 7.4, 10 mM $MgSO_4$, 0.5 mM EDTA, 1 mM ascorbic acid	30 min 37°C	500 μl	GF/C presoaked with 0.5% PEI	27.9	1.2	Kohen *et al.* (1996)
	[^{3}H]LSD (0.1–10 nM)			90 min 37°C			3.1	0.47	
HEK-293 stably transfected with r 5-HT6R	[^{3}H]LSD (0.163–20 nM)	10 μM 5-HT	50 mM Tris–HCl, pH 7.4, 10 μM pargyline, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.1% ascorbic acid	60 min 37°C	200 μl	GF/B presoaked with 0.3% PEI	1.55	2.28	Boess *et al.* (1997)
	[^{3}H]5-HT (0.325–40 nM)			120 min 4°C			12.6	2.76	
HEK-293 stably transfected with r 5-HT6R	[^{3}H]Ro 63-0563 (0.31–40 nM)	10 μM methiothepin	50 mM Tris–HCl, pH 7.4, 10 μM pargyline, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.1% ascorbic acid	80 min RT	200 μl	GF/B presoaked with 0.3% PEI	6.8	2.17	Boess *et al.* (1998)

(*continued*)

Table III (*continued*)

Cell line or tissue	Radioligand	Nonspecific binding	Buffer	Incubation time and temperature	Incubation volume	Filters	K_d (nM)	B_{max} (pmol/mg protein)	Ref
HeLa stably transfected with h 5-HT6R							4.96	1.59	
Rat striatal membranes					1000 µl		11.7	0.175	
Porcine striatal membranes					1000 µl		8	0.13	
HeLa stably transfected with h 5-HT6R	[^{125}I] SB258585 0.1 nM+ cold SB258585 (0.1–25 nM)	10 µM methiothepin	20 mM HEPES, 3 mM $MgCl_2$, 2 mM ascorbate, ph 7.4	45 min 37°C	500 µl	GF/B presoaked with 0.3% PEI	0.8	6.1	Hirst *et al.* (2000)
Rat, pig, and human brain membranes			50 mM Tris–HCl, pH 7.4, 10 µM pargyline, 5 mM $MgCl_2$, 0.5 mM EDTA, 5 mM ascorbic acid				2,8, 2,8, 1,3	0.173, 0.181, 0.15 in rat and pig striatum and human caudate, respectively	
HeLa stably transfected with h 5-HT6R	[^{3}H]LSD (0.05–10 nM)		20 mM HEPES, 3 mM $MgCl_2$, 2 mM ascorbate ph 7.4				1.5	3.9	
HeLa stably transfected with h 5-HT6R	[^{3}H]LSD (0.163–20 nM)	10 µM 5-HT	50 mM Tris–HCl, pH 7.4, 10 µM pargyline, 5 mM $MgCl_2$, 0.1% ascorbic acid	60 min 37°C	200 µl	GF/B	Not reported		Bos *et al.* (2001)
COS-7 transiently transfected with h 5-HT6R	[^{3}H]LSD (0.25–25 nM)	10 µM clozapine	50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.2% ascorbic acid	60 min RT	1000 µl	?	8.6	8.2	Purohit *et al.* (2003)
	[^{3}H]5-HT (0.5–100 nM)						35	1.3	

(*continued*)

Table III (*continued*)

Cell line or tissue	Radioligand	Nonspecific binding	Buffer	Incubation time and temperature	Incubation volume	Filters	K_d (nM)	B_{max} (pmol/mg protein)	Ref
Rat striatal membranes Mouse striatal membranes Human caudate putamen membranes	[^{125}I] SB258585 0.1 nM	10 µM methiothepin	50 mM Tris–HCl, pH 7.4, 10 µM pargyline, 5 mM $MgCl_2$, 0.1% ascorbic acid	45 min 37°C	500 µl	GF/B presoaked with 0.3% PEI	See text		Hirst *et al.* (2003)
Postmortem cortex of AD patients	[125I] SB258585 0.1 nM+ cold SB258585 (1–10 nM)	10 µM SB-214111	50 mM Tris–HCl, pH 7.4, 10 µM pargyline, 5 mM $MgCl_2$, 0.1% ascorbic acid	45 min 37°C	500 µl	GF/B	See text		Garzia-Alloza *et al.* (2004)
HEK-293 stably transfected with r 5-HT6R COS-7 transiently transfected with h 5-HT6R and r 5-HT6R	[^{3}H]LSD (2.5–10 nM)	5 µM methiothepin	50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 0.5 mM EDTA	60 min 37°C	200 µl	96-well filterplates	Not reported		Romero *et al.* (2006)

radioligands seems to label a different number of receptors in the same cell line, which probably reflects the presence of different binding sites in the receptor or a different receptor–radioligand interaction; (3) given the many different methods, caution has to be taken in comparing results obtained in different conditions.

An example of saturation analysis to label native 5-HT6 receptor is given in the paper by Hirst *et al.* (2003) in which the use of binding of [^{125}I]SB258585 revealed a marked difference in the distribution pattern of 5-HT6 receptors in different species, being widely expressed and highly enriched in the basal ganglia in rat and human brain, but very less expressed in the mouse brain with no evidence of enrichment in the basal ganglia.

Saturation analysis with [^{125}I]SB258585 was also used to assess, in postmortem frontal and temporal cortex of Alzheimer's patients, the density of 5-HT6 receptor, which was significantly reduced to about 50% compared with control patients (Garcia-Alloza *et al.*, 2004).

D. Competition Analysis

Competitive binding experiments measure the binding of a single concentration of labeled ligand (usually used at its K_d) to a given receptor in the presence of various concentrations of the unlabeled ligand. The concentration of unlabeled ligand causing a half maximal inhibition of the radioligand binding is defined as IC_{50}. K_i is the parameter, deriving from IC_{50}, which takes into account the concentration of radioligand used, and it is a measure of the cold ligand affinity for that given receptor (see Frey and Albin, 2001 for a review).

As for saturation analysis, many different competition analysis protocols on 5-HT6 receptors are described in the literature. For screening purposes, the majority of studies evaluate ligand affinities on cells expressing the recombinant receptor either transiently or stably expressed, rather than on native receptors, which imply the use of a selective radioligand and are less controllable in terms of receptor expression. Only few papers report affinity values against [^{3}H]5-HT, whereas most of them report the affinities against [^{3}H]LSD and very few with other more selective radioligands (Boess *et al.*, 1998; Dupuis *et al.*, 2008; Hirst *et al.*, 2000; Routledge *et al.*, 2000). As already mentioned, the use of a different cell line, the human rather than the rat variant of the receptor and different radioligands can influence the results. For example, 5-HT and its analogues display higher affinities against radiolabeled 5-HT, while ergoline compounds do so against radiolabeled LSD. See Table I for a broad-spectrum comparison of results obtained with different methods.

The *ex vivo* binding assay is a particular kind of competition binding assay to measure the occupancy, by a certain molecule administered *in vivo*, of a receptor binding site of interest. The work of Stean *et al.* (2002) gives an example of such a

test. In this work rats received different doses of SB3571314, a 5-HT6 antagonist, and few hours later they were killed and striatum removed and homogenized. Radioligand binding on tissue homogenates was performed using 0.1 nM [^{125}I] SB258585 and receptor occupancy was calculated by comparing the inhibition of the binding of radioligand observed in tissues derived from drug-treated animals with total binding obtained in vehicle-treated animals. Data analysis showed that SB3571314 inhibited *ex vivo* [^{125}I]SB258585 binding in the rat with an ED_{50} of 4.9 ± 1.3 mg/kg.

E. cAMP Assays

Since 5-HT6 receptor was cloned, it was clear that it was positively coupled to adenylyl cyclase, the enzyme that regulates the intracellular concentration of cAMP (Kohen *et al.*, 1996; Monsma *et al.*, 1993; Ruat *et al.*, 1993). Measurement of intracellular cAMP is thus the functional test for measuring the agonist or antagonist effect of test compounds on the 5-HT6 receptor, and, together with binding, the election test for compound screening. Many protocols of cAMP, taking advantage of a plethora of technologies ranging from radiometric to enzymatic, are described in the literature, all following the general principle that changes in intracellular cAMP are detected by the competition between cellular cAMP and a labeled and detectable form of cAMP.

In this assay, 5-HT acts as full agonist with EC_{50} ranging from 3.2 to 150 nM, according to the different recombinant cell systems used (Table II), whereas LSD is reported to act as partial agonist (Boess *et al.*, 1997; Kohen *et al.*, 1996). Rapid receptor desensitization of the rat 5-HT6 receptor transfected in HEK cells upon activation with 100 μM 5-HT, not accompanied by receptor loss and probably mediated by protein kinase A (PKA), has also been demonstrated using cAMP assay (Max *et al.*, 1995). Romero *et al.* (2006) used cAMP assay to demonstrate that SB271046 and Ro046790, considered as 5-HT6 receptor antagonists, behaved as inverse agonists upon a constitutively active mutant 5-HT6 receptor, thus giving a warning of the dependence of the concept of antagonism or inverse agonism according to the system used.

Besides direct cAMP measurements, intracellular cAMP modulation can be detected via changes in the expression level of a reporter gene, the transcription of which is regulated by the transcription factor cAMP response-element binding protein (CREB), which binds to upstream cAMP response elements. Kohen *et al.* (2001) measured the ability of mouse wild-type and receptor mutants to activate a cAMP responsive reporter gene when transiently expressed in JEG-3 or COS-7 cells, demonstrating that the wild-type 5-HT6 receptor presents strong constitutive activity, which increased in proportion to the amount of receptor transfected.

Although there are no doubts for the positive coupling of 5-HT6 receptor with adenylyl cyclase in recombinant expression systems, in endogenous tissue the situation is less clear. However, in cultured mouse striatal neurons (Sebben *et al.*, 1994) and in pig caudate membranes (Schoeffter and Waeber, 1994), the activation of cAMP accumulation elicited by different pharmacological agents and their potency rank order, together with the exclusion of other receptor involvement, was interpreted as an action mediated by 5-HT6 receptors.

F. GTPγ Assay

The [^{35}S]GTPγS assay measures the level of G protein activation following agonist occupation of a GPCR, by determining the binding of the non-hydrolyzable analogue [^{35}S]GTPγS to Gα subunits (for a review see Harrison and Traynor, 2003).

In the paper of Bymaster *et al.* (2001), this assay was used to determine the antagonism of 5-HT6 receptors by several compounds. 5-HT stimulated binding of [^{35}S]GTPγS to membranes from cells transfected with human 5-HT6 receptors with an EC_{50} value of 43 ± 20 nM, whereas olanzapine and clozapine dose-dependently antagonized the effect of 1 μM 5-HT with IC_{50} values of approximately 600 nM and calculated pK_B values of 7.38 ± 0.16 and 7.42 ± 0.15 nM, respectively. Among other antipsychotics investigated, clozapine antagonized 5-HT receptors with a pK_B of 7.42 ± 0.15 M, ziprasidone was three-fold less potent, and risperidone, quetiapine, and haloperidol were weak antagonists.

Dupuis *et al.* (2008) utilized this assay for a comparative study in which the interaction of a broad range of novel agonists, antagonists, and antipsychotics at rat 5-HT6 receptors stably expressed in HEK293 cells was evaluated (Table II). In this assay, 5-HT and its derivatives, as well as LSD (in contrast with cAMP studies), behaved as full agonists. The novel sulfonyl derivatives, WAY181187 ($pEC_{50} = 9.1$) and WAY208466 ($pEC_{50} = 7.8$), behaved as partial agonists and attenuated the actions of 5-HT. SB271046 and SB258585 and SB399885 abolished activation of Gαs by 5-HT with pK_B values of 10.2, and 9.9 and 10.9, respectively.

V. Conclusions

In vitro binding and functional assays are the first steps in the process of characterization of a receptor and its ligands. We believe that, in the case of 5-HT6 receptors, stricter criteria for performing these studies and harmonization among laboratories should be reached.

In addition, a better understanding of the radioligand–receptor interactions, probably on the basis of different results obtained according to the radioligand used in binding experiments, would be very useful. Furthermore, more physiological conditions (i.e., use of native tissues instead of recombinant systems) for both binding and functional activity studies would be recommended. In fact, concepts such as full rather than partial agonism and antagonism rather than inverse agonism of a particular compound may be highly dependent, as described, on the system used and on the presence of endogenous ligands in the system.

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5-HT$_6$ RECEPTOR SIGNAL TRANSDUCTION: SECOND MESSENGER SYSTEMS

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I. Introduction

The 5-HT$_6$ receptor belongs to the G-protein-coupled receptor (GPCR) family. Like 5-HT$_4$ and 5-HT$_7$ it is positively coupled to adenylyl cyclase, meaning that upon agonist activation cAMP formation is increased. This activity has been demonstrated in recombinant expression systems (Boess *et al.*, 1997;Kohen *et al.*, 1996; Ruat *et al.*, 1993), mouse striatal neurons (Sebben *et al.*, 1994), or pig caudate membranes (Schoeffter and Waeber, 1994). A constitutive activity of the mouse receptor has been proposed (Kohen *et al.*, 2001), and due to the high degree of conservation of the sequence responsible for such activity, the same constitutive activity has been suggested in other species. In fact, a constitutively active mutant of the human receptor has been generated (Purohit *et al.*, 2003).

Recombinant expression systems are well known for their capacity to differentiate between closely related compounds with respect to their intrinsic efficacy. This capability has led to the wide use of these experimental approaches for the high-throughput screening (HTS) of compound libraries or the screening of new chemical entities. It has been a successful strategy, and

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several 5-HT_6 receptor antagonists have been identified and are being developed by different pharmaceutical companies (for review Fone, 2008; Heal *et al.*, 2008; Holenz *et al.*, 2006). However, a compound with partial agonist properties in a recombinant expression system may demonstrate antagonist activity in *in vivo* integrated systems (Hoyer and Boddeke, 1993). Partial agonism can probably be observed in most systems for such compounds, whereas full agonism can be seen in very efficiently coupled systems and silent, competitive antagonism, in very poorly coupled receptor systems. Therefore, a reliable molecular pharmacological characterization of new ligands can only be based on several observations as made in different expression systems and using different read-outs. Indeed, some discrepancies could be found in the literature showing *in vivo* similar results for compounds being classified as agonists or antagonists in cAMP-based assays. 5-HT_6 receptor coupling to $G\alpha s$ has been widely described (Kang *et al.*, 2005; Romero *et al.*, 2006b), but coupling of 5-HT_6 receptors to other $G\alpha$ protein subunits ($G\alpha i/o$ or $G\alpha q$) has also been recently reported using a Scintillation Proximity Assay (SPA)/antibody-immunocapture technique (Dupuis *et al.*, 2008). In addition, the coupling of 5-HT_6 receptors to Ca^{2+} signaling using a chimeric G-protein has been reported (Zhang *et al.*, 2003) and, besides the cAMP, a novel cellular signaling pathway involving interaction with Fyn tyrosine kinase, a member of the Src family of non-receptor protein-tyrosine kinases, has been demonstrated (Yun *et al.*, 2007).

Controversial results have been obtained with different experimental approaches, when comparing the activity of 5-HT_6 receptor antagonists and agonists. Antidepressant activity has been reported not only for antagonists in the tail suspension and forced-swim tests (Wesolowska and Nikiforuk, 2007) but also for agonists in the tail suspension test (Svenningsson *et al.*, 2007). The selective 5-HT_6-receptor agonist LY-586713 caused a bell-shaped dose–response curve on hippocampal BDNF mRNA expression. It also increased the Arc mRNA levels and this effect was blocked by the 5-HT_6 receptor antagonist SB-271046. However, in some brain regions the antagonist was not able to block the agonist effect and, in fact, it induced an increase in Arc expression by itself (de Foubert *et al.*, 2007). Different 5-HT_6 receptor antagonists have been reported to be active in the novel object discrimination (NOD) test in rats (King *et al.*, 2004; Lieben *et al.*, 2005; Schreiber *et al.*, 2007; Woolley *et al.*, 2003), using different experimental approaches (for review Fone *et al.*, 2008). However, very recently, E-6801 (6-chloro-*N*-(3-(2-(dimethylamino) ethyl)-1H-indol-5-yl) imidazo[2,1-b]thiazole-5-sulfonamide), a potent agonist at rat and human 5-HT_6 receptors, has been reported to show activity in this contextual memory model in rats (Kendall *et al.*, published online April 2010). The activity was shown in several experimental conditions. E-6801 was able to improve object recognition after 4 hours natural delay forgetting and to reverse scopolamine-induced impairment. More intriguing were the results obtained

when combining non-active doses of E-6801 and the 5-HT$_6$ receptor antagonist SB-271046, which produced an improvement in NOD. In addition, according to the reported modulatory role of the 5-HT$_6$ receptor on cholinergic and glutamatergic neurotransmission, E-6801, at a non-active dose by itself, was able to improve synergistically the activity of non-active doses of donepezil (an acetylcholinesterase inhibitor) and memantine (a NMDA receptor antagonist) (Kendall *et al.*, published online April 2010).

Therefore, the translation of the activity of a particular compound from the *in vitro* systems to the *in vivo* situation should be done with caution. Receptors can adopt different conformations which may produce different pharmacological effects (Kenakin, 2002a, 2002b).

In this chapter, we will review some reported experimental approaches that can be used for a better functional characterization and classification of 5-HT$_6$ receptor ligands. The results obtained using some representative 5-HT$_6$ receptor agonists and antagonists will be summarized.

II. The cAMP Signal Transduction Pathway

As mentioned before, 5-HT$_6$ receptor is positively coupled to adenylyl cyclase. The measurement of cAMP formation has been the most widely used method for the screening of new compounds and their classification as agonists or antagonists (for review Holenz *et al.*, 2006). Several powerful agonists and antagonists have been identified using this method [(i.e., EMDT (Glennon *et al.*, 2000), WAY-181187 (Cole *et al.*, 2005), Ro 04-6790 (Sleight *et al.*, 1998), SB-271046 (Bromidge *et al.*, 1999), MS-245 (Tsai *et al.*, 2000), or LY-483518/SGS-518 (Piñeiro-Núñez *et al.*, 2005)]. This strategy was also used in our laboratory and as a result, a list of agonists and antagonist were identified (Holenz *et al.*, 2005). However, being aware of the complexity of the GPCR signaling, and some intriguing *in vivo* results we had obtained, some efforts were done trying to correlate the *in vitro* functional classification with the *in vivo* efficacy. First, we analyzed the ability to modulate the Gs-coupled cAMP pathway linked to the 5-HT$_6$ receptor using 5-HT$_6$ rat receptor stably transfected in HEK-293F cells, and Cos-7 cells transiently transfected with either human wild-type or S267K 5-HT$_6$ receptor constructed by site-directed mutagenesis, with the aim of increasing the resolution and having the possibility to measure either positive or negative efficacy (Romero *et al.*, 2006b). Second, we performed the analysis of efficacy at human wild-type and at a constitutively active human S267K 5-HT$_6$ receptor constructed like previously, but using HEK-293F cells instead of Cos-7 cells (Romero *et al.*, 2006a).

A. Rat 5-HT_6 Receptor Expressed in HEK-Cells: Modulation by Forskolin

In the first study, 5-HT_6 receptor-mediated activation and inhibition of adenylyl cyclase activity was monitored by measuring levels of cAMP in 96-well plates by the FlashPlate method. The production of cAMP was modulated by forskolin, as previously described for other receptors like β_2-adrenoceptors, histamine H_2, or 5-HT_7 (Alewijnse *et al.*, 1997; Krobert and Levy, 2002). No agonist-independent rat 5-HT_6 receptor activation in HEK-293F cells was observed, neither in the absence nor in the co-presence of forskolin. However, in co-presence of forskolin, E_{max} and pEC50 values of 5-HT were significantly enhanced. The 5-HT_6 receptor ligand E-6801 displayed at 1 μM partial antagonism of the 5-HT response in the absence of forskolin, but in the co-presence of 1 μM forskolin the cAMP production was maximal and similar to that obtained with 5-HT. The 5-HT_6 antagonists SB-271046 and Ro-04-6790 antagonized the 5-HT activity in a dose-dependent manner, either in the absence or in the presence of forskolin. Both compounds did not show any intrinsic activity at any of the assayed concentrations, neither in the absence nor in the presence of forskolin.

In this system, SB-271046 and Ro-04-6790 would be classified as neutral antagonists, in agreement with previously reported activity (Bromidge *et al.*, 1999; Sleight *et al.*, 1998) so this system was not able to show any difference between these two ligands or any difference in their interaction with the receptor. On the other hand, E-6801 would be classified as partial agonist because it was able to show some antagonism over the 5-HT-induced cAMP production in the absence of forskolin. However, when forskolin was present, E-6801 was revealed as a full agonist, pointing to a differential interaction of this ligand with the 5-HT_6 receptor. Therefore, this first indication of a misleading system for the classification of new ligands was taken into account in the discovery process.

B. Human 5-HT_6 Receptor Expressed in Cos-7 Cells

In the process of refining the discovery cascade, cAMP measurements on Cos-7 cells transiently expressing either human wild-type or mutant 5-HT_6 receptor were performed by HTRF (homogenous time resolved fluorescence) (Gabriel *et al.*, 2003). In accordance with previous observations (Purohit *et al.*, 2003), constitutive activation of the human 5-HT_6 receptor was observed by mutation of its Ser267 to a Lys, and this approach was set up for improving our understanding of the functional activity of 5-HT_6 receptor ligands.

The reference compound SB-271046, like in the rat 5-HT$_6$ receptor, behaved as antagonist at the wild-type human 5-HT$_6$ receptor, as previously reported (Routledge *et al.*, 2000). However, at a constitutively active human S267K 5-HT$_6$ receptor, SB-271046 displayed negative efficacy. Similar inverse agonist activity was obtained with Ro 04-6790, another 5-HT$_6$ receptor antagonist previously reported to be free of intrinsic activity, and thus devoid of inverse agonism activity, on basal cAMP accumulation in HeLa cells stably expressing human 5-HT$_6$ receptor (Sleight *et al.*, 1998). This negative efficacy has been previously reported for two antipsychotics, clozapine and fluphenazine (Purohit *et al.*, 2003). Co-presence of forskolin did not modify the potency of agonist/inverse agonist-dependent cAMP formation in Cos-7 cells. Therefore, the influence of the host cell type may also have a critical role in the effect of forskolin. Finally, E-6801 behaved as a full and highly potent agonist in the Cos-7 expression systems, both at human wild-type and at mutant S267K 5-HT$_6$ receptors. Similarly full agonism was observed at the rat 5-HT$_6$ receptor in the presence of forskolin but partial agonism in basal condition.

Using this experimental approach where constitutive activity is present, the basal cAMP formation reduction was high enough to reveal the intrinsic activity of previously described neutral antagonists. A neutral antagonist should lower 5-HT-induced cAMP levels to basal levels, but SB-271046 and Ro 04-6790 decreased cAMP levels induced by 5-HT beyond the basal level, suggesting a change in the classification of these ligands from neutral antagonists to inverse agonists.

C. Human 5-HT$_6$ Receptor Expressed in HEK-Cells

The native human 5-HT$_6$ receptor does not display constitutive activity in Cos-7 or CHO-K1 cells (Purohit *et al.*, 2003, 2005). In these same cell lines, efficient agonist-independent activity was found when a mutation of the S267K to a Lys near the B^{261}BXXB^{265} CIII-loop motif was introduced (Purohit *et al.*, 2003, 2005). Strong constitutive activity was reported for mouse wild-type 5-HT$_6$ receptor expressed in JEG-3 and Cos-7 cells (Kohen *et al.*, 2001). However, alterations in the same BBXXB CIII-loop motif of the murine 5-HT$_6$ receptor reduce basal activity (Kohen *et al.*, 2001).

Like previously shown in Cos-7 cells, in this third experimental approach, Romero *et al.* (2006a) also reported constitutive activity by the wild-type human 5-HT$_6$ receptor when stably expressed in human embryonic kidney HEK-293F cells (Table I), and an amplification of the 5-HT$_6$ receptor-mediated constitutive activity was obtained by mutation of its S267K near the B^{261}BXXB^{265} CIII-loop

Table I
E_{max}, I_{max}, pEC50, pIC50 Values of 5-HT_6 Ligands for cAMP Response in HEK-293F Cells Stably Expressing Wild-Type Human 5-HT_6 Receptor

Compound	E_{max} (%)	pEC50	I_{max} (%)	pIC50
5-HT	100	9.11 ± 0.01	–	–
Cpd. 1	100 ± 0.4	8.92 ± 0.1	−1.6 ± 0.6	–
E-6801	100.3 ± 1.0			
10.22 ± 0.07	1.11 ± 0.08	–		
WAY-181187	98.2 ± 0.9	9.45 ± 0.09	0 ± 0.7	–
Cpd. 2	94.1 ± 1.0	8.89 ± 0.06	3.7 ± 1.6	–
SB-271046	−100	8.08 ± 0.03	100	6.63 ± 0.02
Ro 04-6790	−89.1 ± 7.5	7.33 ± 0.24	57.9 ± 3.4	5.16 ± 0.04
Ro 66-0074	−111.8 ± 8.7	8.59 ± 0.11	106.9 ± 3.3	7.29 ± 0.06

E_{max}, I_{max}, pEC50 and pIC50 values were derived from cAMP-mediated 5-HT response curves. Data correspond to mean ± s.e.m. values of four to ten independent experiments performed in duplicate.

motif to a Lys. In this study several 5-HT_6 compounds were differentiated on the basis of their intrinsic activity at the constitutively active 5-HT_6 receptor. Qualitatively, the response of the 5-HT_6 receptor ligands was quite similar, but some quantitative differences were shown.

The magnitude of the 5-HT response was similar for both wild-type and mutant 5-HT_6 receptors and was fully antagonized by SB-271046. In contrast to the results obtained in Cos-7 cells, SB-271046 showed negative efficacy in both wild-type and S267K mutant receptor. Similar results were obtained with another antagonist, Ro 04-6790, although much less negative efficacy and potency was obtained in the mutant receptor. Interestingly, these two antagonists have been reported to interact in a different manner at the mouse 5-HT_6 receptor, with virtually no affinity for Ro 04-6790, and no differences for SB-271046 between mouse and rat receptor (Hirst *et al.*, 2003).

The 5-HT_6 receptor agonist E-6801, which behaved as partial agonist at rat 5-HT_6 receptor but as full agonist in the presence of forskolin, and in the Ser267K mutated human 5-HT_6 receptor in Cos-7 cells, showed a potent and efficacious agonist activity at the human wild-type and mutant 5-HT_6 receptor in HEK-297 cells, which was even higher than the potency shown by 5-HT. The concentration–response curves for 5-HT and E-6801, along with 2-(5-methoxy-2-methyl-1H-indol-3-yl)-*N*,*N*-dimethylethanamine (Cpd. 1, see Slassi *et al.*, 2002 compound 54), 5-chloro-2-methyl-3-(1,2,3,6-tetrahydropyridin-4-yl)-1H-indole (Cpd. 2, see Mattsson *et al.*, 2005 compound 18), and WAY-181187 (Cole *et al.*, 2007), are shown in Fig. 1. All the compounds were synthesized at Esteve and used as reference compounds in these studies for comparison with the calcium signaling results presented in the next section.

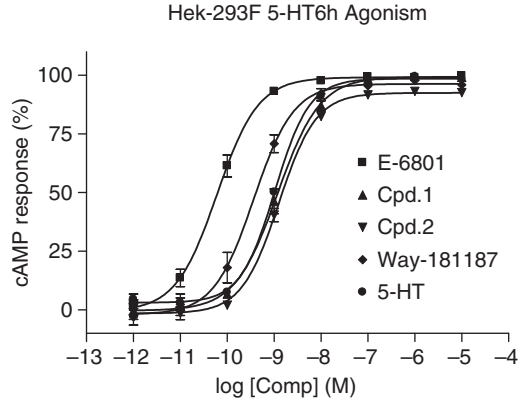

FIG. 1. Modulation of cAMP levels by 5-HT$_6$ agonists at wild-type human 5-HT$_6$ receptor in stably transfected HEK-293F cells. 5-HT$_6$ receptor ligand-mediated cAMP formation in HEK293 cells transiently transfected with human 5-HT$_6$ receptor. cAMP formation was determined after an overnight serum-free incubation in the presence of the indicated ligands using HTRF. Mean dose–response curves ± s.e.m. are shown from five to ten independent experiments performed in duplicate.

Altogether, the capacity to classify 5-HT$_6$ receptor ligands based on their efficacy seems to be enhanced in S267K 5-HT$_6$ mutant, both at rat and at human receptors. This is in agreement with the well-known capacity of constitutively active recombinant expression systems to differentiate closely related compounds in terms of intrinsic efficacy. However, efficacy profiles obtained in such recombinant system could not have a direct correlation with the effect on *in vivo* integrated systems, and thus extrapolation should be done with caution.

The differences found in terms of potency and efficacy in the reviewed studies, either with antagonists or with agonists stress the need of appropriate experimental conditions for monitoring, and more importantly, selecting compounds during the drug discovery process. The results obtained in those studies are in agreement with the suggestion that measures of efficacy in a single recombinant system can be misleading (Kenakin, 2010) and that studies of "agonist selectivity" are needed, like the well-accepted binding selectivity profile usually performed in the lead compound selection processes (Baker, 2010). Moreover, the intrinsic activity of a particular compound is also system-dependent, and false negatives, in terms of efficacy, may demonstrate intrinsic efficacy in a different experimental condition. In this way, experimental work is supporting early theoretical predictions indicating that neutral antagonists are a minority (Kenakin, 2004) and that most of the compounds identified as neutral antagonists have inverse agonism activity to some extent. A quantitative analysis of the magnitude of the efficacy of inverse agonists should be done instead of the more widely used qualitative classification (Giraldo, 2010).

III. Functional 5-HT_6 Receptor/Gα15 Interaction: Calcium-Based Signaling

Besides the cAMP pathway, successful application of a chimeric G protein strategy to the functional characterization of 5-HT_6 receptor ligands has been shown (Zhang *et al.*, 2003). Results showed that the 5-HT_6 receptor can be coupled to Ca^{2+} signaling using a Gαq/Gαs chimera, and based on agonist potencies, the coupling efficiency was found to be similar to that observed when the native coupling to Gαs was evaluated in the cAMP accumulation studies.

We investigated 5-HT_6 receptor ligand responses at a human 5-HT_6 receptor stably expressed in human embryonic kidney HEK-293F cells using a Gα15 protein-mediated Ca^{2+} pathway as functional read-out (unpublished results). Ca^{2+} responses were determined in presence of 5-HT_6 receptor ligands upon overnight serum-free incubation using FLIPR (Fluorometric Imaging Plate Reader). Ca^{2+} formation was expressed as a percentage of the respective maximal Ca^{2+} formation as induced by 1 μM 5-HT versus basal. Mean E_{max} and pEC50 values $\pm$ s.e.m. are summarized in Table II.

We compared 5-HT and the reported 5-HT_6 receptor agonists previously shown in Fig. 1, Cpd. 1 (Slassi *et al.*, 2002 compound 54), Cpd. 2 (Mattsson *et al.*, 2005 compound 18), E-6801 (Holenz *et al.*, 2005), and WAY-181187 (Cole *et al.*, 2007), besides the 5-HT_6 receptor antagonists SB-271046 (Bromidge *et al.*, 1999), Ro 04-6790 (Sleight *et al.*, 1998), and Ro 66-0074 (Riemer *et al.*, 2003). As expected, whereas stably transfected HEK-293F/h5-HT_6 cells did not display a 5-HT-mediated Ca^{2+} response, co-expression with a Gα15 protein resulted in a potent 5-HT-mediated Ca^{2+} response. A comparison between 5-HT-ligand-mediated Ca^{2+} responses in HEK-293F/h5-HT_6 cells co-transfected with a Gα15 protein is illustrated in Fig. 2.

Table II
E_{max}, I_{max}, pEC50 and pIC50 Values of 5-HT_6 Ligands for cAMP and Ca^{2+} Responses in HEK-293F Cells Stably Expressing the Human 5-HT_6 Receptor

	Calcium response			
	E_{max} (%)	pEC50	I_{max} (%)	pIC50
5-HT	100.0	8.59 $\pm$ 0.07	–	–
Cpd. 1	90.3 $\pm$ 6.0	7.71 $\pm$ 0.16	–	–
Cpd. 2	23 $\pm$ 3	6.80 $\pm$ 0.21	96 $\pm$ 2	7.26 $\pm$ 0.18
E-6801	80 $\pm$ 7	7.70 $\pm$ 0.3	–	<6
WAY-181187	45 $\pm$ 3	6.80 $\pm$ 0.1	–	<6
SB-271046	5.5 $\pm$ 0.7	<6	100.0	6.68 $\pm$ 0.05
Ro 04-6790	6.4 $\pm$ 2.2	<6	85.9 $\pm$ 9.1	7.2 $\pm$ 0.1
Ro 66-0074	9.4 $\pm$ 6.0	<6	105.6 $\pm$ 3.0	7.94 $\pm$ 0.08

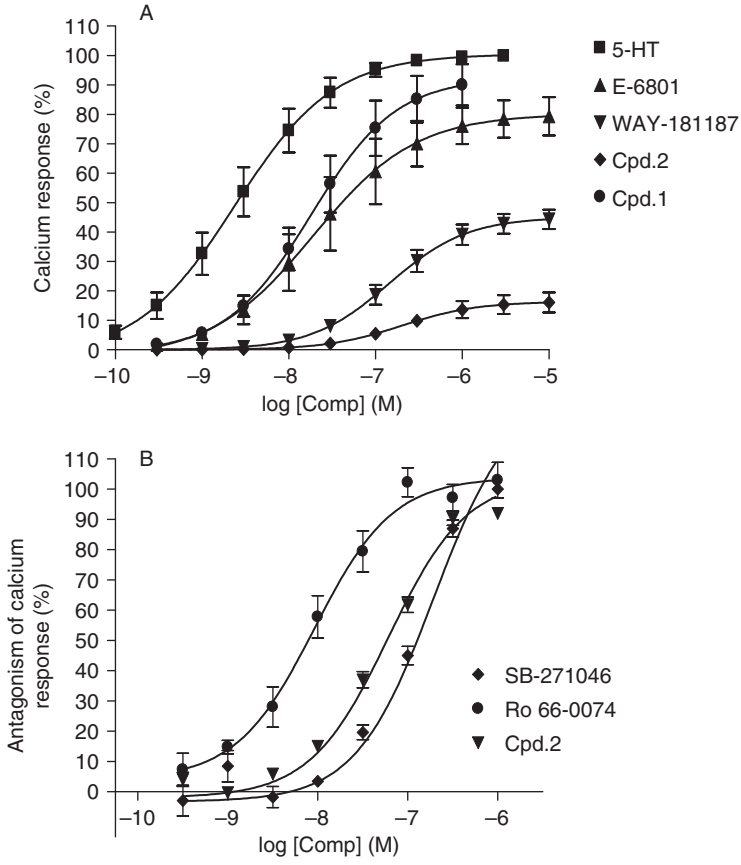

FIG. 2. Modulation of Ca^{2+} responses by 5-HT ligands at 5-HT$_6$ receptors in Gα15 protein transfected HEK-293F/h5-HT$_6$ cells. HEK-293F cells stably transfected with human 5-HT$_6$ receptor were co-transfected with Gα15 protein. Ca^{2+} responses were determined in the presence of the indicated ligands upon overnight serum-free incubation using FLIPR. Dose–response curves are shown as mean $\pm$ s.e.m. from four to six independent experiments performed in duplicate. (A) Ligand-mediated responses at the indicated concentrations. Ca^{2+} formation is expressed as a percentage of the respective maximal Ca^{2+} formation as induced by 1 µM 5-HT versus basal. Mean E_{max} and pEC50 values $\pm$ s.e.m. are summarized in Table II. (B) Antagonism of 5-HT-mediated (10 nM) Ca^{2+} response by indicated ligands. Ca^{2+} formation is expressed as a percentage of the respective maximal inhibition by SB-271046 of Ca^{2+} formation as induced by 10 nM 5-HT. Mean I_{max} and pIC50 values $\pm$ s.e.m. are summarized in Table II.

Interestingly, maximal Ca^{2+} response of Cpd. 1 was similar to that of 5-HT while its potency was lower as compared to 5-HT, and E-6801 was also less potent but also less efficacious. Paradoxically, it behaved as a full agonist when

cAMP formation was analyzed. Compound 2 fully antagonized the 5-HT-induced Ca^{2+} response with potency between SB-271046 and Ro 66-0074 (Fig. 2B and Table II) and similar to Ro 04-6790. We also observed that SB-271046 and Ro 66-0074 antagonized, with similar potency, both 5-HT-mediated cAMP and Ca^{2+} responses. However, Ro 04-6790 appeared to be more potent inhibiting Ca^{2+} response (Table II) than the cAMP response (Table I). Therefore, we suggest that the pharmacology of cAMP and Ca^{2+} responses as mediated by the $5\text{-}HT_6$ receptor in HEK-293F cells is dissimilar. This contrasts with a previous report (Zhang *et al.*, 2003) where coupling efficacy of Ca^{2+} responses via a Gαq/Gαs chimera protein was found to be similar to that observed when native coupling to Gαs was evaluated by cAMP accumulation studies. We used a Gα15 protein instead of a Gαq/Gαs chimera protein to monitor Ca^{2+} responses and this can account for the apparent discrepancy as the use of a different Gα protein may result in different coupling efficacy when comparing cAMP and Ca^{2+} signaling. However, in our opinion, the results of these studies are largely conditioned by the selection of $5\text{-}HT_6$ receptor ligands being investigated. In our hands, the herein analyzed $5\text{-}HT_6$ receptor ligands Cpd. 2, WAY181187, and Ro 04-6790 display distinct pharmacological responses when cAMP and Ca^{2+} signaling pathways are compared.

Differential stimulus-mediated activation pathways can occur through a strength-of-signal type of mechanism (see Kenakin, 1995; Maudsley *et al.*, 2005), i.e., a highly efficacious agonist (i.e., 5-HT and to a lesser extent Cpd. 1 and E-6801) might activate two pathways (cAMP and Ca^{2+} responses), whereas a weaker agonist (i.e., Cpd. 2) may activate only the more sensitive one (cAMP response). On this basis, other agonists may be full agonists for the cAMP and partial for the Ca^{2+} response (i.e., WAY-181187). Interestingly, WAY-181187 was recently classified as a partial agonist in a GTPγS-based assay (Dupuis *et al.*, 2008), in agreement with the Ca^{2+} response shown here. Other examples of ligand-selective GPCR regulation include ligands that promote coupling to one G protein pool while antagonizing coupling to another. The histamine H_3 ligands GT-2331 and proxifan showed weak partial agonist activity and were more effective antagonizing the *R*-α-methylhistamine-mediated increase in intracellular Ca^{2+} levels. Otherwise, they were equally efficacious agonists compared with *R*-α-methylhistamine in signaling through endogenous Gαi to inhibit forskolin-stimulated cAMP production (Krueger *et al.*, 2005). The gonadotropin-release hormone (GnRH) receptor antagonist Ant135-25 not only acts as an antagonist with respect to Gq coupling by the GnRH receptor but also functions as an agonist in cellular contexts where the receptor is coupled to Gi (Maudsley *et al.*, 2004). Similarly, the β_2-adrenergic receptor antagonist ICI-118-551, which behaves as an inverse agonist for coupling to Gs, was recently found to act as an agonist for β_2-adrenergic receptor coupling to Gi (Gong *et al.*, 2002). These examples of GPCR function

suggest that antagonism of receptors can be permissive (see Kenakin, 2005), in that some, but not all, receptor-mediated signals can be blocked. This is different from the conventional view that antagonists occupy the endogenous agonist-binding site to produce blockade of effect and yield a uniform result, namely an inoperative (with respect to the agonist) receptor. In these terms, Kenakin (2005) proposed that these antagonists would be termed as non-permissive in that they do not allow any agonist signal to pass. The herein presented results for Ro 66-0074 and SB-271046 suggest that these ligands act as non-permissive 5-HT$_6$ receptor antagonists for both cAMP and Ca^{2+} responses, while Cpd. 2 is rather a permissive 5-HT$_6$ receptor antagonist for the Ca^{2+} response. Besides agonist-selective receptor signaling (Kenakin, 1995), an antagonist may also block selectively a subset of the signaling pathways elicited by an agonist. This phenomenon reported as signal-selective antagonism (see Maudsley *et al.*, 2005) has been documented previously for cholecystokinin-B receptor with L 365,260 which antagonized all receptor responses while RB 213 was very different as antagonist toward inositol phosphate production and arachidonic acid release (Pommier *et al.*, 1999). In the results reported here, Ro 04-6790 showed a more potent inhibition of Ca^{2+} response than that observed when analyzing cAMP formation. Similarly, SB-271046 appeared as a more potent antagonist of the 5-HT-mediated Ca^{2+} response *via* a chimeric Gαq/αs protein (Zhang *et al.*, 2003). Therefore, Ro 04-6790 do not always antagonize the 5-HT$_6$ receptor-mediated response in the same way and it can also be considered as an example of signal-selective antagonism *via* the 5-HT$_6$ receptor.

Pharmacological differences in 5-HT$_6$ receptor-mediated signaling may also be indicative of the presence of alternative receptor conformations (see Kenakin, 2002). Interestingly, we found differences in the kinetics of the Ca^{2+} activation profile by Cpd. 2 as compared to either 5-HT or Cpd. 1 (Fig. 3). This is likely to reflect two distinct receptor conformations; one for Cpd. 2 and another one for both 5-HT and Cpd. 1. Similar observations on kinetic Ca^{2+} profiles have previously been reported for distinct series of dopaminergic agonists interacting with the dopamine D2 short receptor (Pauwels *et al.*, 2001). This can be explained if the receptor conformations by 5-HT and Cpd. 1 are also different; however, the resolution capacity of the current observations with the Ca^{2+} kinetics does not allow the possibility to obtain such a conclusion. Moreover, the chemical structure of Cpd. 1, but not of Cpd. 2, resembles that of 5-HT. Either clear differences in efficacy or relative potency offer evidence for the presence of alternative receptor conformations (Kenakin, 2003). Thus, one possible explanation for the different 5-HT$_6$ receptor-mediated signaling systems by Cpd. 2 and Cpd. 1 is the presence of different ligand-bound active state conformations that couple differentially to the signaling systems. This could occur by differential ligand-selection

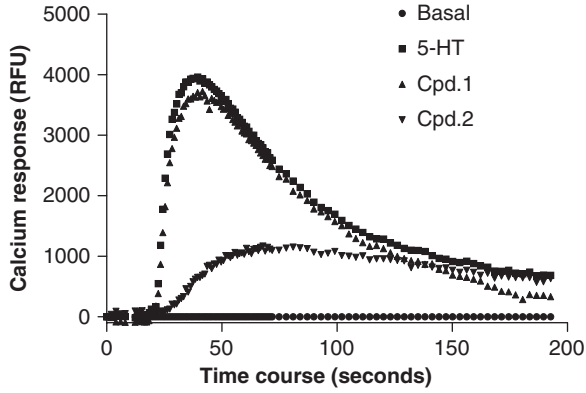

FIG. 3. Time course of 5-HT, Cpd. 1, Cpd. 2 mediated Ca++ responses in Gα15 protein transfected HEK-293F/h5-HT_6 cells. HEK-293F cells stably transfected with h5-HT_6 receptor were co-transfected with Gα15 protein. Ca++ responses were determined in presence of indicated ligands (1 μM) upon overnight serum-free incubation using FLIPR. Time–response curves are shown as mean ± s.e.m. from three experiments performed in duplicate.

or ligand-induction of multiple active states that are capable of differential signaling. Unfortunately, the herein investigated number of compounds is too small and do not allow to provide evidence for a distinct rank order of agonist potency between both signaling pathways.

Very recently, an optimized assay system using HEK293 cells transiently co-transfected with the Gαs-coupled 5-HT_6 receptor and the chimeric G-protein $G\alpha_{qG66Ds5}$ provided another possible functional method for HTS using Ca^{2+} response instead of cAMP (Kim *et al.*, 2008).

The finding that selective ligands can induce two or maybe more functionally distinct receptor conformations suggests the possibility to develop compounds that change the quality as well as the quantity of efficacy as discussed by Kenakin (2002). Furthermore, it appears that the terms "agonist" and "antagonist" for a given receptor ligand are highly dependent on the experimental model system and in particular on the effector pathway that is monitored. Maudsley *et al.* (2005) recently suggested that all ligands that productively engage a GPCR have the potential to be "pluriprotean," acting as agonist or antagonist depending on the signaling function and the nature of the cellular environment.

In conclusion, data presented here support the view that the pharmacology of the 5-HT_6 receptor agonists and inverse agonists/antagonists may change depending on the experimental read-out (i.e., either cAMP or Ca^{2+} signaling pathway) that is monitored.

IV. Functional 5-HT$_6$ Receptor/Fyn Tyrosine Kinase Interaction

Fyn is a member of the Src family of non-receptor protein-tyrosine kinases (Semba *et al.*, 1986). It is primarily localized in the cytoplasmatic leaflet of the plasma membrane where it phosphorylates tyrosine residues on key targets involved in a variety of different signaling pathways. Two main isoforms have been identified, with a preferential distribution of isoform 1, Fyn(B), in the brain and isoform 2, Fyn(T), in T-cells. High expression of Fyn(B) has been reported in neurons, glia (or oligodendrocytes), and several central nervous system deficiencies (i.e., impaired memory or defective long-term potentiation) were demonstrated in fyn$^-$ mutant mice. Fyn has been described to play a role in Alzheimer's disease (Bhaskar *et al.*, 2005; Chin *et al.*, 2005), addiction (Boehm *et al.*, 2003; Lee and Messing, 2008), pain (Abe *et al.*, 2005), or allergy (Parravicini *et al.*, 2002; Rivera and Gilfillan, 2006). It has also been described a role of Fyn in the signaling pathways downstream several receptors, i.e., 5-HT$_4$, 5-HT$_7$, D$_1$, D$_2$, or NMDA (Dunah *et al.*, 2004; Hattori *et al.*, 2006; Norum *et al.*, 2003; Schumann *et al.*, 2009).

Recently, a Fyn interaction with the 5-HT$_6$ receptor was described (Yun *et al.*, 2007), suggesting a novel cellular mechanism of signal transduction for this receptor. In this work, using co-localization assays, 5-HT$_6$ receptor was demonstrated to be physically associated with Fyn, and their activities reciprocally modulated through a specific interaction between the carboxy terminus of 5-HT$_6$ receptor and the Fyn-SH3 domain. Fyn increased the surface expression of 5-HT$_6$ receptor, which in turn increased Fyn phosphorylation. More recently, a physical interaction between 5-HT$_6$ receptor and the Jun activation domain-binding protein-1 (Jab-1), using different experimental approaches, has also been described (Yun *et al.*, 2010). In this study, Jab-1 was shown to be involved in the modulation of the expression and activity of the 5-HT$_6$ receptor, and 5-HT$_6$ receptor has been shown in turn to have an effect over the distribution of Jab-1.

It is well known that 5-HT$_6$ receptor activation leads to the activation of the gene expression regulator CREB (Silva *et al.*, 1998). CREB is a constitutive transcription factor with several representatives. CREB1 is regulated by the phosphorylation in position Ser133, promoting gene transcription (Meyer and Habener, 1993; Montminy *et al.*, 1990). This phosphorylation can be induced by different protein kinases, including the cAMP-dependent protein kinase (PKA) (Sheng *et al.*, 1991). As previously mentioned, 5-HT$_6$ receptor activates the MEK1-ERK signaling pathway through Fyn protein (Yun *et al.*, 2007), and it has been recently reported that the ERK signaling seems to be involved in the memory enhancing properties of some 5-HT$_6$ receptor antagonists (Marcos *et al.*, 2010).

There is evidence supporting an influence of 5-HT_6 receptor on cholinergic neurotransmission either *in vivo* or *in vitro*. Initial studies using antisense oligonucleotides designed to block the translation of 5-HT_6 receptors (Bourson *et al.*, 1995) or using 5-HT_6 receptor antagonists (Bentley *et al.*, 1999; Sleight *et al.*, 1998) showed an increase in the number of yawns or stretches in rats. These behaviors are highly dependent on the cholinergic system and were reversed by the muscarinic antagonists, atropine, and scopolamine. At neurochemical level, using the microdialysis technique in freely moving rats (Riemer *et al.*, 2003), the selective 5-HT_6 receptor antagonist Cpd. 11 (4-(2-Bromo-6-pyrrolidin-1-ylpyridine-4-sulfonyl)phenylamine) increased acetylcholine release, suggesting that 5-HT_6 receptors mediate inhibition of the serotoninergic input to the cholinergic innervation of the frontal cortex. Blockade of 5-HT_6 receptors by Cpd. 11 reduces such inhibitory input. More recently, the involvement of 5-HT_6 receptor in the modulation of acetylcholine release has been reported using an *in vitro* experimental approach (Marcos *et al.*, 2006)

Based on the above-mentioned results, we decided to evaluate the influence of inhibiting either cAMP- or Fyn-signaling pathway on the levels of *in vitro* acetylcholine release (*manuscript in preparation*). Previous studies showed that E-6801 was able to increase acetylcholine release in rat frontal cortex using brain slices (data not shown). In a second study we further confirmed this activity, and compared it with the activity of the reference 5-HT_6 receptor antagonist SB-271046. Intriguingly, both compounds were able to increase acetylcholine release in rat frontal cortex (Fig. 4A). The activity of both compounds was sensitive to the presence of PD 98,056, an inhibitor of the ERK signaling, and to the presence of PP2, a Fyn kinase inhibitor. In the presence of both inhibitors the increase in acetylcholine release induced either by SB-271046 or by E-6801 was blocked (Fig. 4B).

These results point to a cAMP-independent mechanism responsible for acetylcholine release that may partially explain the controversial results obtained either with agonists and with antagonists in cholinergic-mediated activities such as memory improvement in NOD (Kendall *et al.*, 2010).

V. Discussion

Early phases of drug discovery are driven by the characterization, raking, and selection of new molecules based on their affinity and functionality on a selected target. Among those targets, GPCRs are specially relevant because they regulate a huge number of physiological processes and it has been demonstrated that they are easily druggable. Moreover, the number of GPCRs encoded in the human genome is around 1000 (Marchese *et al.*, 1999) and many prescription drugs target GPCRs (Drews, 2000).

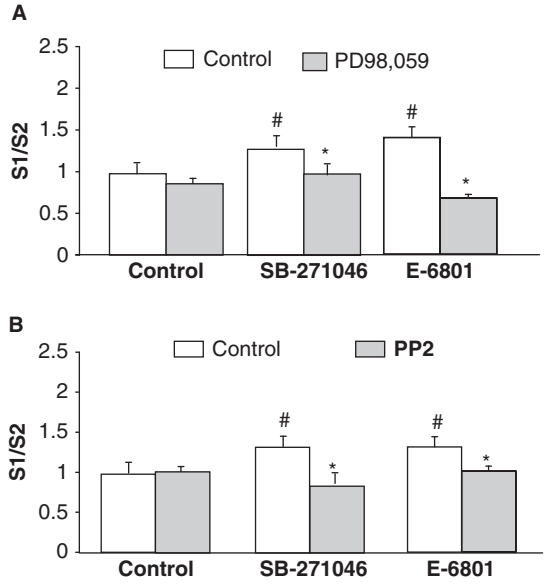

FIG. 4. *In vitro* acetylcholine levels ratio in presence of the 5-HT$_6$ receptor antagonist SB-271046 or the 5-HT$_6$ receptor agonist E-6801, and the inhibition of the effects on Ach release in the presence of (A) the inhibitor PD 98,059 or (B) the fyn inhibitor PP2.

The strategy for hit finding usually focuses on a single functional system, using a single experimental system and a single read-out. However, single-target pathway pleiotropy has been identified, based on the coupling to more than one G-protein, for an increasing number of receptors. Ligands may activate some, but not all, associated cellular pathways (for review see Kenakin, 2008). This selective G-protein activation has received different names including stimulus trafficking, biased agonism, or functional selectivity.

Since the initial *two-state model* of Katz, where a ligand binds to the receptor resulting in the formation of a ligand–receptor complex that generates an active receptor conformation, the models trying to describe the complexity of the GPCRs signal transduction have evolved. The next step was a *simple ternary complex model*, which includes a G protein as a part of the ligand-receptor-transducer complex. This ternary model leads to a *full ternary complex model* and the *extended ternary complex model*, trying to provide a model to explain the high and low affinity states of the receptor and the receptor–transducer complex (De Lean *et al.*, 1980) as well as the discovery of constitutively active seven transmembrane receptor mutants (Samama *et al.*, 1993). Later on, trying to cover the discovery of distinct active and inactive receptor conformations for transducer-bound receptor, the *cubic ternary complex model* was defined (Weiss *et al.*, 1996). More recently,

the need to include the mentioned biased agonism or stimulus trafficking, that is, the fact that receptors can signal through different pathways with different efficacies, the *multiple signaling-competent receptor conformations model* has been proposed (for review see Rajagopal *et al.*, 2010). All these efforts in defining a model are driven by new technological capabilities and the associated increasing knowledge, and point out the complexity of the receptor–signal transduction system relationships, stressing the caution to be taken when discrepancies between *in vitro* classification and *in vivo* activities appear.

When using recombinant systems we should know where it is situated in the *pharmacological volume scale* (Kenakin, 2003). Depending on the efficacy of the coupling system being assayed, a weak partial agonist can demonstrate a competitive antagonism profile in a poorly coupled assay. In a more efficient receptor coupling system, either *in vitro* or *in vivo*, the same partial agonist may be an unexpected agonist, but if the system is much less efficient an agonist may behave as partial agonist or even as an antagonist. The affinity, efficacy, number of receptors present, efficiency of the receptor–effector coupling, effector response measured, and any desensitization that occurs within the time-frame of the measurement should play a role in the final *in vitro* functional profile. But even having all these parameters into account, other factors, like the receptor distribution, splice variants, or the levels of the endogenous ligand may influence the final *in vivo* functional activity.

Several experimental approaches have been reviewed in this chapter, including the more widely used cAMP measurements under distinct experimental conditions (presence/absence of forskolin, rat/human 5-HT_6 receptor, HEK293/CHO cell lines or constitutively active 5-HT_6 receptor mutant), calcium signaling in a chimeric G protein recombinant system, and the fyn kinase pathway. In agreement with the running theories of selective functional efficacy, 5-HT_6 receptor ligands have demonstrated to behave differentially depending on the system and changes in their classification as agonists, partial agonists, antagonists, or inverse agonists have been shown. The 5-HT_6 receptor agonists E-6801 which was initially classified as a partial agonist demonstrated to behave as a full agonist in most of the assayed conditions, but the activity through the fyn kinase pathway is similar to that of the 5-HT_6 receptor inverse agonist SB-271046, pointing to a possible explanation for the *in vivo* similarities found for both compounds.

VI. Conclusion

5-HT_6 receptor functionality is being revealed to be much more complex than initially defined. Based on the existing data, and depending on the agonist used, different selective receptor active states that activate different cellular

pathways may be achieved. Besides Gs-mediated cAMP formation other messenger systems may be involved. The full characterization of the functional profile of 5-HT$_6$ receptor is still pending, and new players in the signaling cascade of 5-HT$_6$ receptor could take the stage in the near future.

The drug discovery process may be highly benefited from this complexity, in terms of quantity and quality of new molecules if, besides selective binding affinity, the selective intrinsic efficacy is taken into account early during the hit finding phase.

Dedicated to Gonzalo Romero, *in memoriam*

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ELECTROPHYSIOLOGY OF 5-HT6 RECEPTORS

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Serotonin (5-HT) exerts its diverse physiological and pharmacological effects through actions on multiple receptor subtypes. One of the newest members of this family is the 5-HT6 receptor, a subtype localized almost exclusively in the central nervous system (CNS) and enriched in brain regions associated with cognition and behavior. With the recent development of selective 5-HT6 receptor drugs, potential functional roles are starting to be identified. The high affinity of a wide range of psychiatric drugs for the 5-HT6 receptor, together with its abundant expression in limbic and cortical regions, has prompted researchers to focus on cognitive and affective disorders. Blockade of 5-HT6 receptors exerts anxiolytic and antidepressant-like effects and leads to an improvement of cognitive performance in a wide variety of learning and memory paradigms. Though these effects seem to be mediated by 5-HT6 receptor-dependent regulation of glutamatergic and cholinergic neurotransmission, to date only a couple of electrophysiological studies have been performed in order to directly investigate the effect of this receptor on neuronal activity.

Here, we will present an overview of the electrophysiological studies performed *in vivo* and *in vitro* to investigate the physiology of 5-HT in different mammalian brain areas, particularly the cortex, striatum, and hippocampus. Then the few available electrophysiological data on the effects of 5-HT6 receptor activation will be discussed in detail. Moreover, the use of electrophysiological approaches for preclinical pharmacology studies on recently developed 5-HT6 receptor drugs will be considered.

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I. Introduction

A large number of serotonin (5-HT) receptors have been identified over the past 10 years. They are currently divided into seven classes (5-HT1 to 5-HT7), based on structural, transductional, and functional features. The existence of a great number of splice and editing variants for several 5-HT receptors, their possible modulation by accessory proteins and chaperones, as well as their potential to form homo- or heteromers suggest an even greater degree of functional diversity. These differences among the subclasses of 5-HT receptors in coupling and signaling, editing, splicing, and tissue distribution presumably serve to finely tune the cellular responses to 5-HT, such as each form of the receptor is probably linked to an exquisitely specific response to 5-HT. Accordingly, a great number of animal and human studies indicate that the 5-HT system regulates emotions, behavioral control, and cognition in a very complex manner. This, in turn, led to the hope that the clinical management of a number of disorders might significantly benefit from subtype-selective serotonergic agents, and indeed experimental evidence suggests that 5-HT receptors may represent therapeutic targets for neurologic and psychiatric diseases. In particular, the 5-HT system innervates brain areas involved in learning and memory processes. These processes underlie normal human behavior, as well as the pathophysiology of addiction, anxiety, depression, schizophrenia, and neurodegenerative diseases (e.g., Parkinson's and Alzheimer's diseases). Hence the search for drugs acting at specific 5-HT receptors aimed at either reversing cognitive deficits or improving residual cognitive function. One of the newest members of the 5-HT receptor family is the 5-HT6 subtype, mostly expressed in brain regions associated with cognition and behavior. With the development of selective 5-HT6 receptor antagonists, preclinical studies in rodents and primates have started elucidating the function of this receptor subtype in more detail. In a wide variety of learning and memory paradigms, blockade of 5-HT6 receptors leads to an improvement of cognitive performance and also results in anxiolytic and antidepressant-like activity. These actions are likely mediated by the reported enhancement of cholinergic, glutamatergic, noradrenergic, and dopaminergic neurotransmission. Moreover, a preliminary report showed that the 5-HT6 receptor antagonist SB-742457 showed cognitive enhancing properties in Alzheimer's disease patients (for review, see Hannon and Hoyer, 2008; King *et al.*, 2008; Kroeze *et al.*, 2002; Upton *et al.*, 2008).

II. Electrophysiological Effects of Serotonin

Much progress has been made in associating the function of many 5-HT receptors with specific physiological responses in the mammalian brain, owing to the development of drugs characterized by 5-HT receptor subtype selectivity.

A noteworthy contribution has derived from some electrophysiological studies performed both *in vivo* and *in vitro* that started to appear around the early 1990s. Important pieces of information on the physiological role of serotonin and its receptor subtypes on specific neuronal cell populations have come from preclinical pharmacology studies, aiming at evaluating the efficacy of novel, putative pharmacological agents (Dremencov *et al.*, 2009; Gronier and Rasmussen, 2003; Haddjeri *et al.*, 1998; Jolas *et al.*, 1994; Marchetti *et al.*, 2004; Passani *et al.*, 1994; Pineyro *et al.*, 1994; Schechter *et al.*, 1990). Many others came from investigations directly designed to study the potential role of 5-HT in the pathophysiology of human disorders. In particular, much effort was conveyed on the study of prefrontal cortex (PFC) neurons. Schizophrenia patients show an abnormal synchronous activity of the PFC, as well as elevated 5-HT1A and reduced 5-HT2A receptor numbers. Moreover, neuronal excitability and synaptic function in the PFC seem to be altered during the development of addictive behaviors, as well as in psychiatric disorders. Puig *et al.* (2010) investigated the role of 5-HT in cortical synchrony by means of *in vivo* recordings from anesthetized rats. These authors found that 5-HT, released by electrical stimulation of the dorsal raphe nucleus (DRN), regulates the frequency and the amplitude of slow (<2 Hz) waves in the PFC via 5-HT2A receptors. Indeed, a discrete subpopulation of pyramidal cells of the PFC is strongly excited by 5-HT2AR activation, leading to an increase in glutamatergic recurrent network activity (Beique *et al.*, 2007). Moreover, electrical stimulation of the DRN also modulated gamma (30–80 Hz) rhythms through both 5-HT1A and 5-HT2A receptors, inducing an overall decrease in the amplitude of gamma oscillations. Most fast-spiking interneurons of the PFC were inhibited by 5-HT through 5-HT1A receptors, while a minority was activated by 5-HT2A receptors. As these interneurons are involved in the generation of gamma waves in the PFC, these authors concluded that 5-HT shapes the frequency and amplitude of slow waves through 5-HT2A receptors, and modulates the amplitude of gamma oscillations by affecting the activity of fast-spiking interneurons through both 5-HT2A and 5-HT1A (Puig *et al.*, 2010). Indeed, several lines of evidence have shown that 5-HT1A and 5-HT2A receptors often have opposing actions on common substrates in PFC. For instance, activation of 5-HT1A receptors results in pyramidal neuron inhibition by increasing potassium currents (Araneda and Andrade, 1991) and decreasing calcium currents (Penington and Kelly, 1990). In contrast, 5-HT2A receptor stimulation leads to neuronal excitation (Beique *et al.*, 2007) by suppressing potassium currents (Andrade, 1998). Moreover, activation of 5-HT2A receptors has been shown to enhance glutamatergic synaptic activity (Aghajanian and Marek, 1999; Beique *et al.*, 2007; Marek and Aghajanian, 1999), while activation of 5-HT1A receptors inhibits *N*-methyl-D-aspartate (NMDA) receptor currents (Yuen *et al.*, 2005). More recently, activation of 5-HT2A/C receptors was found to significantly attenuate the effect of 5-HT1A on NMDA receptor currents (Yuen *et al.*,

2008), suggesting that serotonin, via 5-HT1A and 5-HT2A/C receptor activation, regulates NMDA receptor functions in PFC neurons in an opposite manner. Moreover, the effect of co-activation of 5-HT and NMDA receptors on PFC pyramidal neuron excitability was investigated, by measuring the level of action potential firing elicited by depolarizing current injection (Zhong *et al.*, 2008). In the presence of NMDA, the 5-HT1A agonist 8-OH-DPAT reduced the number of action potentials, whereas the 5-HT2A/C agonist α-Me-5HT significantly enhanced it. Both agonists were ineffective in the absence of NMDA. The 8-OH-DPAT effect on firing was mediated by inhibition of protein kinase A (PKA), whereas the α-Me-5HT effect was mediated by activation of protein kinase C (PKC). Both 5-HT1A and 5-HT2A/C receptor-mediated modulation of neuronal excitability involved the extracellular signal-regulated kinase (ERK) (Derkach *et al.*, 1989) in opposite manners, in that 5-HT1A decreased, whereas 5-HT2A/C increased, the activation of ERK in an NMDA-dependent manner. Interestingly, in animals acutely exposed to stress, the enhancing effect of 5-HT2A/C on firing was reported to be lost, while the decreasing effect of 5-HT1A on firing was still intact (Zhong *et al.*, 2008). A similar bidirectional modulatory effect on neuronal responses has been reported in the macaque visual cortex. *In vivo* electrophysiological experiments have shown modulatory effects of 5-HT1B and 5-HT2A receptor agonists on the responses of primary visual area neurons (Watakabe *et al.*, 2009). The effect of 5-HT1B activation tended to be facilitative for neurons with a high firing rate, and suppressive for those with a low firing rate, whereas 5-HT2A activation showed opposite effects.

While activation of 5-HT2A receptors has been shown to enhance glutamatergic synaptic activity in the PFC in physiological conditions (Aghajanian and Marek, 1999; Beique *et al.*, 2007; Marek and Aghajanian, 1999), recent work showed that repeated cocaine administration resulted in an attenuation of this effect (Huang *et al.*, 2009). Interestingly, repeated cocaine administration was not associated with any changes in the levels of 5-HT2A receptors or regulator of GTP-binding protein signaling, thus suggesting that cocaine-induced inhibition of 5-HT2A receptor-mediated enhancement of glutamatergic transmission may be caused by an impaired coupling of 5-HT2A receptors with GTP-binding proteins during cocaine withdrawal (Huang *et al.*, 2009). A similar enhancing effect of 5-HT on glutamatergic postsynaptic potentials/currents was characterized in layer V pyramidal neurons from different cortical areas by means of intracellular and whole-cell recordings in rat brain slices (Aghajanian and Marek, 1997). These authors suggested that 5-HT, via 5-HT2A receptors, may enhance spontaneous glutamatergic synaptic events through a tetrodotoxin (TTX)-sensitive focal action in the apical dendritic field involving both pre- and postsynaptic mechanisms, the latter involving 5-HT-mediated enhancement of a subthreshold TTX-sensitive sodium current. A novel mechanism by which 5-HT2 receptors have been reported to regulate synapses is by evoking endocannabinoid release, similarly to

other types of Gq/11-coupled receptors, and in turn activating presynaptic cannabinoid type 1 (CB1) receptors. Indeed it has been shown that activation of postsynaptic 5-HT2 receptors expressed on soma and dendrites of inferior olive neurons causes the release of endocannabinoids retrogradely activating presynaptic CB1 receptors, and, in turn, suppressing glutamate release (Best and Regehr, 2008).

The enhancing effect of 5-HT on spontaneous glutamatergic synaptic events is selectively observed in some neuronal populations. By means of intracellular recordings performed with electrodes containing biocytin, in order to label and identify the recorded cell, Lambe *et al.* (2000) found that pyramidal cells in layer V of the frontal cortex showed the greatest 5-HT-induced increase in both the frequency and amplitude of spontaneous excitatory postsynaptic currents, whereas only a small portion of neurons in layer II/III showed an increase in spontaneous glutamatergic current frequency, and these events were not affected by 5-HT in layer VI. Moreover, in layer II pyramidal neurons of the pyriform cortex (Gellman and Aghajanian, 1994; Sheldon and Aghajanian, 1990) and dentate gyrus of the hippocampus (Piguet and Galvan, 1994), 5-HT2A receptors were reported to induce an increase in spontaneous inhibitory postsynaptic potentials.

As discussed for cortical areas, in the hippocampus, 5-HT exerts both receptor- and neuron-specific actions. In the dentate gyrus, by means of conventional intracellular recordings from brain slices, Piguet and Galvan (1994) found that 5-HT hyperpolarizes granule cells via postsynaptic 5-HT1A receptors and increases spontaneous gamma-aminobutyric acid (GABA) release from inhibitory interneurons via the activation of 5-HT3 and/or 5-HT2 receptors. In the CA1 area, 5-HT1B receptors are responsible for the presynaptic inhibition of local excitatory synapses exerted by 5-HT (Mlinar *et al.*, 2003). Moreover, patch-clamp recordings from CA1 slices showed that 5-HT1B receptors, activated by endogenous 5-HT, released by 3,4-methylenedioxymethamphetamine (MDMA), reduced the excitatory synaptic transmission between pyramidal neurons (Mlinar and Corradetti, 2003). Some authors also investigated the role of 5-HT1A receptors on CA1 pyramidal neuron function. Activation of 5-HTlA receptors leads to membrane hyperpolarization and inhibition of cell firing (Andrade and Nicoll, 1987). By means of extracellular and intracellular recordings from slices, Schmitz and co-workers showed that 5-HT acts at presynaptic 5-HT1A receptors to reduce calcium entry and thereby glutamatergic synaptic transmission. Moreover, these authors showed that 5-HT1A activation is also involved in the indirect modulation of inhibitory postsynaptic potentials by acting on inhibitory interneurons (Schmitz *et al.*, 1995a, 1995b).

The thalamus represents another example of the complexity of actions of 5-HT in the brain, as serotonergic inputs have been shown to act differentially across the thalamus in a complex manner involving direct and indirect

mechanisms. Monckton and McCormick (2002) examined the action of 5-HT in several different regions of the ferret dorsal thalamus, using *in vitro* slice preparations and intracellular recording techniques. In nearly all nuclei examined, the predominant action of serotonin was a hyperpolarization and inhibition of tonic firing. The magnitude of the hyperpolarizing response decreased with age and varied greatly across and somewhat within nuclei. This hyperpolarizing response was elicited through both a direct mechanism, involving 5-HT1A receptor and an increase in potassium conductance, and an indirect mechanism, via excitation of local interneurons, causing an increase in the frequency and amplitude of spontaneous inhibitory postsynaptic potentials occurring in thalamocortical neurons. A very recent characterization study further confirmed this complexity by analyzing the effects of 5-HT on relay cells in various first-order and higher-order thalamic nuclei using rat thalamic brain slices and whole-cell, current- and voltage-clamp, recordings (Varela and Sherman, 2009).

Another level of the complex modulatory role exerted by 5-HT is represented by the regulation of synaptic plasticity. In the rat visual cortex an increase in 5-HT levels is correlated with the developmental decrease in long-term potentiation (LTP). *In vitro* extracellular recordings of field potentials evoked in layer II/III by stimulating the underlying layer IV have shown an NMDA receptor-dependent LTP induced by theta-burst stimulation in slices from 3-week-old rats; this form of plasticity was absent in slices from 5-week-old rats (Kim *et al.*, 2006). Parachloroamphetamine-mediated 5-HT depletion restored LTP in slices from older rats. Moreover, the restored LTP was inhibited by 5-HT, as well as by co-application of 5-HT1A and 5-HT2 receptor agonists. These observations suggested that NMDA receptor-dependent LTP was specifically inhibited in the rat visual cortex by the increase in 5-HT levels observed during the critical period causing co-activation of 5-HT1A and 5-HT2 receptors (Kim *et al.*, 2006).

Similar to what was observed in the visual cortex, in the rat hippocampus 5-HT has been reported to modulate LTP. *In vitro* extracellular recordings from rat hippocampal slices showed that 5-HT was able to block LTP induced by primed or theta burst stimulation in the CA1 area (Corradetti *et al.*, 1992; Staubli and Otaky, 1994). Conversely, controversial experimental evidence exists about *in vivo* 5-HT modulatory effect on LTP in the dentate gyrus. While in a previous study LTP recorded from the dentate gyrus was reduced after selective depletion of 5-HT (Bliss *et al.*, 1983), a more recent investigation showed that *in situ* administration of 5-HT4 agonists dose-dependently inhibited both basal synaptic transmission and LTP (Kulla and Manahan-Vaughan, 2002). Another *in vivo* study suggested that the induction of LTP at perforated path-dentate gyrus synapses is promoted by activation of 5-HT2C receptors in the basolateral amygdala (Abe *et al.*, 2009).

Electrophysiological techniques have been useful to clarify the mechanisms of the physiological effects of 5-HT receptor subtype activation. An increase in

potassium conductance or a decrease in calcium currents has been shown to mediate the hyperpolarizing effects of specific 5-HT receptor subtypes (Araneda and Andrade, 1991; Penington and Kelly, 1990; Sprouse and Aghajanian, 1987), whereas a decrease in potassium conductance mediates the depolarizing effects (Andrade, 1998; North and Uchimura, 1989). In intralaminar and midline thalamic neurons, 5-HT7 receptor activation was shown to inhibit the calcium-activated potassium conductance that is responsible for the slow afterhyperpolarization (sAHP) following a spike discharge, an effect mediated by the cAMP second-messenger cascade (Goaillard and Vincent, 2002). Moreover, in the anterodorsal nucleus of the thalamus, this receptor subtype also elicits a membrane depolarization by modulating the hyperpolarization-activated nonselective cation current (Ih), through a cAMP-dependent but PKA-independent mechanism (Chapin and Andrade, 2001). Two parallel studies showed that activation of postsynaptic 5-HT7 receptors depolarizes globus pallidus neurons by enhancing Ih (Chen *et al.*, 2008; Hashimoto and Kita, 2008). Recent studies investigated the ion currents involved in the excitatory effect exerted by 5-HT2C receptor activation on striatal interneurons (Blomeley and Bracci, 2009; Bonsi *et al.*, 2007). Voltage-clamp experiments from rat brain slices revealed that 5-HT2C-mediated effects involved blockade of potassium channels in both cholinergic and fast-spiking interneurons. In the latter neuronal population Kir channels were shown to be inhibited by 5-HT2C receptor activation (Blomeley and Bracci, 2009). In cholinergic interneurons, though three different 5-HT receptor subtypes contributed to the modulatory effect on potassium current, the analysis of the 5-HT-sensitive current recorded after HCN channel blockade by either ZD 7288 or cesium suggested that 5-HT may act on Kir channels, while the residual cesium-insensitive potassium conductance was possibly carried by Kleak channels (Bonsi *et al.*, 2007). Similarly, closure of Kleak channels mediated the 5-HT-induced effects in dorsal vagal neurons and trigeminal and spinal cord motoneurons (Hopwood and Trapp, 2005; Hsiao *et al.*, 1997; Kjaerulff and Kiehn, 2001), and 5-HT has been reported to reduce a Kir conductance in neurons from caudal raphe and nucleus accumbens as well as motoneurons of the spinal cord (Bayliss *et al.*, 1997; Kjaerulff and Kiehn, 2001; North and Uchimura, 1989).

III. Anatomy and Physiology of 5-HT6 Receptors

The 5-HT6 receptor has only recently been discovered (Monsma *et al.*, 1993; Ruat *et al.*, 1993). Autoradiographic binding studies in the rat brain using the highly selective 5-HT6 receptor antagonist radioligand [^{125}I]SB258585 have demonstrated a high level of specific binding within striatum, nucleus accumbens,

islands of Calleja, and olfactory tubercle, moderate level in the hippocampal formation, cerebral cortex, thalamus, hypothalamus, and substantia nigra, and very low levels in the globus pallidus, cerebellum, other mesencephalic regions, and the rhombencephalon. These results are in accordance with mRNA and immunolabeling localization studies, overall showing an almost exclusive localization within the CNS, and a particular abundance in regions playing a key role in cognitive processes (for review, see: Hannon and Hoyer, 2008; Upton *et al.*, 2008). Indeed, both rat and human 5-HT6 receptor mRNA is primarily postsynaptically located in the striatum, amygdala, nucleus accumbens, hippocampus, cortex, and olfactory tubercle. Immunohistochemical studies have demonstrated the highest 5-HT6 receptor expression in the striatum, nucleus accumbens, olfactory tubercle, and cortex, with moderate expression in the amygdala, hypothalamus, thalamus, cerebellum, and hippocampus. Electron microscopic analysis revealed receptor staining primarily on dendritic and cilia processes, with little expression on cell bodies.

5-HT6 receptors show desirable characteristics for preclinical studies (for review, see: Hannon and Hoyer, 2008; Upton *et al.*, 2008). Preclinical and clinical studies on 5-HT6 receptor antagonists showed an absence of unwanted peripheral side effects, likely due to the apparent lack of expression of this receptor subtype in the periphery. Moreover, the human gene has 89% sequence homology with the rat ortholog and both the pattern of expression of 5-HT6 receptors as well as the potency of 5-HT6 receptor antagonists are similar between human and rat.

Activation of the 5-HT6 receptor indirectly regulates a variety of neurotransmitters, including acetylcholine (ACh), glutamate, and dopamine, in a brain-region-specific manner (for review, see: King *et al.*, 2008). A postsynaptic localization of 5-HT6 receptors is further supported by studies with the 5-HT neurotoxin 5,7-dihydroxytryptamine (5,7-DHT), which did not alter either hippocampal or striatal 5-HT6 mRNA (Gerard *et al.*, 1996). Interestingly, 5-HT6 receptor seems to co-localize extensively with glutamic acid decarboxylase. Indeed, within the striatum, 5-HT6 receptor mRNA is highly expressed on GABAergic medium spiny neurons (Ward and Dorsa, 1996) (Fig. 1). Conversely, both immunohistochemical analysis and studies lesioning cholinergic neurons with 192 IgG-saporin suggested a low level of 5-HT6 receptor expression on cholinergic neurons (Marcos *et al.*, 2006; Ward and Dorsa, 1996). However, an investigation utilizing a combination of pharmacological, electrophysiological, and molecular approaches demonstrated unequivocally the presence and functionality of 5-HT6 receptors in striatal cholinergic interneurons (Bonsi *et al.*, 2007) (Fig. 1). This study investigated the effects of 5-HT on these neurons in rat brain slices, by means of both conventional intracellular and whole-cell patch-clamp recordings. During current-clamp recordings, bath-applied 5-HT induced a dose-dependent membrane depolarization and increased the frequency of ongoing action

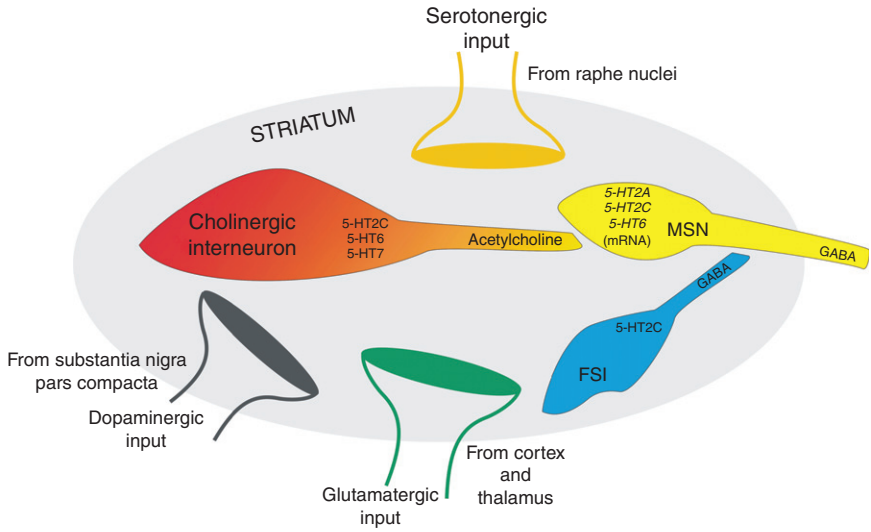

FIG. 1. Striatal neuronal subtype-specific expression of functionally characterized 5-HT receptors. Cartoon of a simplified representation of the physiology of striatum. Three neuronal subtypes are represented: GABAergic fast-spiking interneurons (FSIs), cholinergic interneurons, and medium spiny neurons (MSNs), the GABAergic projection neurons. The recently characterized functional expression of 5-HT receptor subtypes in FSIs (Blomeley and Bracci, 2009) and cholinergic interneurons (Bonsi *et al.*, 2007) is depicted. Similar electrophysiological studies on MSNs are still lacking.

potential firing. This effect was also observed upon 5-HT reuptake blockade with either citalopram or fluvoxamine, which causes an increase in endogenous 5-HT level. The depolarizing response to 5-HT was still observed in the presence of TTX, or blockers of both ionotropic glutamate receptors and GABAA receptors, indicating that 5-HT was acting at postsynaptic sites. In voltage-clamped neurons, 5-HT application induced an inward current, whose reversal potential was close to the potassium equilibrium potential. Accordingly, the involvement of potassium channels was confirmed both by increasing extracellular potassium concentration and by blockade of potassium channels with barium. Single-cell reverse transcription polymerase chain reaction (RT-PCR) profiling demonstrated the presence of 5-HT2C, 5-HT6, and 5-HT7 receptor mRNAs in identified cholinergic interneurons (Fig. 1). Accordingly, the depolarization/inward current induced by 5-HT was partially mimicked by the 5-HT2 receptor agonist 2,5-Dimethoxy-4-iodoamphetamine hydrochloride (DOI), and antagonized by both ketanserin and the selective 5-HT2C antagonist RS102221, whereas the selective 5-HT3 and 5-HT4 receptor antagonists tropisetron and RS23597-190 had no effect. The depolarizing response to 5-HT was also reduced by the selective 5-HT7 receptor antagonist SB269970 and

mimicked by the 5-HT7 agonist 5-CT, which, in the presence of the 5-HT1B antagonist isamoltane, induced an inward current. Similarly, the depolarizing response to 5-HT was reduced by the selective 5-HT6 receptor antagonist SB258585. Moreover, application of 5-HT, in the presence of RS102221 plus SB269970 to selectively activate 5-HT6 receptor, caused an increase in input resistance and induced an inward current. The digitally subtracted current induced by 5-HT in the presence of RS 102221 and SB 269970 showed a linear current–voltage relationship, with a mean reversal potential of ~−90 mV. These data are consistent with the major involvement of a reduction of potassium conductance in the 5-HT6 receptor-induced inward current (Bourson *et al.*, 1998). The 5-HT response was attenuated by U73122, blocker of phospholipase C, and by SQ22,536, an inhibitor of adenylyl cyclase. These results suggest that 5-HT released by serotonergic fibers originating in the raphe nuclei has a potent excitatory effect on striatal cholinergic interneurons. Growing evidence indicates that the striatal serotonergic innervation contributes to motor function. In Parkinson's disease, striatal levels of 5-HT fall in parallel with those of dopamine, potentially contributing to motor and affective symptoms (Halliday *et al.*, 1990; Sandyk and Fisher, 1988). Moreover, selective 5-HT reuptake inhibitors, widely used to treat depression, have been reported to induce a variety of movement disorders, including tremor, parkinsonism, and dystonia (Caley, 1997; Leo, 1996). These data shed further light on the involvement of 5-HT6 receptor in the control of cholinergic transmission in the striatum. Previous reports suggested that 5-HT6 receptor blockade might induce an increase in ACh release, or, alternatively, that interactions between 5-HT6 and muscarinic receptors expressed on spiny neurons in the striatum might modulate GABA neurotransmission (Bourson *et al.*, 1998; Gerard *et al.*, 1997). However, the molecular and electrophysiological identification of functional 5-HT6 receptors depolarizing cholinergic interneurons suggests a different mechanism for this modulatory effect in the striatum. In particular, these observations demonstrate that 5-HT6 receptor activation contributes to 5-HT-induced excitatory effect on striatal cholinergic interneurons, mainly by acting on potassium currents, and support the existence of an endogenous serotonergic tone directly modulating striatal cholinergic function (Bonsi *et al.*, 2007). This modulatory activity is likely contributing to influence the overall striatal output. Indeed, though cholinergic interneurons account for a small portion of the entire neuronal population, the striatum is one of the brain areas with the highest ACh content (Izzo and Bolam, 1988). Accordingly, striatal cholinergic transmission has been implicated as a key player in striatal function (synaptic plasticity and motor learning), and dysfunction (increased ACh release in Parkinson's disease and dystonia, fall in striatal cholinergic markers in Huntington's disease, and progressive supranuclear palsy) (Pisani *et al.*, 2007) (Fig. 2).

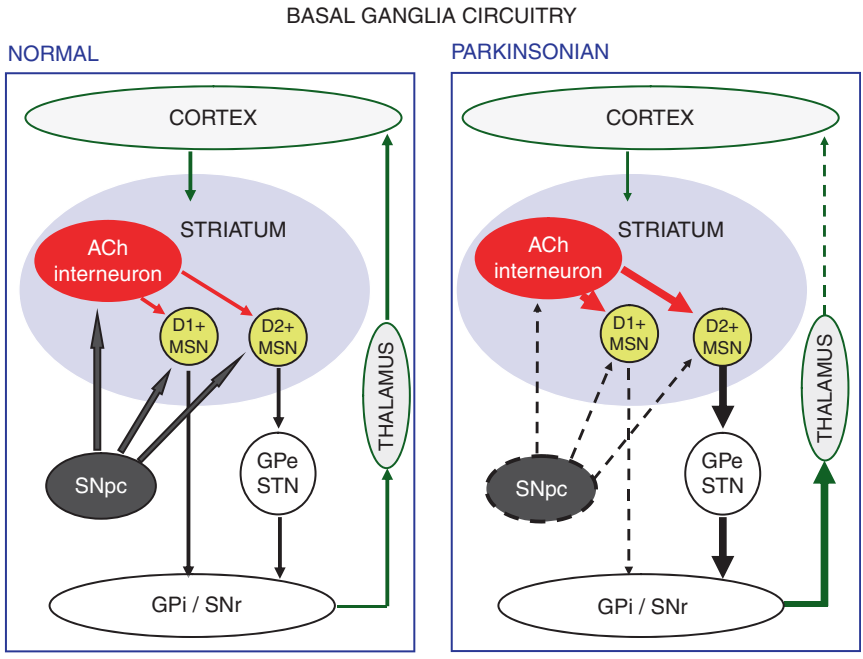

FIG. 2. Schematic representation of the basal ganglia circuitry. The striatum is the main input station of the basal ganglia circuitry. In several pathologic conditions its acetylcholine (ACh) content becomes altered, leading to an unbalanced GABAergic output and, eventually, to abnormal movement control. In Parkinson's disease, the reduced dopaminergic input from the degenerating substantia nigra pars compacta (SNpc) to the striatum leads to an increased ACh release from cholinergic interneurons, and a disinhibition of medium spiny neurons (MSNs) projecting to the external portion of the globus pallidus (GPe) and the subthalamic nucleus (STN). This causes, in turn, an increased inhibitory output from the basal ganglia output stations (internal portion of the globus pallidus, GPi, and substantia nigra pars reticulata, SNr) to the thalamus, and a reduced excitatory drive to the cortex, resulting in hypokinesia and parkinsonism.

Several microdialysis studies (for review see: Upton *et al.*, 2008) have demonstrated that 5-HT6 receptor blockade by systemic administration of selective 5-HT6 receptor antagonists is able to increase the release of ACh, glutamate, dopamine, and noradrenaline in freely moving rats, whereas 5-HT6 receptor activation increases GABA levels. As several studies showed that 5-HT6 receptors are expressed on GABAergic neurons, it has been suggested that blockade of these receptors, by removing the GABAergic inhibition on downstream neurons, may result in enhanced neurotransmission of glutamate, and, possibly, contribute to an increased ACh release. A recent electrophysiological investigation confirmed previous neurochemical observations showing that the selective 5-HT6

agonist, WAY-181187, increased the frequency of spontaneous GABA release in area CA1, as assessed by measuring spontaneous inhibitory postsynaptic currents by means of whole-cell patch-clamp recordings from brain slices (West *et al.*, 2009). This increase in GABA spontaneous transmission was prevented by the selective 5-HT6 antagonist SB-399885. Moreover, these authors investigated the effect of 5-HT6 receptor activation on long-term potentiation, utilizing extracellular recordings. WAY-181187 had no effect on baseline synaptic transmission. Conversely, the selective 5-HT6 agonist attenuated LTP induced by theta burst stimulation. This effect was dose-dependently blocked by the selective 5-HT6 antagonist, SB-399885. These effects of the 5-HT6 receptor in hippocampal area CA1 may underlie the cognition enhancing effects of 5-HT6 antagonists (West *et al.*, 2009). Indeed, 5-HT6 receptor has been implicated in the regulation of cognitive function, as antagonists of the 5-HT6 receptor improve cognitive performance in a number of preclinical models and have recently been found to be effective in Alzheimer's disease patients.

IV. Preclinical Pharmacology of 5-HT6 Receptor Modulators

As soon as the 5-HT6 receptor was discovered, antipsychotics and antidepressants were found to have high affinity for this new receptor subtype. Afterward, a number of potent and selective 5-HT6 receptor antagonists became available. Conversely, there is not a large availability of selective agonists to date (Hannon and Hoyer, 2008; Upton *et al.*, 2008).

The ability of 5-HT6 receptor blockade to elevate cholinergic neurotransmission in the PFC and the dorsal hippocampus observed in *in vivo* studies is in line with the ability of 5-HT6 receptor antagonists to reverse learning and memory deficits induced by cholinergic antagonists. However, the procognitive properties of 5-HT6 receptor antagonists might be due to the glutamate-enhancing effect of 5-HT6 receptor antagonism, as well. In fact, SB-271046 also induces the release of the excitatory neurotransmitter glutamate in the dorsal hippocampus and both glutamate and aspartate in the frontal cortex (Dawson *et al.*, 2000; 2001). These different mechanisms of action of 5-HT6 receptor antagonists should be activated independently, and therefore it is possible that 5-HT6 receptor antagonists might be effective also in case of cholinergic neuron demise, such as in Alzheimer's disease (for review see: Upton *et al.*, 2008). Moreover, a study suggested that 5-HT6 receptor antagonists might induce dopamine release in the striatum through the modulation of ACh (Sleight *et al.*, 1998). A multidisciplinary study characterized the action of a potential antipsychotic, FMPD (6-fluoro-10-[3-(2-methoxyethyl)-4-methyl-piperazin-1-yl]-2-methyl-4*H*-3-thia-4,9-diaza-benzo[*f*]azulene),

that shows nanomolar affinity for the 5-HT6 receptor (Rasmussen *et al.*, 2005). These authors showed that FMPD inhibited the *ex vivo* binding of the 5-HT6 receptor antagonist [^{125}I]SB258585 to striatal 5-HT6 receptors. Furthermore, they performed *in vivo* electrophysiological recordings from the A9/A10 dopamine neurons of anesthetized rats. Extracellular recordings of dopamine neurons showed that either acute or chronic administration of FMPD did not change the number of spontaneously active A9 dopamine neurons; conversely, it did change the number of spontaneously active A10 dopamine neurons. Acute treatment increased the number of spontaneously active A10 dopamine neurons, whereas chronic administration of FMPD decreased it. However, this study did not investigate whether this effect of FMPD was attributable selectively to its agonist activity at 5-HT6 receptor.

To this aim, *in vitro* electrophysiological studies are better suited to pharmacologically characterize the effects of a novel putative 5-HT6 drug. For example, in light of the recent characterization of the excitatory effect of 5-HT6 receptor activation on striatal cholinergic interneurons (Bonsi *et al.*, 2007), we have recently tested the effect of a novel, putative 5-HT6 receptor agonist (ST 1936) by performing electrophysiological recordings of cholinergic interneurons from rat striatal slice preparations (Tassone A., Borsini F., and Pisani A., unpublished observations). Bath-applied ST 1936 caused a dose-dependent depolarization/inward current in striatal cholinergic interneurons, coupled to an increase in membrane resistance. This excitatory effect was prevented by pre-incubation of the slice with the selective 5-HT6 antagonist SB258585. Further voltage-clamp analysis suggested that the inward current induced by ST 1936 was mediated by the closure of potassium channels, in line with previous observations (Bonsi *et al.*, 2007).

V. Conclusions

Many medications currently in use are active at 5-HT receptors and/or 5-HT transporters or metabolizing enzymes. Drugs acting at 5-HT6 receptor subtype might be of particular interest, as, for example, 5-HT6 receptor antagonists have been generally well tolerated in all of the human and preclinical studies reported to date. However, though some likely models have been proposed, for example, for the procognitive effects of 5-HT6 antagonists, the cellular mechanisms are far from having been elucidated, yet. Moreover, in the field of 5-HT6 receptor pharmacology, some discrepancies are still evident. For example, purported agonists and antagonists at 5-HT6 receptor have been reported to share some pharmacological similarities in their potential antidepressant and cognitive effects (Branchek and Blackburn, 2000; Carr *et al.*, 2010; Geldenhuys

and Van der Schyf, 2009; Hirano *et al.*, 2009; Svenningsson *et al.*, 2007; Wesolowska, 2007; Wesolowska and Nikiforuk, 2007; Wesolowska *et al.*, 2007). Methodological issues might partly account for these discrepancies. It is noteworthy that heterologously expressed 5-HT6 receptor has been reported to undergo fast desensitization, without receptor downregulation (Max *et al.*, 1995); thus, 5-HT6 agonists may show an antagonistic action at longer times.

In light of these observations, a great effort in physiology studies is desirable in order to better clarify the potential of 5-HT6 receptor drugs as therapeutic tools.

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GENETIC VARIATIONS AND ASSOCIATION

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I. 5-HT6 Receptor Human Gene

In 1996, 14 different mammalian serotonin [5-hydroxytryptamine (5-HT)] receptor genes have been cloned; all but one (5-HT3, a ligand-gated ion channel) of them were members of the G protein-coupled receptor superfamily (Hoyer *et al.*, 1994;Humphrey *et al.*, 1993) and three of them (5-HT4, 5-HT6, and 5-HT7) have been found to stimulate adenylyl cyclase via Gs coupling. The first of these to be cloned is the rat 5-HT6 (Monsma *et al.*, 1993). Although little was known regarding its function, when expressed in mammalian cells, the rat 5-HT6 receptor showed high affinity for several therapeutically important antidepressant and antipsychotic drugs, in particular the atypical antipsychotic clozapine and related compounds (Monsma *et al.*, 1993; Roth *et al.*, 1994). Therefore, because of its potential interest to psychopharmacology (Schoeffter

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and Waeber, 1994) and the history of profound species differences in drug affinities of 5-HT receptors (Adham *et al.*, 1992), it has been considered important for the cloning and also the characterization of the human homolog gene.

In this chapter we will give information about the human HTR6 gene structure, its evolution, and its expression profile both in the brain and in peripheral tissues. We will describe also the regulatory regions like binding sites for transcription factors (TFs) or for miRNA. Finally, because of the involvement of HTR6 gene in mood regulation and in cognitive function, we have also discussed its role as a genetic risk factor in neurodegenerative or psychiatric disorders, including Alzheimer's disease (AD), schizophrenia, and mood disorders.

II. Cloning and Mapping of the Human 5-HT6 Receptor Gene

The cloning of the human 5-HT6 receptor was reached by a screening of the human caudate cDNA library with a probe obtained from the rat 5-HT6 gene labeled with [α-^{32} P] dCTP. Such screening permitted the identification of four positive human clones (HB16a, HC21a, HB9a, and HB14c clone) with high sequence homology to the rat 5-HT6 receptor: the clone HB16a containing '—S 1.4 kb of 5′-untranslated region (UTR) and the first 204 bp of the presumed receptor open reading frame (ORF); the clone HC21a, a 1.3 kb fragment starting at bp 135 of the ORF containing part of an apparent unspliced intron beginning at bp 715; and finally the two identical clones HB9a and HB14c, extending from what later proved to be bp 890 in the ORF through the 3′-end of the coding region and containing ~1.4 kb of 3′-UTR. However, among these fragments, there was a missing region extending from bp 715 to 889 and spanning at least one intron that was then identified by screening a human genomic library, which allowed the identification of three positive clones. One of them, the MTIAI clone, as it contains the entire 5-HT6 gene, was selected for further investigation. In particular, its sequencing revealed the presence of two putative introns of 1.8 kb and 193 bp located at bp 714 and 873, respectively, separated by a 159 bp exon. Such characterization of the human HTR6 gene revealed that the gene is made up of two introns and three exons; the cds region of the human HTR6 cDNA is 1322 bp long, encoding a 440-amino-acid polypeptide with a molecular size of 46.96 kDa. Finally the comparison of cDNA and genomic sequences for HTR6 gene identified a synonymous RsaI polymorphism at bp 267 (tyrosine in position 89; Fig. 1A), which was the first single-nucleotide polymorphism (SNP) identified within HTR6 gene and also the most studied in the context of genetic association studies (see Section VIII).

The mapping of the human 5-HT6 gene on chromosome 1, region 1p35-p36, was possible by using rodent hybrid cells containing different portion of human

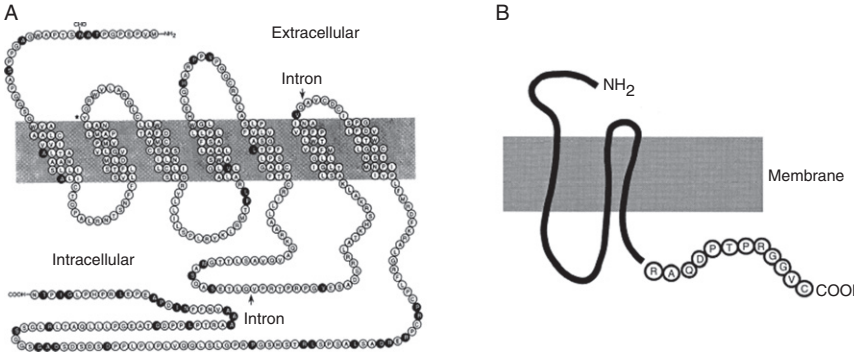

FIG. 1. (A) Deduced aminoacid sequence and structure of the human wild-type 5-HT6 receptor. The position of the two introns is marked with an arrow. The asterisk indicates the position of the RsaI polymorphism. (B) Predicted conformation of the truncated 5-HT6 receptor coded by the transcript variant that shows only 10 amino acids at the C-end as consequence of the frameshift.

chromosomes (Kohen *et al.*, 1996) and using human cDNA clone (HB14c) as probe for HTR6 gene. After high-stringency washing, only hybrids retaining the 1p35-1p36 portion of human chromosome 1 showed positive hybridization.

III. Comparison of Human and Rat 5-HT6 Receptor Gene Sequences

During the comparison of the nucleotide sequences of the human and the rodent HTR6 gene sequence previously published by Monsma *et al.* (1993), Kohen *et al.* (1996) noticed an apparent frameshift at bp 1034, so that they decided to resequence the rat clone (St-B17). This time they used a dideoxynucleotide chain termination method with polymerase chain reaction (PCR) and Taq polymerase as compared to another dideoxynucleotide chain termination method with Sequenase, which is the technique originally used by Monsma *et al.* (1993) to sequence the rat clone St-B17. Interestingly the PCR-based method was found to be less vulnerable to compressions and strong stops, thus yielding better sequence data, especially for the very G/C-rich 5-HT6 clones. In fact, using this more sensitive method they discovered in the rat sequence described by Monsma *et al.* (1993) the presence of several frameshift errors especially occurring in G/C-rich areas. After reconsidering these errors, they compared the corrected rat 5-HT6 sequence with the nucleic acid sequence of the human and revealed a homology of 85%. Also the predicted polypeptides of the human and rat receptors are 89% identical and 95% similar with conservative substitutions. The two receptors differ mostly in their C-terminal region, with the human receptor being 2 amino acids longer than its rat homologue (440 vs. 438 amino acids).

IV. Gene Expression Profile of 5-HT6 Receptors

The first evidence of the distribution of 5-HT6 receptors comes from a Northern blot study conducted by Kohen *et al.* (1999) and subsequently by gene expression analyses in real-time PCR conducted by Hirst *et al.* (2003). The distribution of 5-HT6 receptor and the expression profile of HTR6 gene are similar in rodents and in humans, but completely different as compared to mice. In particular, within the human central nervous system, the protein levels of 5-HT6 receptor reach the highest levels in the caudate nucleus and putamen, suggesting an even distribution of this receptor subtype throughout the dorsal striatum. Specific high levels can be observed in the nucleus accumbens, and substantially lower levels in globus pallidus, cerebral cortex, hippocampus, thalamus, and cerebellum. A similar pattern can be observed in the brain of rodents where the highest protein levels are detected in the striatum and in the nucleus accumbens, whereas fivefold lower levels are observed in cerebral cortex and thalamus and eightfold lower levels in hippocampus and cerebellum (Hirst *et al.*, 2003).

In contrast, only a very low level of 5HT6 receptor can be detected in the mouse brain regions besides a notable lack of enrichment, also in the striatum.

This distribution pattern of 5-HT6 receptor is also supported by gene expression data. In fact, mRNA data of the HTR6 gene indicated that in the human brain HTR6 mRNA expression is 6–10-fold higher in caudate nucleus, putamen, and nucleus accumbens than in cerebral cortex and hippocampus. Low levels are detectable in the amygdala, hypothalamus, and thalamus. Similarly for the rat brain, HTR6 mRNA expression is highest in the nucleus accumbens and striatum and the levels are approximately ninefold lower in the amygdala and cerebral cortex and approximately 20-fold lower in the hippocampus, hypothalamus, and thalamus. No HTR6 mRNA has been detected in rat cerebellum, medulla oblongata, pons, dorsal root ganglia, and spinal cord.

Based on its abundance in extrapyramidal, limbic, and cortical regions, it has been suggested that the 5-HT6 receptor plays a role in functions like motor control, emotionality, cognition, and memory.

The complexity of this serotonin receptor gene has increased with the identification of variant transcript generated by alternative splicing (Olsen *et al.*, 1999). In fact the HTR6 gene generates, above the conventional transcript, also a splicing variant, which is characterized by a 289 bp of deletion.

This region of 289 bp corresponds to the area from transmembrane IV through the third intracellular loop. As a result of deletion and subsequent frameshift, a truncated receptor is generated, and it is characterized by the first three transmembrane domains and 10 unique amino acids at its C-end.

This variant transcript shows a completely different expression pattern as compared to the normal transcript. In particular the variant is detected only in

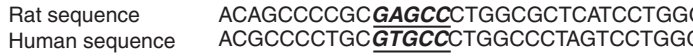

FIG. 2. Sequence comparison of the 5-HT6 gene region in rats (top) and human (bottom) containing the GTGCC donor site in human used to generate the human splice variant. Note the lack of consensus donor site in the rat sequence. Human and rat sequences have been downloaded from GenBank using NM_000871.1 and NM-024365 and as NCBI reference sequence.

caudate and substantia nigra, whereas the normal 5-HT6 transcript has a more heterogeneous expression profile as it is detected in different brain regions (Olsen *et al.*, 1999). The existence of tissue-specific regulation of alternatively spliced transcripts may suggest an important biological function and provide a mechanism that enables specialized cells such as neurons and antibody-producing cells to generate different proteins in response to environmental stimuli (Andreadis *et al.*, 1987). The alternative splicing is frequently conserved in evolution, even though some data suggest the existence of splicing pattern, like for the HTR7 gene, which is unique to certain species. This is also the case for HTR6. In fact, the splicing variant of HTR6 gene missing the 289 bp is not detected in the rat or in the mouse. This is mainly due to the sequence of the human HTR6 gene that is slightly different as compared to rat and mouse. In fact, it is well known that the alternate usage of donor or acceptor sites normally associated with an intron/exon boundary is absolutely required for the generation of alternatively spliced products. This splicing is consistent with intron/exon organization of the human HTR6 receptor gene and utilizes a cryptic upstream donor site and the normal 3′-acceptor site of the first intron. However, in rodents, in contrast to the alterative 5′-donor site (C/**GT**GCC) observed in the human transcript, the homologous donor site in the rat sequence is different (C/**GA**GCC) (Fig. 2) Accordingly, this inconsistency does not conform to the widely accepted GT/AG rule (Mount, 1982), and, therefore, the absence of the rat 5-HT6 splicing variant is due to the lack of this consensus 5′-donor site in the rat gene sequence.

V. 5-HT6 mRNA Levels in Peripheral Tissues

The first indication of HTR6 mRNA expression in human peripheral tissues coming from Hirst *et al.* (2003) revealed no detection of HTR6 mRNA levels in any of the following human peripheral tissues: heart, liver, lung, skeletal muscle, kidney, pancreas, spleen, small intestine, placenta, testis, stomach, prostate, or uterus. However, these data have been subsequently integrated and updated by recent studies conducted by using more sophisticated technologies and approaches whose data are available in online public databases like GeneNote

(http://bioinfo2.weizmann.ac.il/cgi-bin/genenote/home_page.pl) or BioGPS (http://biogps.gnf.org/#goto=welcome).

In particular, GeneNote and BioGPS are databases based on DNA array experiment performed on the Affymetrix HG-U95 set A–E or using gene atlas U133A and they contain data on *human* genes and their expression profiles in *healthy* tissues and in cancer tissues.

The using of these databases allows the identification of the (1) expression profile (*tissue vector*) for each gene in the human genome; (2) gene and tissue clustering based on expression profiles; and (3) a full genome ranking procedure according to the gene's tendency for tissue specificity, from tissue-specific to housekeeping genes.

For example, if one enters in the database web page as a key word "HTR6," one can obtain information regarding the gene expression profile of the 5-HT6 receptor for 12 normal human tissues hybridized against Affymetrix GeneChip HG-U95A-E (GeneNote data) and for 22 normal human tissues and hybridized against HG-U133A (GNF data).

As we can observe in Fig. 3, downloaded from GeneNote database, the 5-HT6 receptor is well expressed in several brain regions including the olfactory tubercle, cerebral cortex (frontal and entorhinal regions), nucleus accumbens, striatum, caudate nucleus, hippocampus, and the molecular layer of the cerebellum. However, relatively high levels of HTR6 gene expression can also be observed in several peripheral tissues, in particular in heart, skeletal muscles, and tongue.

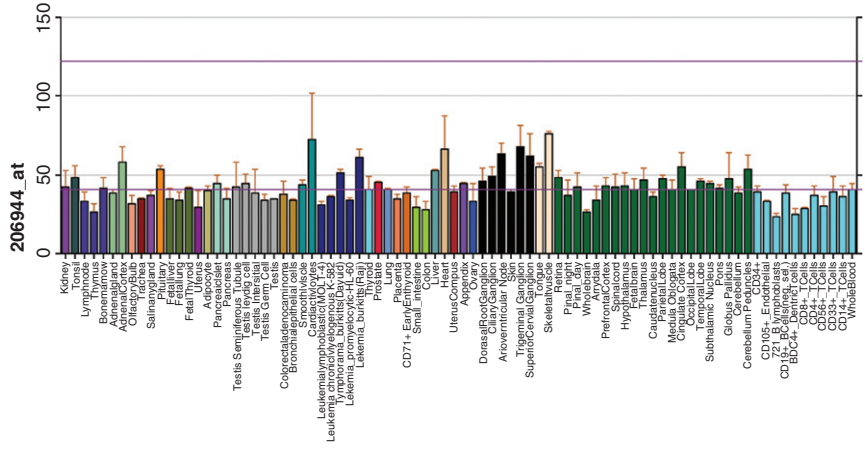

FIG. 3. Gene expression profiles of 5-HT6 mRNA levels in different human normal tissues downloaded from BioGPS (see http://biogps.gnf.org/#goto=genereport&id=3362).

VI. Orthologs of HTR6 Gene

Orthologs are genes in different species that evolved from a common ancestral gene by speciation, and, in general, orthologs retain the same function during the course of evolution. Identification of orthologs is a critical process for reliable prediction of gene function in newly sequenced genomes.

The identification of different HTR6 orthologs is possible by using different databases (HomoloGene, euGenes, SGD, and MGD, with further links to FlyBase and WormBase), where one can have access to the sequence of different genes in different species. Through the comparison of the sequence of the same gene in different species it is possible not only to determine the degree of homology but also to build up up a gene tree for that specific gene (see the gene tree for HTR6, ensembl database, http://www.ensembl.org/Homo_sapiens/Gene/Compara_Tree?collapse=1862045,1862036,1861928,1862098,1862101,1861738,1861746,1861731,1862024,1862116,1862062,1862112;db=core;g=ENSG00000158748;r=1:19991780-20006055;t=ENST00000289753;time=1276958802734.734). Ensembl gene trees are generated by the Gene Orthology/Paralogy prediction method pipeline and they aim to represent the evolutionary history of gene families, i.e., genes that diverged from a common ancestor. Homologies in Ensembl are determined from gene trees. Gene trees are constructed using the longest protein for every gene in every species in Ensembl. The display shows the maximum-likelihood phylogenetic tree representing the evolutionary history of gene families. These trees are reconciled with their species tree, generated by TreeBeST, having their internal nodes annotated for duplication or speciation events.

To date 43 orthologs of HTR6 gene receptor have been identified in different species with different percentages of homology, and genes with the highest similarity to the human HTR6 receptors have been found in dog, rat, chimpanzee, cow, mouse, and chicken (See Table I).

VII. Regulation of 5HTR6 Gene and Pathway Interaction

The regulation of the HTR6 gene is quite complex as the gene contains regions that are recognized by several TFs. In particular, as also shown in Fig. 4, there are two sequences upstream the transcription start site of HTR6 gene that are recognized by TFs neuron-restrictive silencer factor (NRSF) form 1 and form 2. These factors act as transcriptional repressors of neuronal genes in non-neuronal tissues. They are members of the Kruppel-type zinc finger TF

Table I
ORTHOLOGS FOR HTR6 GENE FROM 5 SPECIES

Organism	Gene	Description	Human similarity	NCBI accessions
Dog *(Canis familiaris)*	HTR6	5-hydroxytryptamine (serotonin) receptor 6	89.67 89.77	487402 XM_544528.2 XP_544528.2
Chimpanzee *(Pan troglodytes)*	HTR6	5-hydroxytryptamine (serotonin) receptor 6	98.94 99.32	469199 NM_001034092.1 NP_001029264.1
Cow *(Bos taurus)*	HTR6	5-hydroxytryptamine (serotonin) receptor 6	89.7 89.77	512167 XM_589622.3 XP_589622.2
Mouse *(Mus musculus)*	HTR6	5-hydroxytryptamine (serotonin) receptor 6	86.91 88.58	15565, NM_021358.2 NP_067333.1 AF134158 AK134977
Chicken *(Gallus gallus)*	HTR6	5-hydroxytryptamine (serotonin) receptor 6	70.07 64.93	430019

Table I contains different columns:
Organism: The names of the homologous species, using both scientific and popular terminology.
Gene: The symbol for the gene in the homologous species.
Description: Its description.
Human similarity: The percent similarity to the human gene.
NCBI accessions: Links to the sequences for the gene in NCBI databases including GenBank and Entrez Gene.

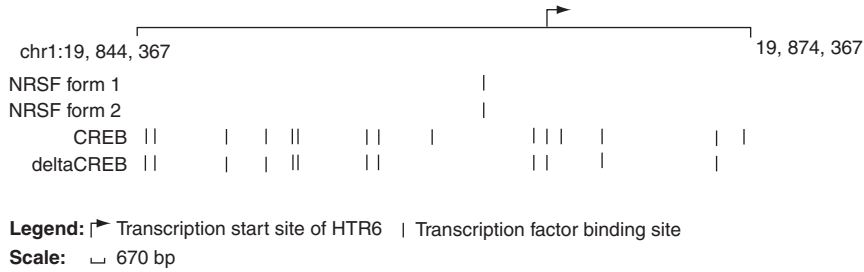

FIG. 4. Transcription factors and their binding sites on HTR6 gene.

family and repress the transcription by binding a DNA sequence element called the neuron-restrictive silencer element. The protein is also found in undifferentiated neuronal progenitor cells, and it is thought that this repressor may act as a master negative regular of neurogenesis.

Moreover, both upstream and downstream the transcription start site of HTR6, we can observe the presence of several binding sites for cyclic AMP response element-binding (CREB) and delta CREB that are TFs belonging to the leucine zipper family of DNA-binding proteins. These proteins after being phosphorylated by several protein kinases bind, as a homodime, the cAMP-

responsive element and induce the transcription of the target genes in response to hormonal stimulation of the cAMP pathway.

The regulation of HTR6 gene is also under control of a large numbers of cisRED atomic motifs and small *mRNAs* (miRNA). The cisRED atomic motifs are short conserved regulatory elements (typically 7–30 bp) where a TF binds to DNA. In particular, while the regulatory motifs are frequently located in or near promoter regions, in human and other mammalian genomes the cisRED atomic motifs are found experimentally as far as 50 kb upstream of a gene, as well in introns and in 3′-downstream regions. To date thousands of these cisRED motifs have been identified and ranked by a genome-scale computational system and are deposited in a database (www.cisred.org).

The miRNA are a class of small, single-stranded RNAs involved primarily in the negative regulation of gene expression as they act as adaptors that employ a silencing complex to target mRNAs by selective base pairing, primarily in the 3′-UTR region. Target interaction does not require perfect complementarity between microRNA and mRNA sequences, although near-perfect base pairing in a small region at the 5′-end (positions 2–8) of the microRNA (sometimes termed "seed") appears to be one of the key determinants of target recognition. The reduction of target gene expression appears to be achieved by one or both of two mechanisms: inhibition of translation initiation or degradation of the mRNA. To date several resources provide microRNA target predictions based on sequence complementarity to target sites with emphasis on perfect base pairing in the seed region and sequence conservation (see *TargetScan*, *PicTar*, *TargetRank*). Other target prediction methods are based on calculations of mRNA secondary structure and energetically favorable hybridization between microRNA and target mRNA (*RNAhybrid, STarMir*). To date, 50 miRNAs are predicted to be involved in the regulation of 5-HTR6 gene expression (see the list on ensemble database at http://www.ensembl.org/Homo_sapiens/Gene/Regulation?g=ENSG00000158748;r=1%3A19991780-20006055;t=ENST00000289753). Among these miRNA, the miR-145 and miR-192 are also involved in the regulation of genes that play a pivotal role in neurogenesis, cell cycle control, and cell proliferation, suggesting also an involvement of HTR6 in these processes.

Recently, there is a growing interest in discovering polymorphisms within miRNA sequence, also referred to as miRSNPs, which are normally located to predicted miRNA target sites within the 3′-UTR of mRNAs. Such SNPs have the potential to affect the efficiency of miRNA binding on its target site, and create or destroy binding sites. In fact, the specific binding between the miRNA and its target mRNA is established by the complementarity of the so-called miRNA seed site (positions 2–7 from the 5′end of the mature miRNA) and the target sequence in the 3′-UTR of the mRNA. Therefore, a single-nucleotide change of the miRNA target site of the mRNA can considerably affect the translational regulation of the encoded protein and it may be responsible for functional variation

Table II
List of SNPs within the Sequence miRNA Predicted to Target HTR6

MIRNA ID	ACCESSION	CHROMOSOME	UCSC VARIATION
hsa-mir-554	MI0003559	1	rs79661940
hsa-mir-28	MI0000086	3	rs78547906
hsa-mir-620	MI0003634	12	rs77703604, rs10549054, rs5801168, rs3043743, rs34380284, rs34551929
hsa-mir-518d	MI0003171	19	rs73602910, rs74704964
hsa-mir-423	MI0001445	17	rs6505162
hsa-mir-193a	MI0000487	17	rs60406007
hsa-mir-631	MI0003645	15	rs5745925
hsa-mir-639	MI0003654	19	rs45556632
hsa-mir-639	MI0003654	19	rs35149836
hsa-mir-125a	MI0000469	19	rs12975333
hsa-mir-516b-2	MI0003167	19	rs10670323, rs33953969, rs10583889

(Bandiera *et al.*, 2010). In particular, if the SNP enhances the targeting of the miRNA or creates a new target site in the 3′-UTR of the mRNA, there will be a gain-of-function effect, and, accordingly, less protein is expected to be translated. On the other hand, a loss-of-function effect can occur when the SNP decreases or abolishes the targeting of the miRNA on the target.

Interestingly, as reported in Table II, several SNPs have been found located in some of the predicted miRNA that bind and regulate 5HT-6 expression (the entire list of miRNA is available on http://www.ensembl.org/Homo_sapiens/Gene/Regulation?g=ENSG00000158748;r=1%3A19991780-20006055;t=ENST00000289753). Therefore, it is possible that as a consequence of the presence of miRSNPs within an miRNA, the resulting gene dysregulation could play a critical role both in the susceptibility and in the pathogenesis of different disorders (Murray *et al.*, 2010) including cancer (Ryan *et al.*, 2010), psoriasis (Chatzikyriakidou *et al.*, 2010), and also psychiatric diseases (Xu *et al.*, 2010).

VIII. Genetic Variation within HTR6 Gene

During the cloning and characterization of the 5-HT6 receptor, Kohan and colleagues identify a synonymous RsaI polymorphism at bp 267 (C267T) within the coding region of HTR6 gene, which does not cause a change in the amino acid sequence (Kohen *et al.*, 1996). Subsequently, other SNPs have been identified and a list of all of them and their position is deposited in databases (http://hapmap.ncbi.nlm.nih.gov) and represented in Fig. 5.

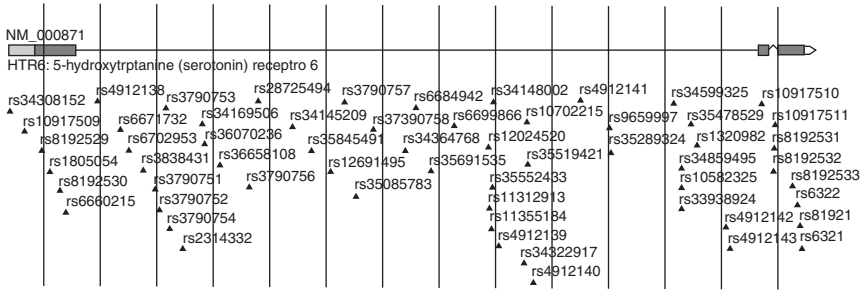

FIG. 5. List of SNPs within HTR6 gene and their position (http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap3r3_B36/#search).

Among all of them, the C267T and the trinucleotide repeat $(GCC)_{2/3}$ located in the 5′-UTR of the gene are the most studied in the contest of psychiatric and neurodegenerative disorders, especially because the rs1805054 may affect translation through the secondary structure and stability of the mRNA and because the rs1805054 has been found in tight linkage disequilibrium with the $(GCC)_{2/3}$ (Vogt *et al.*, 2000). However, the frequencies of the C267T are different in different populations (Table III) and this needs to be taken into account when genetic association studies are performed.

Regarding the trinucleotide repeat polymorphism, only the frequency in Japanese population was reported. We have available data on the frequency of the $(GCC)_{2/3}$ polymorphism in a sample of 200 Italian controls (see Table III); in the same table we also added the frequency of the C267T polymorphism in the same Italian sample.

IX. C257T and Alzheimer's Disease

Alzheimer's disease (AD) is a serious disorder clinically characterized by progressive dementia and cognitive decline. It is well known that the serotoninergic system plays an important role in cognitive processes and it has been implicated in the pathogenesis of AD as well (DeMichele-Sweet and Sweet, 2010; Geldenhuys and Van der Schyf, 2009). Among genes involved in the serotoninegic transmission, the HTR6 gene is one of those involved also in cognitive function (Kohen *et al.*, 1996; Sleight *et al.*, 1998). Moreover, preclinical studies conducted in rodents demonstrated that 5-HT6 receptor plays a physiological role in the modulation of cholinergic neurotransmission during memory acquisition and formation. In addition, 5HTR6 receptor densities have been found

Table III
Genotype and Allele Frequencies of the rs1805054 Polymorphism in Different Populations

Population	Genotype frequencies						Allele frequencies Ref-allele			
rs1805054	Genotype	Freq.	Genotype	Freq.	Genotype	Freq.	Allele	Freq.	Allele	Freq.
Italian sample (our study)	C/C	0.73	C/T	0.24	T/T	0.03	C	0.85	T	0.15
CEU (C)	C/C	0.74	C/T	0.26	T/T	0	C	0.87	T	0.13
CHB (H)	C/C	0.57	C/T	0.36	T/T	0.07	C	0.75	T	0.25
JPT (J)	C/C	0.47	C/T	0.41	T/T	0.12	C	0.68	T	0.32
YRI (Y)	C/C	0.69	C/T	0.28	T/T	0.03	C	0.83	T	0.17
ASW (A)	C/C	0.61	C/T	0.316	T/T	0.07	C	0.772	T	0.23
CHD (D)	C/C	0.62	C/T	0.367	T/T	0.009	C	0.807	T	0.19
GIH (G)	C/C	0.70	C/T	0.277	T/T	0.02	C	0.842	T	0.16
LWK (L)	C/C	0.75	C/T	0.218	T/T	0.036	C	0.855	T	0.15
MEX (M)	C/C	0.77	C/T	0.228	T/T	0	C	0.886	T	0.11
MKK (K)	C/C	0.71	C/T	0.256	T/T	0.032	C	0.84	T	0.16
TSI (T)	C/C	0.76	C/T	0.216	T/T	0.029	C	0.863	T	0.14
(GCC)2/3 repeat										
Asian (Japan)	(2/2)	0.11	(2/3)	0.44	(3/3)	0.46	2-Allele	0.33	3-Allele	0.67
Caucasian (Italy)	(2/2)	0.06	(2/3)	0.19	(3/3)	0.75	2-Allele	0.16	3-Allele	0.84

Population descriptors:
ASW (A): African ancestry in Southwest USA
CEU (C): Utah residents with Northern and Western European ancestry from the CEPH collection
CHB (H): Han Chinese in Beijing, China
CHD (D): Chinese in Metropolitan Denver, Colorado
GIH (G): Gujarati Indians in Houston, Texas
JPT (J): Japanese in Tokyo, Japan
LWK (L): Luhya in Webuye, Kenya
MEX (M): Mexican ancestry in Los Angeles, California
MKK (K): Maasai in Kinyawa, Kenya
TSI (T): Tuscan in Italy
YRI (Y): Yoruban in Ibadan, Nigeria

reduced in the brain of AD patients, which predicted the receptor may be involved in non-cognitive symptoms (Garcia-Alloza *et al.*, 2004). Therefore, because of the involvement of HTR6 in AD pathogenesis, to date, several studies aimed to investigate whether the C267T polymorphism in HTR6 gene could represent a risk factor for AD.

The first study conducted in a Taiwanese population (Tsai *et al.*, 1999b) showed a significant difference in the genotype and allele frequencies of the C267T polymorphism between 92 AD patients and the 94 controls ($P = 0.006$ and $P = 0.023$, respectively), suggesting for the first time that the individuals with the 267C allele had increased risk of AD. Subsequently, as a deficit in serotonergic neurotransmission is also involved in the pathogenesis of major depression, Liu *et al.* (2001) tried to find out whether the 267C allele, above to represent an increased susceptibility risk for AD, could also be associated with the development of depressive symptoms in AD patients. They confirmed their previous observation supporting the 267C allele as a genetic risk factor for AD (145 patients, 104 controls), but they failed to prove that the 267C allele was associated with depressive symptoms.

The association between the 267C allele and AD, although in part replicated in 2004 by Kan *et al.*, where they found an association between the C/T genotype and late-onset Alzheimer's disease (105 LOAD patients and 130 controls, odds ratio (OR) = 2.10, $P = 0.014$) in a Chinese population, has not been confirmed by other studies, conducted in Caucasian population (Alvarez-Alvarez *et al.*, 2003; Orlacchio *et al.*, 2002; Thome *et al.*, 2001).

However, as the genotypic and allelic frequencies of the C267T SNP differ in Chinese and Taiwanese populations as compared to the Caucasian population (Tables III and IV), it is possible that the association with AD may depend on differences between the C267T allele distributions in different populations.

Therefore, because the genetic association findings in AD have been conducted only in these populations, studies in other populations in the world may be necessary to clarify the role of HTR6 gene in AD. Moreover, a more exhaustive identification and analysis of other polymorphisms and/or mutations within this gene could demonstrate or exclude the implication of this receptor in the increased susceptibility to AD.

X. C267T and Schizophrenia

Serotonergic transmission plays an important role in the pathogenesis of schizophrenia and also in its treatment, as most of the antipsychotic drugs show a high affinity for all the serotonin receptors, including 5-HT6. Moreover, the

Table IV

Gene Association Studies Conducted on the C267T Polymorphism and AD in Different Populations (http://www.alzgene.org/geneoverview.asp?geneid=69)

Study	Population	SNP	Sample	Sample size	T-allele	C-allele	T/T frequency	T/C frequency	C/C frequency	Finding
Caucasian										
Alvarez, 2003	Spain	rs1805054	AD	173	0.07	0.93	1 (0.006)	23 (0.133)	149 (0.861)	NEGATIVE
			CTRL	102	0.12	0.88	3 (0.029)	18 (0.176)	81 (0.794)	
Orlacchio, 2002	Italy	rs1805054	AD	303	0.15	0.85	6 (0.025)	60 (0.253)	171 (0.722)	NEGATIVE
			CTR	100	0.15	0.86	5 (0.050)	19 (0.190)	76 (0.760)	
Thome, 2001	Italy	rs1805054	AD	71	0.16	0.84	2 (0.028)	19 (0.268)	50 (0.704)	NEGATIVE
			CTR	156	0.16	0.84	2 (0.013)	47 (0.301)	107 (0.686)	
Asian										
Kan, 2004	China	rs1805054	AD	105	0.17	0.83	0 (0.000)	35 (0.333)	70 (0.667)	POSITIVE
			CTR	130	0.13	0.87	4 (0.031)	25 (0.192)	101 (0.777)	
Tsai, 1999	China	rs1805054	AD	92	0.24	0.76	6 (0.065)	33 (0.359)	53 (0.576)	POSITIVE
			CTR	104	0.34	0.66	5 (0.048)	61 (0.587)	38 (0.385)	

mRNA of the HTR6 gene is expressed predominantly in the limbic and cortical regions, which are well known to play a central role in the pathophysiology of schizophrenia (Monsma *et al.*, 1993). Therefore, it has been suggested that the 5-HT6 receptor gene polymorphism might contribute to the genetic background of this disorder.

In order to better clarify the role of this gene in the susceptibility risk for schizophrenia, several studies investigated the association between the C267T SNP and the pathology. Unfortunately, to date, these studies have not clearly supported the role of HTR6 gene in the susceptibility risk for schizophrenia. In fact, although Tsai *et al.* (1999) demonstrated an association between the T allele of the C267T and schizophrenia in Asiatic population, other researchers in the same year did not support the association in a Japanese population (Shinkai *et al.*, 1999). Also, a few years later, Chiu *et al.* (2001) were not able to replicate in a Taiwanese population the positive association between the C267T polymorphism and schizophrenia previously found in the study of Tsai *et al.* (1999). Subsequently, Ohmori *et al.*, (2001), in research of new SNPs in the 5′-upstream region of the gene, identified a trinucleotide repeat polymorphism $(GCC)_{2/3}$ at a nucleotide position between –1093 and –1085 bp upstream from the translation start site. Interestingly, this SNP has been found in tight linkage disequilibrium with the C267T SNP in Japanese population, where the $(GCC)^3$ allele was usually found with the 267C allele (Ohmori *et al.*, 2001). However, when the $(GCC)_{2/3}$ polymorphism was investigated as a putative susceptibility gene for schizophrenia in a case-control study, the results failed to find a positive association (Ohmori *et al.*, 2001). As other unidentified putative functional polymorphisms in promoter region or in the 5′- or 3′-region of the gene can exert an effect on the transcriptional activity of the receptor, or other SNPs in linkage disequilibrium with C267T or with $(GCC)_{2/3}$ polymorphisms, Vogt *et al.* (2000) decided to sequence the HTR6 gene to identify putative additional genetic variants. In particular the sequencing of exons flanking 5′- and 3′-regions from intron 1 as well as the entire intron 2 enabled to perform a systematic mutation screening of the whole coding region and the exon–intron boundaries of the 5-HT6 gene. The sequence analysis was performed in 45 controls, 46 schizophrenic patients, and 45 bipolar patients and revealed the presence of four variants in the coding region and two variants in intronic sequences (Table V), but none of the variants altered the amino acid composition of the receptor or was located in consensus sequences for splice sites, respectively (Vogt *et al.*, 2000).

Only the 873 + 128A/C variant resulted as particularly interesting as it has been found in strong linkage disequilibrium with 267C/T. However, the genotype distribution and allelic frequencies of all the novel variants as well as the C267T and $(GCC)_{2/3}$ were not different in controls as compared to those in schizophrenic patients, failing to support in a Caucasian

Table V
List of Novel Mutations in HTR6 Gene Identified by Vogt *et al.* (2000) in Caucasian Population

Variant	Nucleotide position	Sequence change	Allele
126G/T	126	G–>T	126G
			126T
267C/T	267	C–>T	267C
			267T
873 + 30CT	873+30	C–>T	873 + 30C
			873 + 30T
873 + 128A/C	873+128	A–>C	873 + 128A
			873 + 128C
1128G/C	1128	G–>C	1128G
			1128C
1367T/G	1367	T–>G	1367T
			1367G

population the association between HTR6 gene and schizophrenia (Vogt *et al.*, 2000).

In summary, as one can also see in Fig. 6, to date only few studies focusing on the C267T polymorphism have been performed on schizophrenia, and only one of them, conducted in an Asian population, found a positive association. It does not necessarily mean that the role of HTR6 gene is ruled out in schizophrenia. In fact, it has to be noticed that only one study has been conducted in Caucasian population, and, also, the sample size was small in all the studies. Therefore, replication studies using a larger sample of both cases and controls and in different populations are needed to better clarify the role of HTR6 gene in schizophrenia.

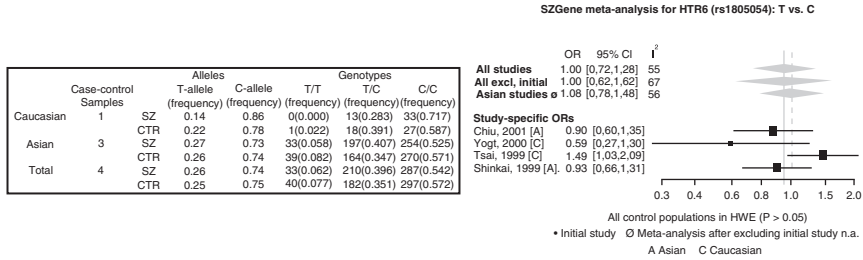

Fig. 6. Allele and genotype frequencies of the rs1805054 in schizophrenic and control individuals in different populations and meta-analyses of the published studies (http://schizophreniaforum.org/res/sczgene/polydetail.asp?geneID=248&studyID=710&ethnicDataID=1045).

XI. C267T Pharmacogenetic Studies of Antipsychotics

As clozapine is an effective atypical antipsychotic with high affinity for 5-HT6 receptor, the hypothesis that clinical response to clozapine could be related to the genetic variant C267T of the HTR6 gene has been tested. However, to date, only a few studies have evaluated this relationship, showing contrasting results. In particular, Yu *et al.* (1999) found a positive, although modest, relationship between the variant C267T within HTR6 gene and the response to clozapine in 99 schizophrenic patients with a history of non-response to typical antipsychotics. In particular, they observed that patients with 267T/T genotype had a better response than other patients, suggesting that the C267T polymorphism may be involved in clozapine response, especially in patients with anxiety or depression symptoms. However, this association was not supported by Masellis *et al.* (2001), who found no evidence for either an allelic or genotypic association of the T267C polymorphism with the response to clozapine.

Besides clozapine, risperidone and other atypical antipsychotics also have high affinity for the 5-HT6 receptor, and several studies have investigated the effects of the C267T polymorphism on risperidone efficacy (Ikeda *et al.*, 2008; Lane *et al.*, 2004). Similarly as for clozapine, for risperidone as well, the results are controversial. In fact, an association between C267T polymorphism and response to risperidone has been shown in 123 schizophrenic inpatients. In particular, when compared to patients with the T/C genotype, those with T/T genotype showed an improvement in positive symptoms ($p = 0.006$) and in the general psychopathology (including anxiety, depression, and cognitive dysfunctions) ($p = 0.005$). No influence of the T267C polymorphism was found on negative symptoms, suggesting a specific effect of the C267T polymorphism on positive symptoms and general psychopathology (Lane *et al.*, 2004). However, the power of C267T in predicting the clinical response was not confirmed in a group of 120 first-episode psychoses patients treated with risperidone monotherapy for 8 weeks (Ikeda *et al.*, 2008).

Therefore, replication studies in larger samples are needed to confirm the role of HTR6 gene as a predictive genetic factor associated with the treatment outcome in schizophrenia.

XII. C267T and Mood Disorders

Abnormalities in serotonergic neural transmission are hypothesized to be involved also in the pathophysiology of mood disorders such as bipolar disorder and major depressive disorder (Harmer, 2008; Kato, 2007; Le Francois *et al.*, 2008;

Levinson, 2006). Among the most recently discovered serotonin receptors, the 5-HT6 receptor is relatively abundant in the limbic area and it shows high affinity for several antidepressant drugs. Moreover, several preclinical studies have shown that the brain regions where 5-HT6 receptors are located are related to the serotonergic control of mood regulation (Gerard *et al.*, 1997), and that 5-HT6 receptor antagonists exert an antidepressant effect in the forced swim test and tail-suspension test, which are commonly employed to screen antidepressant drugs (Kulkarni and Dhir, 2009; Wesolowska and Nikiforuk, 2007; Wesolowska *et al.*, 2007).

Moreover, the genomic region where HTR6 gene map, 1p36-35, has been shown to be closely related to the susceptibility for bipolar disorder (Cheng *et al.*, 2006; Schumacher *et al.*, 2005; Vazza *et al.*, 2007) and major depression (Nash *et al.*, 2004) suggests that the HTR6 gene may confer an increased risk for mood disorders.

To date, several genetic studies have investigated the association between polymorphisms within the HTR6 gene and bipolar disorders and major depression. Similarly to schizophrenia and AD, also for mood disorders the most studied polymorphism is the C267T. The first evidence comes from a study conducted by Hong *et al.* (1999), where they genotyped the C267T polymorphism in 139 patients with mood disorders (62 with bipolar disorder and 77 with major depression) and 147 controls in a Chinese population. However, the results showed no significant differences in genotype or allele frequencies between controls and patients with bipolar disorders or major depression. Since the publication of this study, only Vogt *et al.* have reported a positive association between the C267T and bipolar patients (45 bipolar patients and 46 controls and replication study in an independent sample of 105 bipolar patients and their parents) in a Caucasian population (Vogt *et al.*, 2000), but it has not been supported by a recent study conducted in Japanese population involving a large sample of 1007 bipolar patients and 1753 controls (Fukuo *et al.*, 2010).

Vogt *et al.* (2000) is the only study reporting a positive association with C267T and bipolar disorder, and it is also the only study conducted in Caucasian population. It has to be noticed that the frequencies of C267T polymorphism in the sample used by Vogt *et al.* (2000) are different as compared to the other studies; therefore, it is possible that the heterogeneity of the results may have resulted from different ancestries, and, hence, other replication studies in larger samples are needed to clarify the role of this polymorphism in bipolar disorder.

Also the data coming from genetic association studies in major depression do not support the role of HTR6 gene as a risk factor for an increased susceptibility to develop the disorder. The first evidence comes from a study conducted in 1999 where Hong *et al.* (1999) found a negative association between C267T and depression (139 patients and 147 controls) in an Asian population. The same negative finding was then replicated by other two studies conducted in different populations: the first one in Korean population (Lee *et al.*, 2005) and the second

one in Finnish population (Illi *et al.*, 2009). Although the sample size used in all these studies was small, the negative association has been recently replicated by Fukuo and colleagues (2010) in a large cohort of 447 depressed patients and 1753 controls in a Japanese population.

XIII. C267T and Pharmacogenetic Studies of Antidepressants

Genes involved in the regulation of the serotonin signaling system are potential target for investigation not only for the etiology of mood disorder but also for the treatment response, especially to serotonin reuptake inhibitors. To date several studies investigated whether the C267T polymorphism could predict the antidepressant treatment outcome, but the results obtained are not promising.

In particular, only Lee *et al.* (2005) found a significant difference in the treatment response in some Hamilton Depression Rating Scale (HAM-D) scores (sleep, activity, somatic anxiety, and total score) between genotypes in 91 Korean depressed patients after 8 weeks of treatment. Specifically, the heterozygote group (CT genotype) had significantly better treatment response than the homozygote group (CC + TT genotypes) in the somatic-anxiety subcategory and the total score of HAM-D. Unfortunately, these promising results have not been replicated in other independent samples and other populations (Illi *et al.*, 2009; Wilkie *et al.*, 2009; Wu *et al.*, 2001).

Therefore, further replication studies in larger and more heterogeneous samples, also involving other polymorphisms within the gene, are needed to better explore the role of HTR6 gene in treatment response to major depression.

XIV. Conclusion

To date, only a few gene association studies have been conducted on the HTR6 gene and most of them have been focused only on the C267T polymorphism. Unfortunately, they did not answer the question if the HTR6 gene can exert an important role as susceptibility gene for complex pathologies, in particular for psychiatric disorders, and as a useful predictive genetic marker for pharmacogenetic studies.

Only the possibility to have access to large databases containing data coming from genome-wide association studies in different populations or the opportunity to use next-generation sequencing approaches will allow defining the role of the genetic variation within HTR6 gene in these complex phenotypes.

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PHARMACOKINETICS OF 5-HT$_6$ RECEPTOR LIGANDS

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I. Introduction

Although serotonin (5-HT) was discovered in the late 1940s, a continuous interest of the scientific community in this area has led to the identification of seven families of 5-HT receptors. At present, many serotoninergic agents acting at 5-HT$_{1–4}$ receptors are marketed worldwide (i.e., buspirone, sumatriptan, risperidone, and ondansetron) whereas no therapeutic compounds acting at 5-HT$_{5–7}$ receptors are present in the marketplace. In the last two decades, the 5-HT$_6$ receptor population, identified by molecular cloning in rat (Monsma *et al.*, 1993; Ruat *et al.*, 1993), mouse (Unsworth and Molinoff, 1994), and human (Kohen *et al.*, 1996), has shown most progress in the synthesis of selective 5-HT$_6$ agonists and antagonists (and imaging radiotracers). Many 5-HT$_6$ receptor compounds have been used as pharmacological tools in this arena, but other compounds have also entered clinical trials for the treatment of central nervous system (CNS) and eating disorders. In spite of the advances that have led to the development of selective 5-HT$_6$ receptor compounds, information on the adsorption, distribution, metabolism, and excretion (ADME) of these agents is still limited in literature. The ADME of selective 5-HT$_6$ receptor compounds have been investigated only partially and it is difficult to obtain more than minimal information from the published results.

In this chapter, after a description of the role of ADME (i.e., pharmacokinetics) in the research and development of CNS therapeutics, we will provide information on physicochemical properties and ADME results of preclinical and clinical studies available on 5-HT$_6$ receptor compounds.

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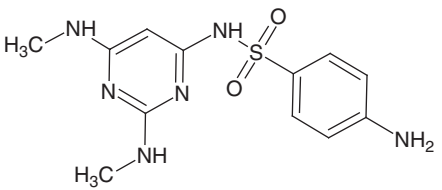
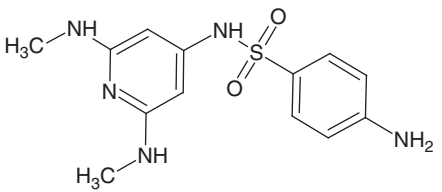
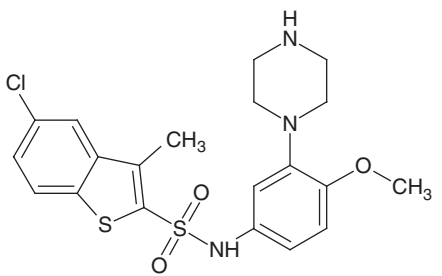
During the research and development of novel therapeutic agents, all the compounds that have shown high-activity *in vitro* biological screening are submitted to pharmacokinetic and metabolic evaluation in order to optimize pharmacokinetic and pharmacological properties. Therefore, scientists in pharmacokinetic arena are crucial for the success or failure of a potentially marketable drug. In fact, the inclusion of ADME investigation at the earlier stages of drug development has been shown to reduce failure because of poor pharmacokinetic features at the clinical stage from 40% to 10% (Kelly, 2009). Besides, to have good pharmacokinetic properties, such as high oral bioavailability, linear predictable pharmacokinetics, low plasma protein binding, appropriate half-life, metabolic stability, and low or no enzyme inhibition/induction, a big challenge for CNS therapeutic candidates is to possess high blood–brain penetration properties so as to ensure adequate concentration at its site of action (Kelly, 2009). Putting aside anatomical and physiological details of the blood–brain barrier (BBB), the major issue is to evaluate the mechanisms of brain penetration of active CNS therapeutic candidates. In studies of BBB permeation, though diffusion through the lipid bilayer is considered to be the dominant process, the role of carrier-mediated influx and efflux of drugs from the brain is being intensively emerging. Estimating the contribution of passive diffusion or active transport processes of drug into brain is necessary for understanding the disposition of drugs in this organ. However, such information about 5-HT_6 receptor compounds is not readily available in current literature.

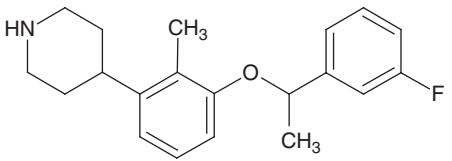
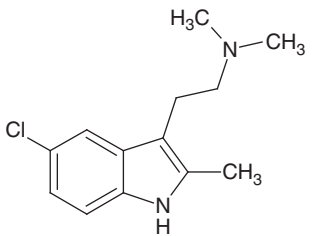
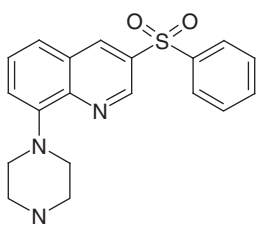
Recently, in an excellent perspective, Hitchcock and Pennington (2006) have identified the following physicochemical parameters as strong factors in brain penetration, particularly for drugs that permeate passively trough the BBB. These parameters include molecular weight (MW), polar surface area (PSA), lipophilicity (expressed as the calculated logarithm octanol/water partition coefficient—*c* log *P*; or as the calculated logarithm octanol/buffer pH 7.4 partition coefficient—*c* log *D*), and hydrogen bond donor (HBD). For these parameters a range of values have been suggested: MW < 500; PSA < 90 Å; *c* log *P* 2–5; *c* log *D* 2–5; HBD < 3 (Hitchcock and Pennington, 2006). In order to check if 5-HT_6 receptor compounds fall in the range of reported values, we have calculated, using ACD Labs version 12.0 (Advanced Chemistry Development Inc., Toronto, ON, Canada), the physicochemical properties of some 5-HT_6 receptor compounds and shown them in Table I. Interestingly, the calculated values fall in the above ranges, indicating a possibility of their passive brain penetration. However, the complex structure of CNS makes brain penetration a very complicated feature that cannot be only rationalized on the basis of physicochemical parameters but is also influenced by other biological processes such as influx and efflux transport mechanisms.

TABLE I
PHYSICOCHEMICAL PROPERTIES OF SELECTED 5-HT_6 RECEPTOR LIGANDS

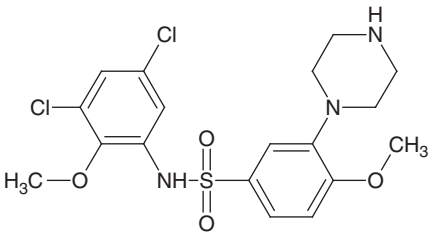
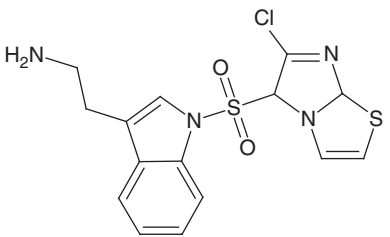
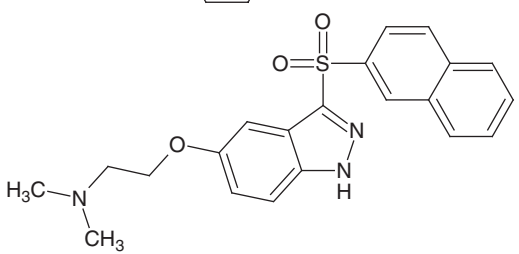
Compound	Funct.	Structure	MW (<500)[a]	log *P* (2–5)[a]	log *D* pH 7.4 (2–5)[a]	PSA (Å^2) (<90)[a]	HBD (<3)[a]	Status
Ro 04-6790	Ant.		308.36	1.18	0.34	130.41	2	Tool
Ro 63-0563	Ant.		307.37	2.68	0.89	117.52	5	Tool
SB-271046	Ant.		451.99	2.71	1.32	107.29	2	Phase II (discontinued)

(*continued*)

TABLE I (*continued*)

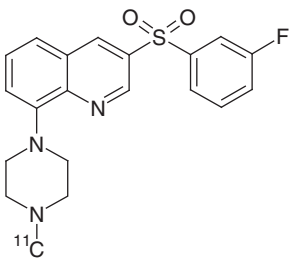
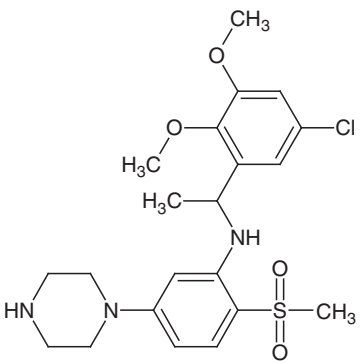
Compound	Funct.	Structure	MW (<500)[a]	log *P* (2–5)[a]	log *D* pH 7.4 (2–5)[a]	PSA (Å^2) (<90)[a]	HBD (<3)[a]	Status
Tolylpiperidine 9h[b]	Ant.		313.41	4.18	1.55	21.26	1	Preclinical
ST1936	Ag.		236.74	3.27	1.43	19.03	1	Preclinical
SB-742457	Ant.		353.44	1.76	0.59	70.68	1	Phase II

(*continued*)

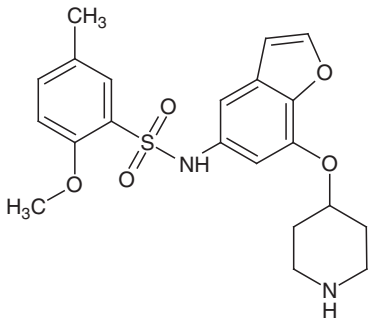
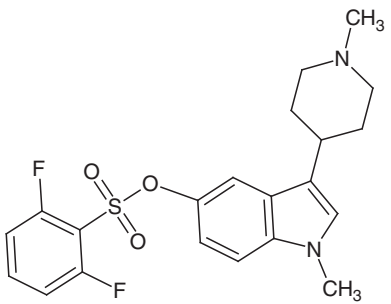
SB-399885	Ant.		446.35	2.87	1.15	88.28	2	Tool
WAY-181187	Ag.		382.89	3.79	3.79	114.37	5	Phase I
SAM-531 (WAY-262531)	Ant.		395.47	3.65	2.55	83.67	1	Phase II

(*continued*)

TABLE I (*continued*)

Compound	Funct.	Structure	MW (<500)[a]	log *P* (2–5)[a]	log *D* pH 7.4 (2–5)[a]	PSA (Å^2) (<90)[a]	HBD (<3)[a]	Status
[^{11}*C*]GSK-215083	Ant.		385.46	2.17	1.56	61.89	0	Tool
PRX-07034	Ant.		453.98	0.88	-0.35	88.28	2	Phase I completed

(*continued*)

BVT-74316	Ant.		416.49	4.92	2.71	98.18	2	Phase I
SGS-518 (LY-483518)	Ant.		420.47	3.55	1.82	59.92	0	Phase II

[a]Optimal values for brain penetration (Hitchcock and Pennington, 2006.
[b]From Singer *et al.* (2009)
Funct. = functionality; Ag. = agonist; Ant. = antagonist; MW = Molecular weight; HBD = hydrogen bond donor; PSA = polar surface area. The values are calculated using ACD Labs version 12.0 (Advanced Chemistry Development Inc., Toronto, ON, Canada).

II. Preclinical Pharmacokinetic Results

A characteristic of 5-HT_6 receptors is their localization, which is exclusively in the brain with high expression in the cerebral cortex, olfactory tubercles, nucleus accumbens, hippocampus, and striatum regions (Reavill and Rogers, 2001). These findings suggest that potential 5-HT_6 receptor therapeutics must have good tissue distribution in brain for the application of the above-mentioned pharmacokinetic properties. As no distribution of 5-HT_6 receptors in peripheral organs has apparently been reported, peripheral tissue distribution of these agents should not be of concern for receptor-mediated adverse side effects. With the above considerations in mind, pharmacokinetics information on lead drug candidates or tool compounds of selective 5-HT_6 receptors (agonists or antagonists) will be reported in this section. However, no pharmacokinetics information will be shown for the marketed drug clozapine and related atypical antipsychotics which have high affinity for the 5-HT_6 receptor, but are not highly selective for this receptor (Roth *et al.*, 1994).

The first selective 5-HT_6 receptor ligands of a series of pyrimidinyl- (Ro 04-6790) and pyridinylsulfonamides (Ro 63-0563) compounds were reported by scientists at Roche in 1998 (Bourson *et al.*, 1998; Sleight *et al.*, 1998). In addition to *in vitro* characterization and pharmacological behavioral observations, plasma and cerebrospinal fluid (CSF) concentrations for these selective 5-HT_6 receptor antagonists were reported in rats (Sleight *et al.*, 1998). After the administration of Ro 04-6790 (30 mg/kg, i.p.) or Ro 63-0563 (10 mg/kg, i.v.), blood and CSF (from the cisterna magna) samples were collected at 0.5, 1, 2, 3, and 6 h after dosing. The plasma and CSF samples were analyzed by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection and a time course of concentration changes for Ro 04-6790 was reported, which indicated a slow elimination of this compound from the body. In fact, 6 h after the administration of Ro 04-6790, plasma and CSF concentrations were only one-third of those found at 0.5 h post-administration. The presence of the compound in the CSF samples indicated that it was able to cross the BBB, although the plasma concentrations exceeded by 3 orders of magnitude over those in CFS (Sleight *et al.*, 1998). Regarding Ro 63-0563, a compound with similar chemical structure as Ro 04-6790, the authors reported that its concentration in plasma was similar that of Ro 04-6790, but undetectable in the CSF samples, indicating its inability to cross the BBB (Sleight *et al.*, 1998). The low brain penetration (<1%) of these first 5-HT_6 receptor ligands was a major weakness for their utility as potential therapeutics. Consequently, a series of Ro 04-6790 analogs were synthesized in a hope for these to have a more favorable brain penetration (Bös *et al.*, 2001). In a structure–activity relationship investigation, the authors concluded that compounds with log *D* values between 2 and 3.5 were shown to have higher brain penetration. Considering that log *D* values for Ro 04-6790 and Ro 63-0563 were 0.34 and 0.89, respectively (Table I),

these low values may be argued to justify their poor BBB penetration. However, contribution of other pharmacokinetic properties (see Section I) should not be ruled out. Unfortunately, no information was reported on brain distribution of other highly lipophilic synthetic compounds (Bös *et al.*, 2001).

About the same time, the group of Glennon, one of the pioneers of the synthesis of 5-HT$_6$ receptor ligands, undertook a structure–affinity investigation of the binding of 2-substituted tryptamine derivatives in human 5-HT$_6$ receptors (Glennon *et al.*, 2000). This work resulted in the recognition of 5-methoxy-2-ethyl-*N*, *N*-dimethyltryptamine or EMDT as the first potent 5-HT$_6$ receptor agonist (Glennon *et al.*, 2000), along with other indolealkylamine compounds (e.g.,MS-245) that also had 5-HT$_6$ receptor antagonist activity (Glennon *et al.*, 2000). Unfortunately, no information on their pharmacokinetics and brain exposure has been reported (Glennon, 2003). It is plausible to assume that the compounds synthesized by Glennon *et al.* were of low metabolic stability as reported for tryptamine derivatives such as *N*,*N*-dimethyltryptamine, 5-methoxy-*N*, *N*-dimethyltryptamine, and bufotenine. These may have been biotransformed tomore polar compounds, through deamination, N-demethylation, O-demethylation, and N-oxidation metabolic pathways (Yu, 2008). It is not surprising that polar functional groups such as amine *N*-oxides and carboxylic acids can have a particularly negative effect on BBB permeability of a compound. Furthermore, for some tryptamine derivatives, such as serotonin, psilocin, bufotenine, and 2-methyl-serotonin, poor or modest brain penetration has been shown (Campiani *et al.*, 1997; Fuller *et al.*, 1995; Kalberer *et al.*, 1962; Verbeuren, 1992). An interesting finding was that the introduction of a sulfonamide motif in the *N*-indole-type structure was optimal for their activity as shown for the MS-245 and SB-271046 5-HT$_6$ receptor antagonists (Routledge *et al.*, 2000; Russel *et al.*, 2001).

The compound SB-271046 was developed by SmithKline Beecham Pharmaceuticals and reached clinical trials in 2002. The preclinical pharmacokinetic properties of SB-271046 were investigated in rat by orally administering the compound formulated in 1% methyl cellulose in aqueous solution, at 10 mg/kg and the volume of 1 mL/kg body weight (Routledge *et al.*, 2000). For the assay of SB-271046 in blood (or plasma) and brain samples, an HPLC tandem mass spectrometry method employing positive-ion electrospray ionization was used reaching a lower limit of quantification of 4.52 ng/mL (or ng/g), after deproteinization with acetonitrile. SB-271046 was rapidly absorbed from the gastrointestinal tract and reached maximum plasma concentration ($C_{max} \sim 1$ μg/mL) in 3 h with a half-life of 4.8 ± 0.1 h. The results showed good oral bioavailability (>80%), low blood clearance (7.7 mL/min/kg), and reduced inhibition of the activity of major human P450 enzymes (Bromidge *et al.*, 1999). Regarding BBB penetration, SB-271046 concentration in rat brain samples was found below the lower limit of quantification, although measurable values (4.5–18.1 ng/g) were detected between 2 and 6 h with the highest levels corresponding to the

time of C_{max}. Based on these data, it was evident that SB-271046 attains poor concentration in the brain and, therefore, it has a moderately brain penetration (10%) (Bromidge *et al.*, 1999). Because SB-271046 is a substrate for P-glycoprotein-(P-gp) mediated efflux protein, it could not accumulate in rat brain. In many cases the action of P-gp (or other efflux transport systems) is counterbalanced by the ability of lipophilic molecule, such as SB-271046 (Table I; log $D = 1.32$, PSA = 107.29, and HBD = 2) to diffuse through the blood-brain barrier. In addition, the P-gp pump expression may be upregulated, especially after chronic exposure of P-gp substrate resulting in an elevated drug resistance and reduced access of this substrate to the site of action within the CNS. Unfortunately, not a single detailed study has been reported on the mechanisms that influence the uptake and efflux of SB-271046 from the brain after acute or chronic administration of the compound.

Interestingly, SB-271046 was not synthesized by the scientists of SmithKline Beecham Pharmaceuticals, but it was found as an unknown compound and at significant concentration in blood after 16 h infusion of SB-258510A in rat. Subsequently, SB-271046 was identified and found to be the *N*-demethyl metabolite of administered SB-258510A. In spite of good pharmacokinetic parameters such as low blood clearance (12.5 mL/min/kg) and moderate brain penetration (18%), this compound (SB-258510A) was not further investigated because the *N*-demethyl metabolite SB-271046 showed a much better pharmacological and pharmacokinetic profile (Bromidge *et al.*, 1999). This is an excellent example in which biological transformation led to the discovery of a lead candidate with superior potential for development as a drug.

In an attempt to further increase brain penetration, Bromidge *et al.* (2001) synthesized a new series of 5-HT_6 receptor antagonists. Although increasing lipophilicity of these compounds resulted in a better brain penetration, their *in vivo* clearance in rats was increased too. Therefore, the compound SB-357134, which showed good oral bioavailability (65%), low clearance (14 mL/min/kg), and reasonable brain penetration (19%), was chosen for further pharmacological evaluation (Bromidge *et al.*, 2001; Stean *et al.*, 2002). Further work by the same group at GlaxoSmithKline Pharmaceuticals resulted in the compound SB-399885, an orally bioavailable and brain-penetrant 5-HT_6 receptor antagonist (Hirst *et al.*, 2006). In rats, following an oral dose of 10 mg/kg, this compound reached peak brain concentrations of 0.18 ± 0.04 μM at 4 h, indicating a passage through the BBB. When compared to SB-271046, it showed a threefold improvement in the brain/plasma ratio, justifying the improved potency of this compound, particularly when considered with oral bioavailability of SB-399885 (52%) versus that of SB-271046 (82%) (Hirst *et al.*, 2006). Additional efforts by medicinal chemists to optimize brain penetration resulted in the development of compound SB-742457. This 5-HT_6 receptor antagonist is actually undergoing Phase II clinical trial studies after having shown good brain/blood ratio (0.5), excellent oral bioavailability (76%), and acceptable half-life (3 h) in rat (Upton *et al.*, 2008).

The researchers at the Wyeth Laboratories (now part of Pfizer) focused the research on the discovery of selective 5-HT$_6$ receptor agonists as tools to better understand the effects of 5-HT$_6$ agonism *in vivo*. This work led to the identification of compound WAY-181187, and to its development in a model for obsessive compulsive disorders (Cole *et al.*, 2007; Schechter *et al.*, 2008). Currently, the compound is in clinical trial for anxiety (Holenz *et al.*, 2006). Preclinical pharmacokinetic parameters were evaluated with an intravenous and oral dose of 1 and 10 mg/kg, respectively, in rat (Cole *et al.*, 2007). Following the intravenous administration of the dose 1 mg/kg formulated in 2% Tween-80, WAY-181187 was characterized by an apparent volume of distribution of 24 L/kg, which is greater than the rat blood volume of 0.054 L/kg (Davis and Morris, 1993), indicating significant extravascular distribution. The plasma systemic clearance (123 mL/min/kg) was approximately twice that of the hepatic blood flow (50–70 mL/min/kg) in rat. The authors suggest involvement of extrahepatic clearance of compound. However, when a physiological interpretation is placed on clearance, clearance from blood is generally a more appropriate measure of organ function than is plasma clearance. In fact, it is whole blood, not plasma or serum, that flows through the organs (e.g., liver) and a relationship between blood and plasma clearance is linked to the value of the blood to plasma concentration ratio (λ) as shown in the following equation: $CL_{plasma} = CL_{blood} \times \lambda$, where CL is clearance. Unfortunately, in the study of Cole *et al.* (2007), no information is reported on λ. Therefore, an extrahepatic clearance may be questionable. When the compound was orally administered in 2% Tween-80/0.5% methyl cellulose at dosage of 10 mg/kg, it was rapidly absorbed and showed a bioavailability of 23%. This was consistent with the high metabolic instability of the compound in rat liver microsomal incubations. Additional information in rat suggested a saturable absorption of the compound when a dose of 100 mg/kg was administered (Cole *et al.*, 2007). In dogs, after intravenous administration of 1 mg/kg, in normal saline at volume of 1 mL/kg, WAY-181187 showed a systemic plasma clearance of 30 mL/min/kg, which is approximately in the range of dog hepatic blood flow that is 40–50 mL/min/kg. The apparent volume of distribution was large (7.9 L/kg) and the oral bioavailability after 5 mg/kg was of 55%. The higher oral bioavailability of WAY-181187 is consistent with a better hepatic microsomal stability of the compound in dogs. Interestingly, an increased oral dose above 5 mg/kg resulted in increased bioavailability, suggesting saturation of hepatic first-pass or metabolic clearance (Cole *et al.*, 2007). Regarding the BBB passage issue, WAY-181187 was considered a moderate brain penetrant as indicated by a brain/plasma ratio of 0.1 and 0.7 after intraperitoneal (3 mg/kg) and oral (100 mg/kg) administration, respectively (Cole *et al.*, 2007). Additional medicinal chemistry of the same group resulted in a 5-HT$_6$ receptor antagonist, known as SAM-531 (or WAY-262531), that is actually in clinical trial for cognitive disorders (Upton *et al.*, 2008). Although SAM-531 is in advanced

development (Phase II trial), to the best of our knowledge, no preclinical or clinical pharmacokinetic information has been published.

Preclinical data on SUVN-623 (generated by Suven Life Sci.) has shown a good oral bioavailability of 75% and 53% in rats and dogs, respectively. This compound exhibited a plasma half-life of 2.2 h in rats and 11.3 h in dogs (Abraham *et al.*, 2009). Furthermore, in the same study, SUVN-623 demonstrated an excellent brain penetration index of 3.8 in rats. Interestingly, Suven's 5-HT_6 receptor antagonists (e.g., SUVN-502) have been claimed to have high brain penetration in rodents. Although, to the best of our knowledge, no pharmacokinetic information has been reported for the compound SUVN-502, it is currently in Phase I/II clinical trials for symptomatic treatment of Alzheimer's disease.

Recently, tolylamine 5-HT_6 antagonist (Table I; compound 9h; synthesized by Pfizer) has been shown to have all pharmacokinetic values desirable for a CNS drug candidate (Singer *et al.*, 2009). It has an excellent brain/plasma ratio of 23 with oral bioavailability of 90%, systemic clearance (66 mL/min/kg), large volume of distribution at steady state (12 L/kg), and moderate plasma half-life (5.9 h) in rat.

More recently, the Sigma-Tau group reported ST1936 as a potent 5-HT_6 receptor agonist with activity in animal pharmacological models for CNS disorders (Bedini *et al.*, 2005; Valentini *et al.*, 2009). After an intraperitoneal dose of 20 mg/kg, the compound attained C_{max} of plasma (534 $\pm$ 26 ng/mL; mean $\pm$ SD) and brain (22,165 $\pm$ 1383 ng/g; mean $\pm$ SD) within 0.25 h (first sampling time; T_{max}) of administration, decreasing thereafter to below the limit of quantification by 8 h after dosing. The mean elimination half-life of ST1936 was approximately 1 h in both plasma and brain. The examination of the area under the curve from zero to infinity (AUC) between brain and plasma resulted in a brain/plasma AUC ratio value of 53, indicating an excellent brain penetration. Similar pharmacokinetic parameters were found for the *N*-demethyl metabolite (ST5523), but, as expected, a lower brain penetration was observed. In fact, the brain/plasma AUC ratio value for this compound was 11. However, this metabolite was not further investigated because it was not more effective than ST1936 in pharmacological experimental studies (Bordi F., personal communication, Sigma-Tau).

The following ones are some selective 5-HT_6 ligands, synthesized by pharmaceutical companies and/or academic researchers, which suffer from lack of pharmacokinetic studies. However, as many of these compounds are under advanced clinical trials, information on preclinical disposition should be available in-house. The compounds include 5-HT_6 receptor partial agonists E-6801 and E-6837, from Esteve (Romero *et al.*, 2006); SGS-518 (antagonist) from Saegis Pharmaceuticals in cooperation with Lilly; SYN-114 and SYN-120, both 5-HT_6 receptor antagonists developed by Synosia Therapeutics in collaboration with Roche; and AVN-211 and AVN-322 antagonists from Avineuro

Pharmaceuticals. PRX-07034 has shown excellent 5-HT$_6$ receptor antagonist properties, but its Phase II trials have been halted for financial reasons.

In the course of drug development, imaging technologies, such as positron-emission tomography (PET), single-photon-emission tomography (SPECT), magnetic resonance (MRI), and ultrasound, have been used to elucidate site-specific receptor occupancy or assess biodistribution of the drug candidate (Willmann *et al.*, 2008). In 5-HT$_6$ receptor occupancy studies, the first compound to be synthesized was the radionuclide SB258585. Although [^{125}I]SB258585 showed a good *in vitro* profile [i.e., high level (>60%) of specific binding] to membranes derived from rat or pig striatum and human caudate putamen, its use as a tool for *in vivo* imaging has been hampered because of its poor brain penetration (Hirst *et al.*, 2000). In order to improve brain penetration, Tang *et al.* (2007) have developed the [^{18}F]12ST05 compound, which is structurally related to *in vitro* selective 5-HT$_6$ receptor antagonists (Zhou *et al.*, 2005). Following intravenous injection of the fluorine-18 labeled compound, PET scan acquisition in cat brain regions showed a rapid accumulation of [^{18}F]12ST05 in striatum, hippocampus, thalamus, cingulated cortex, and cerebellum. This homogenous uptake of [^{18}F]12ST05 in the above brain areas suggests that this radiotracer is not consistent with 5-HT$_6$ receptor distribution (Tang *et al.*, 2007). Recently, a potential PET radioligand, [^{11}C]GSK215083, for site-specific occupancy of 5-HT$_6$ receptors has been evaluated in brain of anesthetized pigs (Martarello *et al.*, 2005). The radionuclide appeared rapidly in the brain reaching the C_{max} at about 20 min, with the highest uptake into the striatum followed by cortical regions and cerebellum. This regional brain concentration is consistent with reported 5-HT$_6$ receptor densities and localization determined by tissue section autoradiography in animals and man. Although [^{11}C]GSK215083 is rapidly metabolized, the contribution to total radioactivity is mainly due to the parent compound (60%), at 30 min post-administration. Therefore, this compound seems to be an appropriate PET radiotracer also for PET studies in human subjects (see Section III). In conclusion, the availability of specific PET tracers facilitates pharmacokinetic investigation in preclinical experiments aimed to obtain important properties of candidate therapeutic agents before entering in Phase I clinical trials.

III. Clinical Pharmacokinetic Results

At the time of this writing, many 5-HT$_6$ ligands, particularly 5-HT$_6$ antagonists, are in Phase I and II clinical trials for CNS diseases (Table I; Upton *et al.*, 2008) and obesity (Table I; Geldenhuys and Van der Schyf, 2009). Although Phase I studies are also aimed at investigating the pharmacokinetics in healthy

volunteers, results on such trials are not often reported. Therefore, published information on the clinical pharmacokinetics of targeted 5-HT_6 receptors compounds is actually very poor.

As highlighted above, the first compound selective on 5-HT_6 receptors to reach the Phase I human trial was SB-271046 for the treatment of cognitive dysfunction in Alzheimer's disease. However, its development has been halted due to low BBB penetration as shown by Cunningham (see www.wales.nhs.uk/sites3/Documents/357/Vin_Cunningham_Cardiff_PET.pdf) using the PET tracer [^{11}C]SB-271046 in man. In fact, the distribution of the compound has shown a brain/plasma ratio <1%, which is much less than that reported in rat (5–14%). As discussed in Section II, SB-271046 is a P-glycoprotein (P-gp) substrate; therefore, at the very first glance, one could surmise that the lower brain distribution in human (<1%) compared to rat (5–14%) is due to species difference in the intrinsic activity of P-gp efflux protein at the BBB. However, one should consider other than P-gp efflux transport also the influx process (passive and active uptake) because the drug concentration in the brain is the net result of many factors. This is a good example of how the use of *in vivo* imaging with PET has produced information on unfavorable pharmacokinetics of a potentially marketable drug.

In contrast to the mentioned direct approach, in which the drug SB-271046 has been radiolabeled with a positron emitter (^{11}C for ^{12}C forming [^{11}C]SB-271046) and performed by dynamic imaging, in indirect studies (or site-specific occupancy studies), the receptor binding profile of the compound of interest is derived from its co-injection with a radiolabeled test ligand that binds to specific receptors. Using the indirect approach, site-specific occupancy studies have been performed with the compound SB-742457. The radioligand [^{11}C]GSK215083 has shown a high non-displaceable binding potential (BP_{ND}) in caudate, putamen, and striatum areas that are consistent with the known distribution of 5-HT_6 receptors. When the 5-HT_6 receptor antagonist SB-742457 was orally administrated (175 mg) 5 h prior the administration of [^{11}C]GSK215083 in two subjects in a Phase II clinical trial, a marked reduction (>40%) of BP_{ND} was observed. This finding suggests that (1) [^{11}C]GSK215083 is a good PET radioligand to image 5-HT_6 receptors in the human brain and (2) binding sites in human brain have been occupied by SB-742457, a 5-HT_6 receptor antagonist (Martarello *et al.*, 2008; Parker *et al.*, 2008).

For SUVN-502, the pharmacokinetic profile during Phase I single or multiple ascending dose studies resulted in an estimate of the parent compound SUVN-502 and its active metabolite M1 in plasma samples obtained up to 72 h after dosing. The compound has been claimed to have favorable pharmacokinetic, safety, and toxicology profile when administered once daily. Unfortunately, pharmacokinetic parameters are not shown in the published poster abstracts (Nirogi *et al.*, 2009).

For the compound SAM-531 (Table I), a selective 5-HT$_6$ receptor antagonist for treatment of cognitive disorders associated with schizophrenia and Alzheimer's disease, clinical studies including (1) mass balance and metabolic disposition of orally administered ^{14}C-labeled SAM-531; (2) safety, tolerability, and pharmacokinetics of single and multiple doses of SAM-531; (3) and potential interaction between verapamil immediate release and a single dose of SAM-531 are under way (see www. pfizerpro.com/clinicaltrials). For other 5-HT$_6$ receptor drug candidates, to the best of our knowledge, no information has appeared in scientific literature on human drug disposition; therefore, we await the pharmacokinetic results of ongoing and future clinical studies.

IV. Concluding Remarks

Over the past decade, advances in molecular biology and neurobiology, *in vitro*/*vivo* pharmacology, medicinal chemistry, and clinical development on 5-HT$_6$ receptors have dramatically increased. An enormous effort in the design of 5-HT$_6$ receptor ligands has resulted in the synthesis of potential therapeutic agents with activity in CNS and obesity disorders (Geldenhuys and Van der Schyf, 2009; Upton *et al.*, 2008). However, few pharmacokinetic data of preclinical and clinical studies have been published. As discussed above, a better understanding of brain disposition of 5-HT$_6$ receptor ligands in animal experiments can lead to improved decisions for clinical studies. For many of the compounds, discussed in this chapter, besides systemic pharmacokinetic parameters, brain penetration information has also been reported (Table II). Unfortunately, the measure of brain penetration has been calculated using the traditional approach, based on total drug concentration ratio between brain and blood/plasma. This could be misleading particularly when a link between tissue exposure (pharmacokinetics) and efficacy (pharmacodynamic) is needed. As it is generally accepted that the unbound drug exerts the pharmacological effect(s), the new approach places emphasis on right balance between free fraction in plasma and brain, and between rate and extent of CNS penetration (Hammarlund-Udenaes *et al.*, 2008; Liu *et al.*, 2008). In order to gain information on unbound drug concentration, a useful *in vivo* tool is the microdialysis method, which is being increasingly applied in preclinical and clinical investigation (Elmquist and Sawchuk, 1997). Although this technique has been widely applied for the evaluation of neurotransmitters in 5-HT$_6$ receptor ligand neurochemical studies (Chapter Neurochemistry; Dr. Dawson), its use for pharmacokinetic studies remains to be explored.

TABLE II

Pharmacokinetic Parameters of Pharmacological Tools or Drug Candidates on 5-HT_6 Receptors

Compound	Species, dose, and route of administration	Parameter	Parameter value	Reference
SB-271046	Rat, N.A., i.v. infusion	CL (mL/min/kg)	7.7	Bromidge *et al.* (1999)
		T/2 (h)	4.8 ± 0.1	
		F (%)	>80	
		CNS penetration (%)	10	
		P-gp substrate	yes	
SB-357134	Rat, 0.6 mg/kg/h, i.v. infusion	CL (mL/min/kg)	14	Bromidge *et al.* (1999)
		F (%)	65	
		CNS penetration (%)	~19	
SB-742457	Rat, 0.5–4.4 mg/kg, oral	*T*/2 (h)	3	Upton *et al.* (2008)
		F (%)	76	
		CNS penetration (%)	50	
WAY-181187	Rat, 1 mg/kg i.v. or 10 mg/kg oral	CL (mL/min/kg)	120	Cole *et al.* (2007)
		F (%)	23	
		V_d (L/kg)	24	
		CNS penetration (%)	10 (i.v.), 70 (os)	
	Dog, 1 mg/kg i.v. or 5 mg/kg oral	CL (mL/min/kg)	30	
		F (%)	55	
		V_d (L/kg)	7.9	
SUVN-623	Rat, N.A.	*T*/2 (h)	2.2	Abraham *et al.* (2009)
		F (%)	75	
		BPI	3.8	
	Dog, N.A.	*T*/2 (h)	11.3	
		F (%)	53	
ST1936	Rat, 20 mg/kg, intraperitoneal	*T*/2 (h)	1	Mancinelli *et al.* (2010)
		CNS penetration (%)	530	
Tolylamine (compound 9h)	Rat, N.A.	CL (mL/min/kg)	66	Singer *et al.* (2009)
		T/2 (h)	5.9	
		F (%)	90	
		V_d (L/kg)	12	
		CNS penetration (%)	230	

CL = systemic clearance; *T*/2 = half-life; *F* = bioavailability; V_d = volume of distribution; CNS = central nervous system; BPI = Brain Penetration Index; N.A. = not available; i.v. = intravenous.

As mentioned in the previous sections, the 5-HT$_6$ receptor compounds are vastly different in their chemical structures (Table I). Therefore, no general rule concerning their metabolism can be given. Besides very few studies in which the formation of metabolite(s) (Mancinelli *et al.*, 2010; Nirogi *et al.*, 2009) is disclosed, the full characterization of the *in vivo* metabolic fate of these drug candidates remains to be elucidated in both animals and humans using the gold-standard, radiolabeled techniques. Some of these 5-HT$_6$ receptor compounds have been probably already investigated in pharmaceutical and/or academic laboratories, but no information has been disclosed to the scientific community. We must remain keenly aware of the lack of other important pharmacokinetic issues, e.g., species- and sex-related differences in metabolism, drug–drug and nutrient–drug interactions, interindividual variability, and interspecies scaling. This information is necessary to assure advances in the development of these new molecular entities.

In conclusion, the exciting time in the field of 5-HT$_6$ receptor ligands has successfully pushed some compounds to the stage of clinical trials. However, until now, few studies have highlighted the pharmacokinetics of these agents in preclinical and clinical studies. We hope ADME results will become available soon.

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