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Position 5.46 of the Serotonin 5-HT_{2A} Receptor Contributes to a Species-Dependent Variation for the 5-HT_{2C} Agonist (*R*)-9-Ethyl-1,3,4,10*b*-tetrahydro-7-trifluoromethylpyrazino[2,1-*a*]isoindol-6(2*H*)-one: Impact on Selectivity and Toxicological Evaluation[S]

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ABSTRACT

Successful development of 5-HT_{2C} agonists requires selectivity versus the highly homologous 5-HT_{2A} receptor, because agonism at this receptor can result in significant adverse events. (*R*)-9-Ethyl-1,3,4,10*b*-tetrahydro-7-trifluoromethylpyrazino[2,1-*a*]-isoindol-6(2*H*)-one (compound **1**) is a potent 5-HT_{2C} agonist exhibiting selectivity over the human 5-HT_{2A} receptor. Evaluation of the compound at the rat 5-HT_{2A} receptor, however, revealed potent binding and agonist functional activity. The physiological consequence of this higher potency was the observation of a significant increase in blood pressure in conscious telemeterized rats that could be prevented by ketanserin. Docking of compound **1** in a homology model of the 5-HT_{2A} receptor indicated a possible binding mode in which the ethyl group at the 9-position of the molecule was oriented toward position 5.46 of the 5-HT_{2A} receptor. Within the human 5-HT_{2A} receptor, position 5.46 is Ser242; however, in the rat 5-HT_{2A} receptor, it is Ala242, suggesting that the potent functional activity in this species resulted from the absence of the steric bulk provided by the -OH moiety of the Ser in the human isoform. We confirmed this hypothesis using site-directed mutagenesis through the mutation of both the human receptor Ser242 to Ala and the rat receptor Ala242 to Ser, followed by radioligand binding and second messenger studies. In addition, we attempted to define the space allowed by the alanine by evaluating compounds with larger substitutions at the 9-position. The data indicate that position 5.46 contributed to the species difference in 5-HT_{2A} receptor potency observed for a pyrazinoisoindolone compound, resulting in the observation of a significant cardiovascular safety signal.

Agonists of the serotonin 5-HT_{2C} receptor have been demonstrated to be effective in the treatment of obesity and are postulated to be useful in the treatment of affective disorders such as schizophrenia (Miller, 2005; Rosenzweig-Lipson et al., 2007). The development of clinically useful 5-HT_{2C} agonists is dependent upon the attainment of selectivity versus the highly homologous 5-HT_{2A} and 5-HT_{2B} receptors, because agonism at each can produce significant central nervous system (CNS) and cardiovascular adverse events (Titeler et al., 1988; Fitzgerald et al., 2000; Rothman et al., 2000).

The 5-HT_{2A} receptor is highly expressed in the CNS, especially within the cortex, but can also be found in the smooth muscle cells of numerous peripheral tissues, where it mediates contractile responses (Appel et al., 1990; Roth et al., 1998; Nagatomo et al., 2004). 5-HT_{2A} agonists such as (+)-norfenfluramine and DOI can produce elevations in blood pressure in the rat and directly constrict renal and carotid arteries in rats and dogs, respectively (Centurión et al., 2001; Ni et al., 2004).Thus, in addition to the oft-cited potential for hallucinogenic adverse events for those compounds possessing 5-HT_{2A} agonist activity, the potential for blood vessel constriction is also a significant event that must be monitored (Kaumann and Levy, 2006).

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); CNS, central nervous system; DOI, 4-iodo-2,5-dimethoxyphenylisopropylamine; GPCR, G-protein-coupled receptor; HEK, human embryonic kidney; FLIPR, fluorometric imaging plate reader; compound **1,** (*R*)-9-ethyl-1,3,4,10*b*-tetrahydro-7-trifluoromethylpyrazino[2,1-*a*]isoindol-6(2*H*)-one; compound **2**, (*R*)-9-isopropyl-1,3,4,10*b*-tetrahydro-7-trifluormethylpyrazino[2,1-*a*]-isoinol-6(2*H*)-one; WT, wild type; TM, transmembrane.

The literature is replete with examples of species variation in the amino acid sequences of G-protein-coupled receptors, including those for the 5-HT_{2A} receptor, affecting compound binding affinity and functional potency. Specifically, a species variation in the fifth transmembrane domain of the 5-HT_{2A} receptor (position 5.46, using the numbering system of Ballesteros and Weinstein, 1995) has been shown by a number of groups to affect the affinity and functional potency of both agonist and antagonist ligands (Kao et al., 1992; Johnson et al., 1993; Almaula et al., 1996; Braden and Nichols, 2007). Position 5.46 is a serine (Ser242) in humans, cynomolgus monkey, and dog receptors, whereas it is an alanine (Ala242) in rodents. More specifically, the affinity of the ergoline antagonist radioligand [^{3}H]mesulergine is lower at the human receptor versus the rat as a result of the serine at 5.46. The affinity of [^{3}H]mesulergine could be made equivalent to the rat after mutagenesis of the human receptor homolog from Ser→Ala (Kao et al., 1992). Additional characterization of this species difference has indicated that the serine may positively contribute to the binding affinities of N1-unsubstituted ergolines (such as ergonovine) and tryptamines. Conversely, the Ser is thought to disrupt the affinities of N1-substituted ergolines (such as mesulergine) and indolamines, which has been postulated to be due to the loss of hydrogen bond formation (Johnson et al., 1993; Almaula et al., 1996).

We have described a novel 5-HT_{2C} agonist chemotype that exhibited significant selectivity versus the human 5-HT_{2A} and 5-HT_{2B} receptors as well as many other GPCRs (Wacker et al., 2007). The lead molecule [(*R*)-9-ethyl-1,3,4,10*b*-tetrahydro-7-trifluoromethylpyrazino[2,1-*a*]isoindol-6(2*H*)-one (compound **1**)] produced significant reductions in food intake in an overnight feeding assay and reductions in body weight after long-term administration to rats. The reductions in food intake could be completely reversed by a selective 5-HT_{2C} antagonist. Further in vitro characterization of the lead molecule at the rat variants of the 5-HT_2 receptor family revealed that the compound demonstrated significantly higher potency versus the rat 5-HT_{2A} receptor while remaining unchanged, relative to human, at the 5-HT_{2B} and 5-HT_{2C} receptors. The significant potency for the compound at the rat receptor resulted in the observation of a ketanserin-sensitive elevation in blood pressure during subsequent toxicological studies. To help understand the species difference, compound **1** was docked in a homology model of the 5-HT_{2A} receptor that implicated position 5.46 within the fifth transmembrane domain of the receptor. We carried out site-directed mutagenesis to confirm this hypothesis. Our data indicate that position 5.46 in the 5-HT_{2A} receptor confers species specific selectivity for the 9-ethyl pyrazinoindolone and that the serine present in the human homolog provides steric bulk that hinders the binding of the compound and may produce a less than optimal confirmation within the receptor pocket to produce second messenger activation. We also attempted to better define the available space presented by the serine to alanine change using additional 9-substituted pyrazinoindolones of greater size. Because the analogous amino acid in the human 5-HT_{2C} receptor is an alanine, we propose that the space-filling serine 242 of the human 5-HT_{2A} receptor represents a mechanism by which to confer 5-HT_{2C} selectivity versus this highly homologous serotonin receptor family member.

Materials and Methods

Site-Directed Mutagenesis and Plasmid Construction. Mutagenesis was carried out using polymerase chain reaction-based methods (the QuikChange site-directed mutagenesis kit from Stratagene, La Jolla, CA) according to the manufacture's protocol. The mutagenesis and sequencing primers were from Invitrogen (Carlsbad, CA). All constructs were sequenced after incorporation into pcDNA3.1 at the BMS Core Sequencing Facility.

Cell Culture. HEK293 cells were stably transfected with cDNAs for the described receptor constructs using Lipofectamine. Clonal selection was conducted under hygromycin and/or G418 resistance. Individual clones were screened by 5-HT-mediated increases in intracellular calcium using a fluorometric imaging plate reader (FLIPR). Cells were maintained in high-glucose Dulbecco's modified Eagle's medium with L-glutamine, HEPES, pyridoxine HCl (Invitrogen) plus 10% dialyzed fetal bovine serum and 0.2 mg/ml G418 and 0.25 mg/ml hygromycin.

Binding Assays. Membranes were prepared from the above cell lines by scraping the cells from cell culture plates in a buffer consisting of 50 mM Tris and 1.0 mM EDTA, pH 7.4. Alternatively, membranes were prepared from either dog or cynomolgus monkey frontal cortex in the same buffer. The cells/tissues were then processed with a Polytron homogenizer (Kinematica, Littau-Lucerne, Switzerland), followed by centrifugation. Radioligand binding assays were carried out in 96-well plates with a total assay volume of 200 μl. Tests compounds (spanning a concentration range of 10,000-fold) and [^{125}I]DOI (at a concentration of 0.4 nM for competition studies) were added to the assay plate in assay buffer (50 mM Tris, 10 mM $MgSO_4$, 0.5 mM EDTA, 0.05% ascorbic acid, and 10 μM pargyline, pH 7.4) followed by the addition of membranes (20 μg protein/well). The assay was then incubated at 37°C for 45 min in the dark followed by rapid filtration on a Packard 96-well harvester (PerkinElmer Life and Analytical Sciences, Waltham, MA) onto Packard Unifilter GF/B filter plates, followed by three washes (50 mM ice-cold Tris, pH 7.4,). After the plates had dried, 40 μl of MicroScint 20 (PerkinElmer Life and Analytical Sciences) was added into each well, the plate was sealed with TopSeal, and counted on a Packard TopCount. Binding data were calculated using Prism (GraphPad Software, San Diego, CA) with K_i values calculated using the Cheng-Prusoff equation. Differences in compound binding affinities across the receptor constructs were assessed by analysis of variance followed by post hoc analysis using a Bonferroni test.

Intracellular Calcium Measurements. Cells were plated in a 96-well black clear-bottomed plates coated with poly-D-lysine (BD BioCoat; BD Biosciences, San Jose, CA) at a density of 5×10^4 cells/well and cultured overnight. Cells were loaded with Fluo-4 AM (Invitrogen) for 1 h at 37°C. After loading, the dye was aspirated, and 150 μl of base buffer (1× Hanks' buffered saline solution with 5 mM glucose and 10 mM HEPES) was added to the wells. The compounds (spanning a concentration range of 10,000-fold) were added to wells and the plate read for 90 s on a FLIPR-TETRA (Molecular Devices, Sunnyvale, CA). Potency and efficacy determinations were made using nonlinear curve fitting with Prism software. Intrinsic activity values were calculated based on the maximal responses produced by serotonin.

Rat Telemetry Studies. Male Sprague-Dawley rats were implanted with radiotelemetry devices (PA-C40 transmitter; Data Sciences International, St. Paul, MN) to measure blood pressure, heart rate, and locomotor activity. The animals were allowed a recovery period of 14 days before testing. Animals were treated with either vehicle (distilled H_2O at pH 4.0) ($n = 5$), a 10 mg/kg dose of compound **1** p.o. in a volume of 5 ml/kg, 1.5 mg/kg ketanserin i.p., or 1.5 mg/kg of prazosin s.c., 30 min before a dose of 10 mg/kg compound **1** p.o. Compound 2 was administered in a volume of 5 ml/kg p.o. The

animals were then monitored for blood pressure, heart rate, and locomotor activity for a period of 24 h. The change in blood pressure for each treatment was calculated as a percentage change from baseline with the use of the following equation: % change = [(average after 2 h to 8 h − baseline)/baseline × 100)].

Homology Model. Chain A from the crystal structure of bovine Rhodopsin (Protein Data Bank entry 1f88) was used as a template for homology modeling. A model of the human 5HT$_{2A}$ receptor was generated using Prime v.1.6 (Schrödinger LLC, New York, NY). GPCR anchor motifs (Kimura and Chasman, 2003) were used to modify the alignment before model building. Because of the vast differences in extracellular loop 2 in rhodopsin and the 5HT$_{2A}$ receptor, and the uncertainty of loop placement, the loop was removed for this study. The active site was refined by docking serotonin using Schrödinger's Induced-Fit Docking protocol (Sherman et al., 2006), and the active site was minimized using OPLS2001. The sequence alignment of the human versus rat species had 91% sequence identity; therefore, the model of human 5HT$_{2A}$ receptor was used as a basis for the rat 5HT$_{2A}$ model. Human residues were mutated to the rat residues and the active site was minimized using Prime.

Test Compounds. All of the isoindolones described herein were prepared at Bristol-Myers Squibb as detailed by Wacker et al. (2007).

Results

Evaluation of compound **1** (Fig. 1) at the rat variants of the 5-HT$_{2C}$ and 5-HT$_{2A}$ receptors revealed that the binding affinity for compound **1** did not change between species for the 2C receptor, whereas the affinity at the 2A receptor improved by >10-fold relative to the human subtype (Fig. 2 and Table 1). More importantly, the functional potency of compound **1** exhibited a leftward shift of approximately 100-fold at the rat receptor when assessed by FLIPR (Fig. 3 and Table 1). Although we did not conduct radioligand or functional studies in a rat 5-HT$_{2B}$ recombinant cell line, when compound **1** was tested in a rat fundus smooth muscle contraction assay, it exhibited potency and efficacy characteristics similar to those of the recombinantly expressed human 5-HT$_{2B}$ receptor (data not shown).

The impact of the greater rat 5-HT$_{2A}$ receptor potency became readily apparent when compound **1** was assessed for effects on blood pressure in a conscious rat telemetry assay. A 10 mg/kg dose of compound **1** produced a statistically significant ($p < 0.01$) increase in blood pressure versus vehicle in normal Sprague-Dawley rats (Fig. 4). A 1.5 mg/kg i.p. dose of ketanserin completely prevented the elevation in blood pressure produced by compound **1**, indicative of a 5-HT$_{2A}$ receptor mediated effect. Because ketanserin also has appreciable α_1-adrenergic affinity, we also tested the ability of the α_1-adrenergic receptor antagonist prazosin to block the blood pressure increase, which it failed to do (data not shown). Compound **1** was found to exhibit no binding

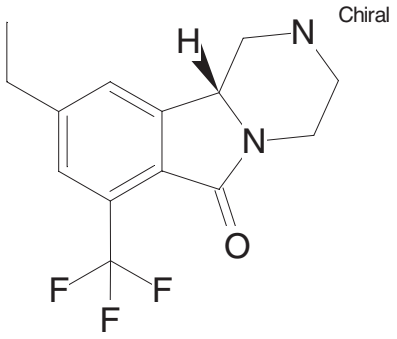


Fig. 1. Compound **1** [(*R*)-9-ethyl-1,3,4,10*b*-tetrahydro-7-trifluoromethylpyrazino[2,1-*a*]isoindol-6(2*H*)-one] as described previously by Wacker et al. (2007).

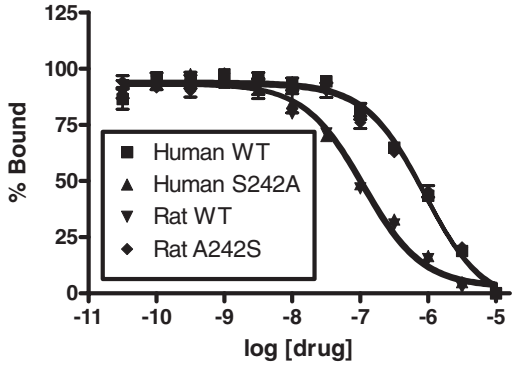


Fig. 2. Competition binding studies with compound **1** in membranes prepared from stable HEK cells expressing the indicated receptor constructs. The data are expressed as a percentage of total [^{125}I] DOI binding. Data are the mean ± S.E.M. of four experiments performed in triplicate.

TABLE 1

5-HT$_{2A}$ and 5-HT$_{2C}$ receptor binding affinities and functional potencies for compound **1**

Binding affinities (K_i) were determined from competition assays with [^{125}I]DOI. The potency (EC_{50}) and maximal efficacy (E_{max}) values were determined by FLIPR assays using Fluo-4 as the indicator dye for intracellular calcium. Intrinsic activities were calculated based on the maximal elevation of fluorescent light units produced by serotonin. The values represent the mean nanomolar values ± S.E.M. of four to six experiments.

5-HT$_{2A}$	
Human	
K_i	568 ± 63
EC_{50}	2565 ± 65
E_{max}	34 ± 1
Rat	
K_i	52 ± 9
EC_{50}	24 ± 3
IA	93 ± 1
5-HT$_{2C}$	
Human	
K_i	18 ± 2
EC_{50}	16 ± 4
IA	100
Rat	
K_i	16 ± 3
EC_{50}	6 ± 1.5
IA	91 ± 3

IA, intrinsic activity.
[a] Data previously reported by Wacker et al. (2007).

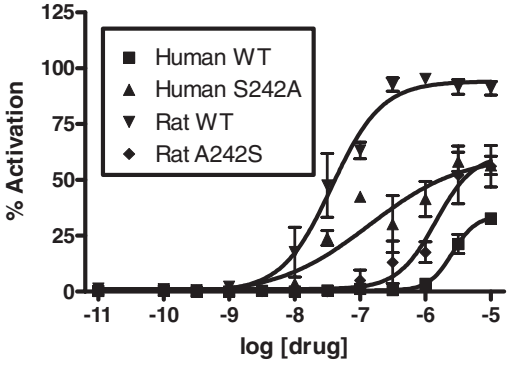


Fig. 3. Stimulation of intracellular calcium signaling in stable HEK cell lines expressing the indicated receptor constructs. The data are expressed as a percentage of the maximal signal produced by serotonin. Data are the mean ± S.E.M. of four experiments performed in triplicate.

affinity to α1-adrenergic receptors at a concentration of 10 μM, as determined in a cortical homogenate binding assay using [^{3}H]prazosin (assay conducted by MDS Pharma Services, King of Prussia, PA). Subsequent evaluation of compound **1** for agonist activity in recombinant cell lines expressing the α_{1a}-adrenergic receptor subtype in a FLIPR assay yielded no response (Supplemental Fig. 1).

To better understand the basis of the species selectivity for compound **1** at the 5-HT$_{2A}$ receptor, as well as to develop a structural basis for understanding selectivity versus the 5-HT$_{2C}$ receptor, we conducted receptor homology modeling studies. Compound **1** was docked into the human 5-HT$_{2A}$ receptor model using Glide SP (Sherman et al., 2006). Assuming that the basic amine of compound **1** is required to interact with Asp3.32 of the receptor, our docking studies revealed that the 9-ethyl moiety of the compound was oriented toward Ser5.46 in the fifth transmembrane domain; in the rat model, it was oriented toward the analogous alanine of position 5.46 (Figs. 5 and 6). These data suggested that the species differences observed with compound **1** were contributed by position 5.46, because the distance between the Ser-OH and the 9-ethyl group of compound were predicted to lie within 1.9 Å of each other. The homology model also indicated that the isoindolone core, specifically the 7-CF$_3$ group, was within van der Waals contact with the hydrophobic box postulated to be important for functional activity at the 5-HT$_{2A}$ receptor (Roth et al., 1997).

To confirm the hypothesis suggested by the modeling data, we mutated Ser5.46 of the human receptor to Ala and mutated Ala5.46 of the rat receptor to Ser. The mutated and wild-type receptor constructs were evaluated in radioligand binding studies using the agonist [^{125}I]DOI and in FLIPR functional assays. As reported previously, the Ser-to-Ala mutation had no impact on the K_d of [^{125}I]DOI binding (Johnson et al., 1993; Braden and Nichols, 2007) and the relative B_{max} values between recombinant cell lines were similar (Table 2). Competition studies revealed a small shift to the right in the affinity of 5-methoxytryptamine when mutating to the rat sequence and a corresponding left shift when mutating to the human sequence, whereas the affinity of 5-HT and tryptamine was not affected by either mutation (Table 3), replicating data published previously (Johnson et al., 1993). The Ser-to-Ala mutation in the human receptor improved the binding affinity of compound **1** by greater than 6-fold, matching that of the WT rat receptor, whereas the Ala-to-Ser mutation in the rat receptor reduced binding affinity by ~10-fold, matching that of the WT human receptor. The potency for the induction of Ca^{2+} influx into the cytoplasm was improved by the Ser-to-Ala mutation but to a greater degree (34-fold) than that observed for the binding (Table 4). Likewise the Ala-to-Ser mutation in the rat receptor reduced the functional potency of compound **1** to match that of the human WT receptor.

Because position 5.46 is Ser in both the dog and cynomolgus monkey (Fig. 5), we expected that the binding affinity of compound **1** to be more similar to that of the human receptor than the rat. Indeed, in membranes prepared from both dog and monkey frontal cortex, compound **1** competed with [^{125}I]DOI, binding with affinities of 500 ± 31 and 508 ± 14 nM, respectively (Fig. 7). Competition binding studies from rat frontal cortex yielded an affinity of 52 ± 3 nM, in excellent agreement with the affinity obtained from the recombinant rat receptor preparation (Fig. 7).

The presence of Ala rather than Ser at position 5.46 in the rat would result in the opening of space, which would reduce the steric clash of the ethyl group in the pocket for improved binding affinity and functional potency. Therefore, we expected other 9-substituted analogs to be similarly affected by the species difference at position 5.46. In addition, we hypothesized that the opening of the binding pocket created by the presence of the alanine would be finite and that the species differences observed with compound **1** would be diminished with larger 9-substituted analogs. Our hypothesis was based on the observation, from the homology model, that the isoleucine of position 4.56 (Fig. 6) may project into the proposed pocket. Indeed, we found that compounds with 9-substitutions similar in size to that of compound **1** (e.g., cyclopropyl-methyl and methoxy) were equally affected by the species difference and the subsequent mutations in binding assays (Table 5). In general, as size of the 9-substitution increased, the degree of species difference became smaller and thus was minimally affected by the mutations. Indeed, the largest substitution examined, the cyclohexyl compound, exhibited almost no differences between the human and the rat receptor in either binding or function (Table 6).

Last, we wished to address the question of whether reducing affinity and potency versus the rat 5-HT$_{2A}$ receptor would result in the attenuation of the blood pressure increase ob-

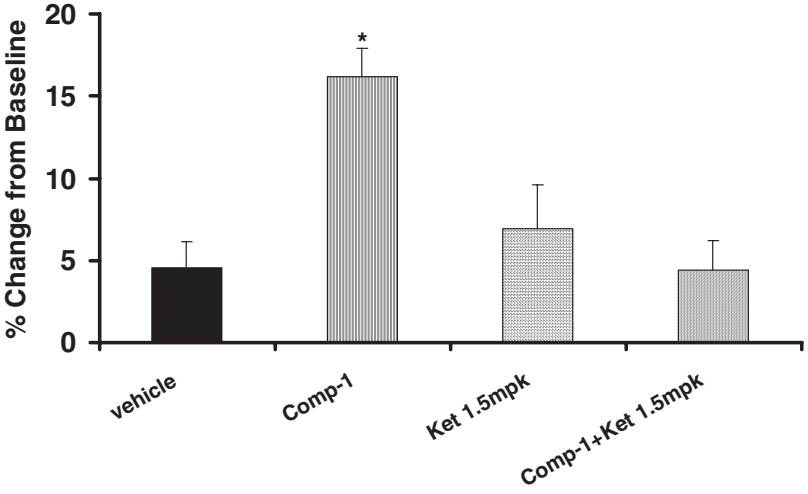


Fig. 4. Effect of compound **1**, ketanserin, and the combination of compound **1** plus ketanserin, on blood pressure in telemeterized Sprague-Dawley rats. Compound **1** produced a statistically significant elevation in blood pressure (p = 0.0009) that was reversed by ketanserin. Compound **1** was dosed by oral gavage, whereas ketanserin was administered intraperitoneally. In the combination arm the ketanserin was administered 30 min before compound **1**. Data are expressed as the mean percentage ± S.E.M. change from baseline from 2 h to 8 h after dose.

served with compound **1**. The 9-isopropyl substituted compound exhibited a 7-fold reduction in functional potency at the rat 5-HT$_{2A}$ receptor relative to compound **1** as well as a lower intrinsic activity. In addition, the binding affinity of the 9-isopropyl analog for the rat receptor was similar to the affinity of compound **1** at the dog and monkey 5-HT$_{2A}$ receptor, suggesting that an observation of no blood pressure increase in the rat for the 9-isopropyl compound might translate to a similar null observation in the dog or monkey for compound **1**. When the 9-isopropyl derivative (termed compound **2** in Fig. 8) was administered to telemeterized rats at a dose of 10 mg/kg p.o., there was no observation of increased blood pressure despite achieving plasma concentrations of drug (data not shown) similar to those of compound **1**.

Discussion

Homology modeling, based on the crystal structure of rhodopsin, has been used for several years in an attempt to understand the structural basis for drug interactions at G-protein-coupled receptors (Filipek et al., 2003). Although critical advances have been made in the crystallization of mammalian GPCRs over the last year (Rosenbaum et al., 2007; Jaakola et al., 2008), crystal structures for members of the 5-HT$_2$ receptor family have yet to be disclosed. Fortunately, one of the conclusions from the crystallization of the β2-adrenergic receptor was that homology modeling, when coupled with site-directed mutagenesis, has been useful in predicting the structure of ligand binding pockets (Rosenbaum et al., 2007). Several rhodopsin-based homology models of the 5-HT$_{2A}$ receptor have been published and used to model the receptor-ligand interactions for compounds such as mesulergine and other various ergolines, tryptamines, benzofuranones, and indoleamines (Chambers and Nichols, 2002; Braden and Nichols, 2007; Aranda et al., 2008). We have also based our homology model on the crystal structure of rhodopsin and the integration of the known literature. Although we did not make an Asp3.32 mutation, we are confident in assuming that the basic amine of compound **1** directly interacts with the counter-ion of the Asp. Using this assumption, we found that compound **1** best docked into the model with the 9-ethyl substitution of the isoindolone oriented toward position 5.46 of the receptor with a distance of 1.9 Å between the Ser-OH and the ethyl of compound **1**. As position 5.46 is one of only three amino acids in the TM regions that differ between the rat and human receptor, the other differences being at position 81 (1.39) Ala in rat versus Thr in human and position 150 (3.27) Ile in rat versus Val in human. We concluded that this change in residue from Ala to Ser was the most likely cause for the species difference for compound **1** and was confirmed by site-directed mutagenesis.

The use of site-directed mutagenesis to infer direct interactions between a compound and an individual amino acid, as it relates to a species-specific difference, is most powerful when the converse mutation is made in both species (Almaula et al., 1996). Based on our binding data, the differences between the human and rat receptor for compound **1** seem to be exclusively dependent upon position 5.46, because the human Ser-to-Ala mutation results in the rat phenotype, and the converse rat Ala-to-Ser change returns the receptor to the human phenotype. The observation was consistent with other members of this chemical class as highlighted by the methoxy and cyclopropylmethyl derivatives (Tables 5 and 6).

Assessment of our data suggests that the loss of binding affinity that occurs from the Ala-to-Ser change is due to a steric clash resulting from the introduction of the Ser -OH group. Therefore, the improved potency and affinity (and potentially the loss of selectivity versus the human 5-HT$_{2C}$ receptor) for the rat receptor is simply due to the increased size of the binding pocket, allowing more space for the ethyl group to bind. The amount of space opened up upon conversion to the alanine seems limited, because larger substitutions, such as the cyclohexyl and isobutyl, are unaffected by the mutagenesis and do not exhibit a species difference. The relatively large cyclopentyl and isopentyl substitutions have

Human 5-HT$_{2A}$	NFVLIGSFV**S**FFIPLTIMVITYFLTIKSLQ
Monkey 5-HT$_{2A}$	NFVLIGSFV**S**FFIPLTIMVITYFLTIKSLQ
Dog 5-HT$_{2A}$	NFVLIGSFV**S**FFIPLTIMVITYFLTIKSLQ
Rat 5-HT$_{2A}$	NFVLIGSFV**A**FFIPLTIMVITYFLTIKSLQ

Fig. 5. Sequence comparison of transmembrane domain 5 of the 5-HT$_{2A}$ receptor from the indicated species. Position 5.46 is highlighted in bold. Sequences were obtained from GenBank and aligned using Sequencher.

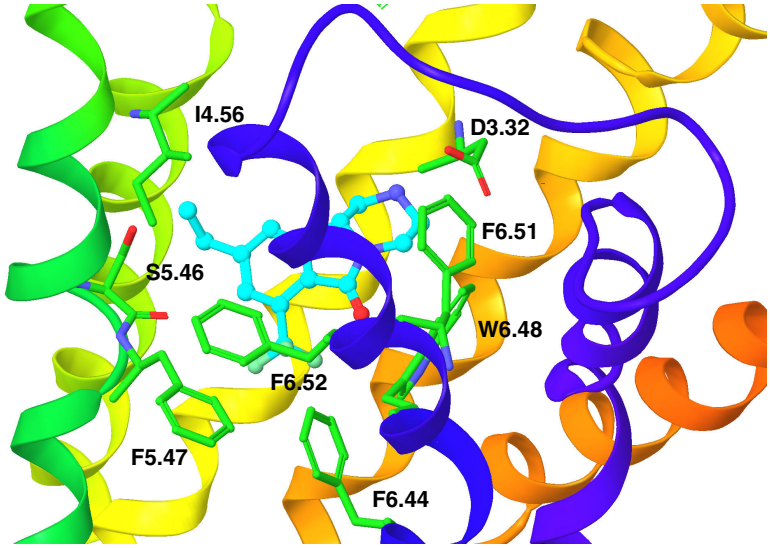


Fig. 6. Docking of compound **1** into the human 5-HT$_{2A}$ receptor homology model using GLIDE SP (Sherman et al., 2006). Residues of note are indicated using the numbering of Ballesteros and Weinstein (1995). Transmembrane domains are denoted by transitioning colors, TM1 being orange and TM7 being purple.

improved affinities upon the Ser-to-Ala mutation, which may be due to the lesser rigidity of these compounds. Based on the homology model, neighboring residue Ile206 (4.56) on TM4, which is oriented toward Ser 5.46, may limit the space available for binding of the larger 9-substitutions. However, we did not validate this observation via mutagenesis.

When one examines the functional data of the various 9-substituted isoindolones, the compounds seem to be less functionally active at the human receptor than predicted by the binding data. Although compound **1** has a binding affinity of 546 nM, the functional potency is 2635 nM with a relatively low intrinsic activity (Table 3). The difference is unlikely to be due to lower receptor expression, because the binding affinities, functional potencies, and intrinsic activities of the three standard ligands 5-HT, tryptamine, and 5-methoxytryptamine were equivalent (Table 3). Our model indicates that the tricyclic structure of the isoindolone lies in relative proximity to the proposed hydrophobic box composed of Phe 5.47, Phe 6.51, Phe 6.52 and Trp 6.48, with the position 7-CF_3 extending into the box region (Choudhary et al., 1995; Roth et al., 1997; Shapiro et al., 2000). It is possible that the presence of the Ser in the human isoform of the receptor may also disrupt the relative position of the isoindolone CF_3 to the box region, or more generally disrupts the potential of the compound to initiate the conformational changes required for receptor activation. It is relatively unlikely that other species differences would account for the increased functional differential between human and rat, because the rat Ala-to-Ser mutation mimics the species difference. It must be acknowledged, however, that the human Ser-to-Ala change does not fully restore the functional potency of the rat. There are only two other species related amino acid differences within the transmembrane domains of the 5-HT_{2A} receptor, a rat threonine to human valine at position 1.39 and a rat isoleucine to human valine at position 3.27. Additional mutagenesis would be required to confirm whether these other amino acid differences would contribute to the functional differences between the rat and human receptor.

Ser239 at position 5.43 of the human 5-HT_{2A} receptor has also been shown to contribute to the affinity and efficacy of various 5-HT_{2A} agonist chemotypes (Braden and Nichols, 2007). Because this residue does not differ among species, it is not contributing to the observed species differences for our 9-substitued isoindolones. We did, however, examine our model to assess the potential for Ser5.43 to contribute to the binding of the 9-isoindolone chemotype to the receptor. Our preferred docking conformation suggests that the -OH group of Ser5.43 lies too distant from the carbonyl group of the isoindolone core to facilitate hydrogen bond formation, primarily due to the restricted conformation of the molecule relative to tryptamine and phenethylamines previously shown to be affected by this residue (Braden and Nichols, 2007). We also found it unlikely that Ser5.43 could contribute to a steric hindrance of the 9-substituted analogs, because it lies one turn above Ser5.46 and is thus too far away to be of significance. Here again, the constrained isoindolone core would not have the conformational freedom to interact with the residue. This prediction would be in agreement with the observations of Braden and Nichols (2007), who found that the rigid LSD molecule also lacked the conformational freedom to associate with Ser5.43.

Because the analogous position of 5.46 in the human 5-HT_{2C} receptor is alanine, our data suggest that the development of selective 5-HT_{2C} receptor agonists could in part be obtained by taking advantage of the steric hindrance provided by the Ser -OH in the 5-HT_{2A} receptor. In the case of compound **1**, the steric clash of the 9-ethyl substituent with the Ser -OH results in the gain of 30-fold binding selectivity,

TABLE 2

Saturation binding analysis of the wild type and mutant human and rat cell lines

Saturation binding studies were conducted using [^{125}I]DOI as the radioligand to determine K_D and B_{max} values for the four cell lines.

Membrane Source	K_D	B_{max}
	nM	*fmol/mg*
h5-HT_{2A} WT	0.12 ± 0.02	264 ± 14
h5-HT_{2A} Ser→Ala	0.17 ± 0.02	727 ± 87
r5-HT_{2A} WT	0.12 ± 0.01	826 ± 94
r5-HT_{2A} Ala→Ser	0.11 ± 0.01	570 ± 144

TABLE 3

Affinities of compound **1** at the wild-type and mutant human and rat 5-HT_{2A} receptors

Binding affinities (K_i) were determined from competition assays with [^{125}I]DOI. The values represent the mean ± S.E.M. of four to six experiments.

Compound	5-HT_{2A}			
	Human		Rat	
	WT	Ser→Ala	WT	Ala→Ser
	nM			
5-HT	18 ± 9	21 ± 5	31 ± 6	23 ± 3
5-Methoxytryptamine	6 ± 0.2	16 ± 1	15 ± 4	8 ± 1
Tryptamine	58 ± 4	84 ± 4	72 ± 8	69 ± 6
Compound **1**	568 ± 63	88 ± 8	52 ± 9	540 ± 33

TABLE 4

Functional potencies of compounds at the wild-type and mutant human and rat 5-HT_{2A} receptors

The potency (EC_{50}) and maximal efficacy (E_{max}) values were determined by FLIPR assays using Fluo-4 as the indicator dye for intracellular calcium. Efficacies were calculated based on the maximal elevation of fluorescent light units produced by serotonin. The values represent the mean ± S.E.M. of four to six experiments.

Compound	5-HT_{2A}							
	Human				Rat			
	WT		Ser→Ala		WT		Ala→Ser	
	EC_{50}	E_{max}	EC_{50}	E_{max}	EC_{50}	E_{max}	EC_{50}	E_{max}
	nM	%	*nM*	%	*nM*	%	*nM*	%
5-HT	4.4 ± 0.9	1.0	6.8 ± 1.4	1.0	2.7 ± 0.5	1.0	2.1 ± 0.5	1.0
5-Methoxytryptamine	6.9 ± 2	83 ± 3	5.2 ± 2.8	72 ± 5	1.4 ± 0.4	93 ± 2	2.7 ± 1.3	83 ± 6
Tryptamine	54 ± 30	80 ± 7	74 ± 25	74 ± 6	13 ± 2	81 ± 3	17 ± 5	68 ± 3
Compound **1**	2565 ± 65	34 ± 1	76 ± 43	64 ± 8	24 ± 7	93 ± 1	1228 ± 934	75 ± 1

which is reduced to 3-fold upon the Ser-to-Ala mutation. The variety of substitutions that can enter the pocket as a result of the opening provided by the alanine is probably limited, however, in that substitutions larger than cyclopropyl begin to lose 5-HT$_{2C}$ affinity and potency (Wacker et al., 2007).

The ability to develop clinically efficacious and safe 5-HT$_{2C}$ agonists for the treatment of obesity has been complicated by the need to obtain significant selectivity versus the highly homologous 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors. Agonism at either of these receptor subtypes has the potential to result in clinically significant adverse events. Fenfluramine was removed from the market in the late 1990s because of an increase in the incidences of heart valve hypertrophy, now believed to be largely the result of 5-HT$_{2B}$ receptor activation by the metabolite norfenfluramine (Fitzgerald et al., 2000). 5-HT$_{2A}$ agonists have long been known to have the potential to evoke hallucinogenic responses in humans (Titeler et al., 1988) and have also been demonstrated to produce contraction in the human vasculature (Kaumann and Levy, 2006).

Compound **1** represents a highly potent and selective 5-HT$_{2C}$ agonist based on in vitro pharmacological assays employing the human isoforms of the 5-HT$_{2C}$, 5-HT$_{2A}$, and 5-HT$_{2B}$ receptors (Wacker et al., 2007). The anorectic effect of the compound could be completely abolished by a 5-HT$_{2C}$ selective antagonist (Wacker et al., 2007), and significant CNS-mediated adverse events were not noted in animals treated with the compound, including 5-HT$_{2A}$-mediated behaviors (K. Miller, unpublished observations). Indeed, further characterization of the compound in a rat fundus assay confirmed the low potency and intrinsic activity observed in the cell line expressing the human homolog of the 5-HT$_{2B}$ receptor.

Because we had not noted any obvious hallmarks of 5-HT$_{2A}$ activation (such as wet dog shakes) (Xu and Miller, 1998), and the compound was selective versus other GPCRs involved in cardiovascular function (Wacker et al., 2007), we carried compound **1** into a telemeterized rat assay of hemodynamic function. As shown in Fig. 4, compound **1** produced a significant rise in blood pressure in the rat that was sensitive to ketanserin. The degree of blood pressure elevation was similar to that observed with other 5-HT$_{2A}$ agonists in rats (Ni et al., 2004). Thus the increase in 5-HT$_{2A}$ receptor potency at the rat receptor was manifested in a significant safety signal. As further confirmation of the 5-HT$_{2A}$ hypothesis, dosing of the closely related 9-isopropyl analog, with reduced rat 5-HT$_{2A}$ activity, resulted in no blood pressure elevation.

We chose ketanserin for our telemetry studies, knowing that it is not a completely selective 5-HT$_{2A}$ antagonist (Centurión et al., 2006). To be sure, ketanserin exhibits significant binding affinity for α1-adrenergic receptors, which are also well known to contribute to blood pressure increases. The in vivo experience both in the literature and in-house with ketanserin in rats was significant relative to other more selective 5-HT$_{2A}$ antagonists; therefore, we chose ketanserin based on this knowledge of doses and plasma concentrations. We are confident in ascribing the blood pressure to the 5-HT$_{2A}$ receptor in that compound **1** exhibits no functional activity at the α_{1a} receptor subtype and did not inhibit prazosin binding at a concentration of 10 μM in a rat brain homogenate assay. Indeed, pretreating rats with prazosin

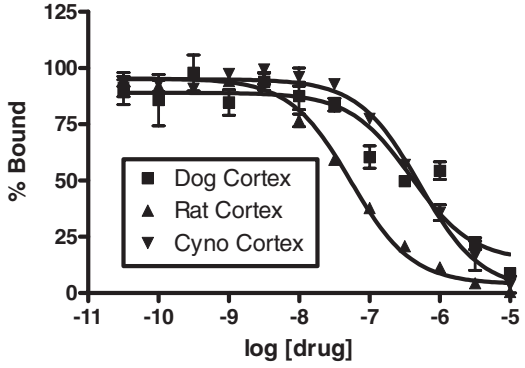
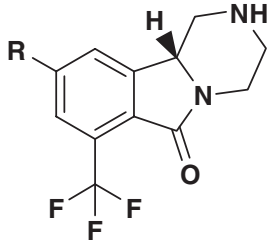


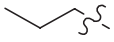
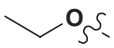
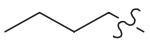
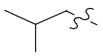
Fig. 7. Competition binding studies with compound **1** in membranes prepared from frontal cortices of the indicated species. The data are expressed as a percentage of total [^{125}I] DOI binding. Data are the mean ± S.E.M. of four experiments performed in triplicate.

TABLE 5

The affect of larger substitutions at position 9 of compound **1** on binding affinity at the human and rat WT receptors and mutant 2A receptors



Binding affinities (K_i) were determined from competition assays with [^{125}I]DOI. The values represent the mean ± S.E.M. of four to six experiments.

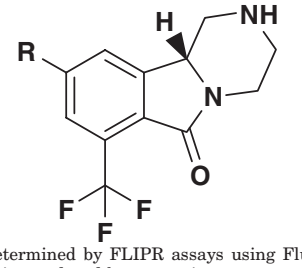
R	5-HT$_{2A}$			
	Human		Rat	
	WT	Ser→Ala	WT	Ala→Ser
	nM			
	568 ± 63	88 ± 8	52 ± 9	540 ± 33
	853 ± 78	332 ± 56	303 ± 12	761 ± 31
	375 ± 29	102 ± 10	97 ± 7	167 ± 39
	1921 ± 161	883 ± 43	744 ± 68	1124 ± 140
	1211 ± 93	543 ± 54	424 ± 30	977 ± 180
	2708 ± 320	563 ± 16	559 ± 23	1504 ± 117
	782 ± 43	114 ± 10	116 ± 11	618 ± 60
	336 ± 36	181 ± 25	194 ± 19	350 ± 88
	1773 ± 131	1428 ± 41	1433 ± 166	2033 ± 154
	900 ± 60	350 ± 14	319 ± 11	877 ± 37
	1218 ± 95	456 ± 15	405 ± 25	1126 ± 75
	990 ± 128	739 ± 53	676 ± 53	1017 ± 58

did not block the blood pressure increase produced by compound **1**. Prazosin has been shown to be more than 200-fold more potent than ketanserin in blocking α1-receptor-mediated increases in blood pressure (Centurión et al., 2006).

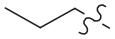
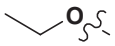
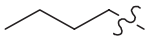
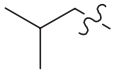
Blood pressure elevations, however small, have impeded the utilization of marketed antiobesity agents such as sibutramine, with a concern that long-term use might actually result in observed increases in morbidity and mortality (Joyal, 2004). Thus it was important to understand the structural basis for the species variation at the 5-HT_{2A} receptor and its impact on compound **1** and to understand the overall implications on structural activity relationships of the chemical series. Our homology modeling and site-directed mutagenesis confirmed that the elevation in blood pressure was likely to be species-specific and that evaluation of compound **1** in higher species might result in a cleaner profile. Indeed, the binding affinity at the monkey and dog 5-HT_{2A} receptors suggested that an acceptable therapeutic window might exist between the efficacious concentrations needed for weight loss and the plasma drug concentration at which a blood pressure elevation might be observed. Indeed, our study with the 9-isopropyl-substituted isoindolone demonstrated that a reduction in 5-HT_{2A} affinity, to a level similar to that seen for the dog and monkey, would result in an observation of no change to blood pressure. Unfortunately, the preclinical development of compound **1** was halted (due to an unanticipated off-target–based toxicity not related to cardiovascular function) before an assessment of the impact of the reduced

TABLE 6

The affect of larger substitutions at position 9 of compound **1** on functional activity at the human and rat WT receptors and mutant 2A receptors



The potency (EC_{50}) and maximal efficacy (E_{max}) values were determined by FLIPR assays using Fluo-4 as the indicator dye for intracellular calcium. Efficacies were calculated based on the maximal elevation of fluorescent light units produced by serotonin.

R	5-HT_{2A}							
	Human				Rat			
	WT		Ser→Ala		WT		Ala→Ser	
	EC_{50}	E_{max}	EC_{50}	E_{max}	EC_{50}	E_{max}	EC_{50}	E_{max}
	nM	%	*nM*	%	*nM*	%	*nM*	%
	2565 ± 70	34 ± 1	76 ± 41	64 ± 8	24 ± 7	93 ± 1	1228 ± 532	75 ± 1
	>10,000	0	1087 ± 331	24 ± 5	256 ± 34	59 ± 3	6187 ± 2203	33 ± 9
	264 ± 27	72 ± 6	71 ± 38	64 ± 3	77 ± 12	77 ± 3	394 ± 4	89 ± 5
	3485 ± 161	13 ± 2	1085 ± 272	20 ± 4	383 ± 128	58 ± 3	2985 ± 711	28 ± 3
	>10,000	0	220 ± 59	27 ± 2	317 ± 88	50 ± 2	788 ± 134	35 ± 1
	>10,000	0	974 ± 122	31 ± 1	179 ± 59	59 ± 2	>10,000	0
	2917 ± 265	34 ± 2	239 ± 50	59 ± 7	40 ± 7	44 ± 2	1801 ± 590	65 ± 10
	>10,000	0	>10,000	0	>10,000	0	>10,000	0
	>10,000	0	>10,000	0	5078 ± 1814	15 ± 5	>10,000	0
	>10,000	0	758 ± 117	10 ± 4	396 ± 64	43 ± 2	6168 ± 2221	13 ± 2
	>10,000	0	909 ± 175	10 ± 1	531 ± 45	44 ± 3	>10,000	0
	>10,000	0	>10,000	0	>10,000	0	>10,000	0

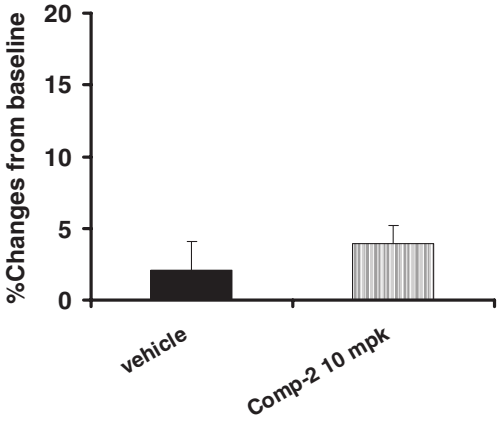


Fig. 8. Effect of compound **2** on blood pressure in telemeterized Sprague-Dawley rats. Compound **2** was dosed by oral gavage. Data are expressed as the mean percentage ± S.E.M. change from baseline from 2 h to 8 h after dose.

5-HT$_{2A}$ affinity on blood pressure in either a telemeterized dog or monkey could be conducted.

We have demonstrated that position 5.46 solely accounts for the observation of species-dependent differences in binding affinity and functional potency at the 5-HT$_{2A}$ receptor for a series of 9-substituted isoindolone compounds. The significant 5-HT$_{2A}$ activity in the rat resulted in the observation of increased blood pressure in a rat cardiovascular study. Because the analogous position in the human 5-HT$_{2C}$ receptor is alanine versus Ser in the human 5-HT$_{2A}$ receptor, our data suggest that substituents oriented toward position 5.46 may provide a structurally based mechanism by which to gain selectivity between these two highly homologous 5-HT receptor family members.

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