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-1,3,4,10b-tetrahydro-7-trifluoromethylpyrazino[2,1-$a$]isoindol-6(2$H$)-one: impact on selectivity and toxicological evaluation

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Running Title: Role of position 5.46 in 5-HT$_{2A}$ species selectivity

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List of Abbreviations: DOI, 4-iodo-2,5-dimethoxyphenylisopropylamine; 5-HT, 5-hydroxytryptamine, serotonin; FLIPR, fluorescent light plate reader; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; ANOVA, analysis of variance; TM, transmembrane
Abstract:
Successful development of 5-HT\textsubscript{2C} agonists requires selectivity vs. the highly homologous 5-HT\textsubscript{2A} receptor as agonism at this receptor can result in significant adverse events. (\textit{R})-9-ethyl-1,3,4,10b-tetrahydro-7-trifluoromethylpyrazino[2,1-a]isoindol-6(2\textit{H})-one (compound 1) is a potent 5-HT\textsubscript{2C} agonist exhibiting selectivity over the human 5-HT\textsubscript{2A} receptor. Evaluation of the compound at the rat 5-HT\textsubscript{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, which could be prevented by ketanserin. Docking of compound 1 in a homology model of the 5-HT\textsubscript{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\textsubscript{2A} receptor. Within the human 5-HT\textsubscript{2A} receptor position 5.46 is S242 however in the rat 5-HT\textsubscript{2A} receptor it is A242, 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 utilizing site directed mutagenesis both through the mutation of the human receptor S242→A as well as the rat receptor A242→S followed by radioligand binding and second messenger studies. Additionally we attempted to define the space allowed by the alanine by evaluating compounds with larger substitutions at the 9-position. The data indicate position 5.46 contributed to the species difference in 5-HT\textsubscript{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; Rosensweig-Lipson et al., 2007). The development of clinically useful 5-HT$_{2C}$ agonists is dependent upon the attainment of selectivity vs. the highly homologous 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors as 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; Nagatomo et al., 2004; Roth et al., 1998). 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 (Ni et al. 2004; Centurian et al. 2001). Thus in addition to the often cited potential for hallucinogenic adverse events for those compound 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).

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, impacting compound binding affinity and functional potency. Specifically a species variation in the 5th transmembrane domain of the 5-HT$_{2A}$ receptor (position 5.46, utilizing the numbering system of Ballesteros and Weinstein, 1995) has been shown by a number of groups to impact 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 (S242) in the human, cynomologous monkey and dog receptors while it is an alanine (A242) in rodents. More specifically, the
affinity of the ergoline antagonist radioligand [3H]mesulergine is lower at the human receptor vs. the rat due to the serine at 5.46. The affinity of [3H]mesulergine could be made equivalent to the rat following mutagenesis of the human receptor homologue 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 have been postulated to be due to the loss of hydrogen bond formation (Johnson et al. 1993, Almaula et al. 1996).

Recently we described a novel 5-HT\textsubscript{2C} agonist chemotype that exhibited significant selectivity vs. the human 5-HT\textsubscript{2A} and 5-HT\textsubscript{2B} receptors as well as many other GPCRs (Wacker et al. 2007). The lead molecule ([(R)-9-ethyl-1,3,4,10b-tetrahydro-7-trifluoromethylpyrazino[2,1-\textsubscript{a}]isoindol-6(2\textsubscript{H})-one]) produced significant reductions in food intake in an overnight feeding assay and reductions in body weight following chronic administration to rats. The reductions in food intake could be completely reversed by a selective 5-HT\textsubscript{2C} antagonist. Further in vitro characterization of the lead molecule at the rat variants of the 5-HT\textsubscript{2} receptor family revealed that the compound demonstrated significantly higher potency vs. the 5-HT\textsubscript{2A} receptor while remaining unchanged, relative to human, at the 5-HT\textsubscript{2B} and 5-HT\textsubscript{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\textsubscript{2A} receptor which implicated position 5.46 within the 5th 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\textsubscript{2A} receptor confers species specific selectivity for the 9-ethyl pyrazinoindolone and that the serine present in the human homologue 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. As 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 vs. 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 methodology (the QuikChange™ Site-Directed Mutagenesis Kit from Stratagene) following the manufacturer’s protocol. The mutagenesis and sequencing primers were from Invitrogen. 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 fluorescent-light polarization reader (FLIPR). Cells were maintained in DMEM high glucose 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 50 mM TRIS, 1.0 mM EDTA (pH 7.4) buffer. Alternatively membranes were prepared from either dog or cynomolgous monkey frontal cortex in the same buffer. The cells/tissues were then homogenized using a Polytron, 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 [125I]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 MgSO4, 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 onto Packard Unifilter GF/B filter plates, followed by three washes (ice cold 50 mM Tris (pH 7.4)). After the plates had dried 40 μl of MicroScint 20 (Packard) was added into each well, the plate sealed with TopSeal, and counted on a Packard Topcount. Binding data was calculated using Graphpad Prism with K_i values calculated using the Cheng-Prusoff equation. Differences in compound binding affinities across the receptor constructs were assessed by ANOVA followed by post-hoc analysis using a Bonferroni test.

**Intracellular Calcium Measurements:** Cells were plated in a 96-well black clear view bottom plate coated with poly-D-lysine (BD BIOCOAT) at a density of 50,000 cells/well and cultured overnight. Cells were loaded with Fluo-4 AM (Molecular Probes) for 1 hr at 37°C. After loading, the dye was aspirated and 150 μl of base buffer (1X Hank’s with 5 mM glucose, 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 seconds on a FLIPR-TETRA (Molecular Devices). Potency and efficacy determinations were done using non-linear curve fitting with Graphpad Prism. Intrinsic activity values were calculated based on the maximal responses produced by serotonin.

**Rat Telemetry Studies:** Male Sprague-Dawley rats were implanted with radio telemetry devices (Data Sciences International, USA; PA-C40 Transmitter) 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 (dH_2O at pH 4.0) (n=5), a 10 mg/kg dose of compound 1 in a volume of 5 ml/kg p.o., 1.5 mg/kg of ketanserin i.p., or 1.5 mg/kg of prazocin, subcutaneously, 30 minutes prior to a p.o. dose
of 10 mg/kg compound 1. 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 hrs. The change in blood pressure for each treatment was calculated
as a percent change from baseline:

\[
\text{%change} = \frac{(\text{average of post 2 hr to 8 hrs} - \text{baseline})}{\text{baseline}} \times 100
\]

**Homology Model:** Chain A from the crystal structure of bovine Rhodopsin (pdb entry:
1F88) was used as a template for homology modeling. A model of the human 5HT\textsubscript{2A}
receptor was generated using Prime v.1.6 (Schrödinger LLC, New York, NY). GPCR
anchor motifs (Kimura, et al. 2003) were used to modify the alignment prior to model
building. Due to the vast differences in ECL2 between Rhodopsin and the 5HT\textsubscript{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 vs. rat species had 91% sequence
identity; therefore the model of human 5HT\textsubscript{2A} receptor was used as a basis for the rat
5HT\textsubscript{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 in Wacker et al. 2007.
Results

Evaluation of ((R)-9-ethyl-1,3,4,10b-tetrahydro-7-trifluoromethylpyrazino[2,1-a]isoindol-6(2H)-one), termed compound 1 (Fig. 1), at the rat variants of the 5-HT2C and 5-HT2A receptors revealed that the binding affinity for compound 1 did not change between species for the 2C receptor while 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). While we did not conduct radioligand or functional studies in a rat 5-HT2B recombinant cell line, compound 1 exhibited similar potency and efficacy characteristics between species when tested in a rat fundus smooth muscle contraction assay to that of the recombinantly expressed human 5-HT2B receptor (data not shown).

The impact of the greater rat 5-HT2A 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 vs. vehicle in normal Sprague-Dawley rats (Figure 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-HT2A receptor mediated effect. As ketanserin also has appreciable α1-adrenergic affinity we also tested the ability of the α1-adrenergic receptor antagonist prazocin to block the blood pressure increase, which it failed to do (data not shown). Compound 1 was found to not exhibit any binding affinity at a concentration of 10μM to α1-adrenergic receptors as determined in a cortical homogenate binding assay using [3H]-prazocin (assay conducted by MDS Pharma Services). Subsequent evaluation of compound 1 for agonist activity in recombinant cell
lines expressing the α1a-adrenergic receptor subtype in a FLIPR assay yielded no response (Suppl. Fig. 1).

In order to better understand the basis of the species selectivity for compound 1 at the 5-HT2A receptor, as well as developing a structural basis to understand selectivity vs. the 5-HT2C receptor, we conducted receptor homology modeling studies. Compound 1 was docked into the human 5-HT2A 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 5th transmembrane domain and 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 as 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, and specifically the 7-CF₃ group, was within van der Waals contact with the hydrophobic box postulated to be important for functional activity at the 5-HT2A receptor (Roth et al., 1997).

In order 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 utilizing the agonist [¹²⁵I]DOI and in FLIPR functional assays. As reported previously, the Ser to Ala mutation had no impact on the Kd of [¹²⁵I]DOI binding (Johnson et al. 1994; Braden and Nichols, 2007) and the relative Bmax 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 while the affinity of 5-HT and tryptamine was not impacted 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 to match that of the WT rat receptor while the Ala to Ser mutation in the rat receptor reduced binding affinity to that of the WT human receptor by ~10-fold. The potency for the induction of Ca\(^{++}\) 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 that of the human WT receptor.

As position 5.46 is Ser in both the dog and cynomologous monkey (Fig. 5), we expected that the binding affinity of compound 1 to 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 \(^{[125]}\)DOI binding with affinities of 500\(\pm\)31 nM and 508\(\pm\)14 nM respectively (Fig. 7). Competition binding studies from rat frontal cortex yielded an affinity of 52\(\pm\)3 nM, in excellent agreement with the affinity obtained from the recombinant rat receptor preparation (Fig. 7).

The presence of Ala compared to 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 analogues to be similarly impacted by the species difference at position 5.46. Additionally, 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 analogues. 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 similar sized 9-substitutions to compound 1 (e.g. cyclopropyl-methyl and methoxy) were
equally impacted by the species difference and the subsequent mutations in binding assays (Table 5). In general as size of the 9-substitution increased, the degrees of species difference became smaller and thus were 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.

Lastly we wished to address the question if reducing affinity and potency vs. the rat 5-HT$_{2A}$ receptor would result in the attenuation of the blood pressure increase observed 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 analogue for the rat receptor was similar to the affinity of compound 1 at the dog and monkey 5-HT$_{2A}$ receptor suggesting 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 orally administered to telemeterized rats at a dose of 10 mg/kg there was no observation of increased blood pressure despite achieving similar plasma concentrations of drug (data not shown) as 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). While critical advances have been made in the crystallization of mammalian GPCRs over the last year (Rosenbaum et al. 2008; and 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. 2008). Several rhodopsin-based homology models of the 5-HT$_2A$ receptor have been previously 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. While 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 others differences being at position 81 (1.39) Ala in rat vs. Thr in human and position 150 (3.27) Ile in rat vs. 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.
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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 it appears as if the differences between the human and rat receptor for compound 1 is exclusively dependent upon position 5.46 as the human S → A mutation results in the rat phenotype and the converse rat A → S 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 (Table 5).

Assessment of our data suggests that the loss of binding affinity that occurs from the change of A → S 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 vs. the human 5-HT2C receptor) for the rat receptor is simply due to the increased size of the binding pocket, allowing for more space for the ethyl group to bind. The amount of space opened up upon conversion to the alanine appears limited as larger substitutions such as the cyclohexyl and isobutyl are unaffected by the mutagenesis and do not exhibit a species difference. As to why the relatively large cyclopentyl and isopentyl substitutions have improved affinities upon the S → A mutation may be due to the fact that these compounds are less rigid. Based on the homology model, neighboring residue Ile 206 (4.56) on TM4 which is oriented toward Ser 5.46 may limit the space available for binding of the larger 9-substitutions. We did not however, validate this observation via mutagenesis.

When one examines the functional data of the various 9-substituted isoindolones it appears that the compounds are less functionally active at the human receptor than predicted by the binding data. While 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 due to lower receptor expression as 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, as the rat A → S mutation mimics the species difference. It must be acknowledged however that the human S → A 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 confirm if 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). As this residue does not differ among species it is not contributing to the observed species differences for our 9-substituted isoindolones. We did however examine our model to assess the potential for S5.43 to contribute to the binding of the 9-isoindolone chemotype to the receptor. Our preferred docking conformation suggests that the -OH group of S5.43 lies too distant relative to 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 impacted by this residue (Braden and Nichols, 2007). We also found it unlikely that S5.43 can contribute to a steric hindrance of the 9-substituted analogues as it lies 1 turn above S5.46 and thus to far away to be of significance. Here again the constrained indolone core would not have the conformational freedom to interact with the residue. This prediction would be in agreement to the observations of Braden and Nichols who found that the rigid LSD molecule also lacked conformational freedom to associate with S5.43 (Braden and Nichols, 2007).

As the analogous position of 5.46 in the human 5-HT\textsubscript{2C} receptor is alanine, our data suggest that the development of selective 5-HT\textsubscript{2C} receptor agonists could in part be obtained by taking advantage of the steric hindrance provided by the Ser -OH in the 5-HT\textsubscript{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, which is reduced to 3-fold upon the S → A mutation. The variety of substitutions that can enter the pocket due to the opening provided by the alanine is likely limited however in that substitutions larger than cyclopropyl begin to lose 5-HT\textsubscript{2C} affinity and potency (Wacker et al. 2007).

The ability to develop clinically efficacious and safe 5-HT\textsubscript{2C} agonists for the treatment of obesity has been complicated by the need to obtain significant selectivity vs. the highly homologous 5-HT\textsubscript{2A} and 5-HT\textsubscript{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 1990’s due to an increase in the incidences of heart valve hypertrophy that is now largely believed to be the result of 5-HT\textsubscript{2B} receptor activation by the metabolite norfenfluramine (Fitzgerald et al. 2000). 5-HT\textsubscript{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<sub>2C</sub> agonist based on in vitro pharmacological assays employing the human isoforms of the 5-HT<sub>2C</sub>, 5-HT<sub>2A</sub> and 5-HT<sub>2B</sub> receptors (Wacker et al. 2007). The anorectic effect of the compound could be completely abolished by a 5-HT<sub>2C</sub> selective antagonist (Wacker et al. 2007) and significant CNS-mediated adverse events were not noted in animals treated with the compound including 5-HT<sub>2A</sub> mediated behaviors (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<sub>2B</sub> receptor.

Based on the fact that we had not noted any obvious hallmarks of 5-HT<sub>2A</sub> activation (such as wet dog shakes) (Xu and Miller, 1998) and the compound was selective vs. 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 figure 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<sub>2A</sub> agonists in rats (Ni et al. 2004). Thus the increase in 5-HT<sub>2A</sub> receptor potency at the rat receptor was manifested in a significant safety signal. As further confirmation of the 5-HT<sub>2A</sub> hypothesis dosing of the closely related 9-isopropyl analogue, with reduced rat 5-HT<sub>2A</sub> activity, resulted in no blood pressure elevation.

We chose ketanserin for our telemetry studies knowing that it is not a completely selective 5-HT<sub>2A</sub> antagonist (Centurian et al. 2006). To be sure, ketanserin exhibits significant binding affinity for α<sub>1</sub>-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<sub>2A</sub> 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<sub>2A</sub>
receptor in that compound 1 exhibits no functional activity at the \(\alpha_{1a}\) receptor subtype and did not inhibit prazocin binding at a concentration of 10 \(\mu\)M in a rat brain homogenate assay. Indeed, pre-treating rats with prazocin did not block the blood pressure increase produced by compound 1. Prazocin has been shown to be more than 200-fold more potent than ketanserin in blocking \(\alpha_{1}\)-receptor-mediated increases in blood pressure (Centurion et al. 2006).

Blood pressure elevations however small have impeded the utilization of marketed anti-obesity 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 may result in a cleaner profile. Indeed the binding affinity at the cynomologous 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 pre-clinical 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 5-HT\(_{2A}\) affinity on blood pressure in either a telemeterized dog or monkey could be conducted.
We have demonstrated position 5.46 solely accounts for the observation of species-dependent differences in binding affinity and functional potency at the 5-HT\textsubscript{2A} receptor for a series of 9-substituted isoindolone compounds. The significant 5-HT\textsubscript{2A} activity in the rat resulted in the observation of increased blood pressure in a rat cardiovascular study. As the analogous position in the human 5-HT\textsubscript{2C} receptor is alanine vs. Ser in the human 5-HT\textsubscript{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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Figure Legends

Figure 1. Compound 1: \((R)-9\text{-ethyl}-1,3,4,10\text{b\text{-tetrahydro-7\text{-trifluoromethylpyrazino[2,1-a\text{-}isoindol-6(2\text{H})\text{-one}} as previously described by Wacker et al. 2007.}

Figure 2. Competition binding studies with compound 1 in membranes prepared from stable HEK cells expressing the indicated receptor constructs. The data is expressed as a percentage of total \([^{125}\text{I}]\) DOI binding. Data are the mean±SEM of four experiments performed in triplicate.

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

Figure 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 while ketanserin was administered i.p. In the combination arm the ketanserin was administered 30 minutes prior to compound 1. Data is expressed as the mean ± SEM percent change from baseline from 2 hr to 8 hr post dose.
Figure 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.

Figure 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 (4). Transmembrane domains are denoted by transitioning colors with TM1 being orange and TM7 being purple.

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

Fig. 8. Effect of compound 2 on blood pressure in telemeterized Sprague-Dawley rats. Compound 2 was dosed by oral gavage. Data is expressed as the mean ± SEM percent change from baseline from 2 hr to 8 hr post dose.
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 fluorescent light polarization reader (FLIPR) assays utilizing 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 ± SEM of four to six experiments.

<table>
<thead>
<tr>
<th></th>
<th>h5-HT$_{2A}$</th>
<th>r5-HT$_{2A}$</th>
<th>h5-HT$_{2C}$</th>
<th>r5-HT$_{2C}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_i$</td>
<td>568±63</td>
<td>2565±65</td>
<td>18±2</td>
<td>16±3</td>
</tr>
<tr>
<td>EC$<em>{50}$ (E$</em>{max}$)</td>
<td>34±1</td>
<td>93±1</td>
<td>100</td>
<td>91±3</td>
</tr>
<tr>
<td>EC$_{50}$ (IA)</td>
<td>52±9</td>
<td>24±3</td>
<td>16±4</td>
<td>16±3</td>
</tr>
<tr>
<td>EC$_{50}$ (IA)</td>
<td>2565±65</td>
<td>18±2</td>
<td>93±1</td>
<td>100</td>
</tr>
</tbody>
</table>

All data expressed as nM

$^1$Data previously reported by Wacker et al. 2007
Table 2.

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

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

<table>
<thead>
<tr>
<th>Membrane Source</th>
<th>$K_D$ (nM)</th>
<th>$B_{\text{max}}$ (fmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>h5-HT$_{2A}$ WT</td>
<td>0.12±0.02</td>
<td>264±14</td>
</tr>
<tr>
<td>h5-HT$_{2A}$ S→A</td>
<td>0.17±0.02</td>
<td>727±87</td>
</tr>
<tr>
<td>r5-HT$_{2A}$ WT</td>
<td>0.12±0.01</td>
<td>826±94</td>
</tr>
<tr>
<td>r5-HT$_{2A}$ A→S</td>
<td>0.11±0.01</td>
<td>570±144</td>
</tr>
</tbody>
</table>
Table 3

Affinities of compound 1 at the wild type and mutant human and rat 5-HT${}_{2A}$ receptors

Binding affinities (Kᵢ) were determined from competition assays with $[^{125}\text{I}]$DOI. The values represent the mean ± SEM of four to six experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>h5-HT$_{2A}$ WT</th>
<th>h5-HT$_{2A}$ S→A</th>
<th>r5-HT$_{2A}$ WT</th>
<th>r5-HT$_{2A}$ A→S</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>18±9</td>
<td>21±5</td>
<td>31±6</td>
<td>23±3</td>
</tr>
<tr>
<td>5-MeO-T</td>
<td>6±0.2</td>
<td>16±1</td>
<td>15±4</td>
<td>8±1</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>58±4</td>
<td>84±4</td>
<td>72±8</td>
<td>69±6</td>
</tr>
<tr>
<td>Compound 1</td>
<td>568±63</td>
<td>88±8</td>
<td>52±9</td>
<td>540±33</td>
</tr>
</tbody>
</table>

All data expressed as nM
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 (Emax) values were determined by fluorescent light polarization reader (FLIPR) assays utilizing Fluo-4 as the indicator dye for intracellular calcium. Percent efficacies (in parentheses) were calculated based on the maximal elevation of fluorescent light units produced by serotonin. The values represent the mean ± SEM of four to six experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>h5-HT_{2A} WT</th>
<th>h5-HT_{2A} S→A</th>
<th>r5-HT_{2A} WT</th>
<th>r5-HT_{2A} A→S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC_{50} (Emax)</td>
<td>EC_{50} (Emax)</td>
<td>EC_{50} (Emax)</td>
<td>EC_{50} (Emax)</td>
</tr>
<tr>
<td>5-HT</td>
<td>4.4±0.9 (1.0)</td>
<td>6.8±1.4 (1.0)</td>
<td>2.7±0.5 (1.0)</td>
<td>2.1±0.5 (1.0)</td>
</tr>
<tr>
<td>5-MeOT</td>
<td>6.9±2 (83±3)</td>
<td>5.2±2.8 (72±5)</td>
<td>1.4±0.4 (93±2)</td>
<td>2.7±1.3 (83±6)</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>54±30 (80±7)</td>
<td>74±25 (74±6)</td>
<td>13±2 (81±3)</td>
<td>17±5 (68±3)</td>
</tr>
<tr>
<td>Compound 1</td>
<td>2565±65 (34±1)</td>
<td>76±43 (64±8)</td>
<td>24±7 (93±1)</td>
<td>1228±934 (75±1)</td>
</tr>
</tbody>
</table>

All data expressed as nM
Table 5.

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

![Chemical structure](image)

Binding affinities (Kₐ) were determined from competition assays with [¹²⁵I]DOI. The values represent the mean ± SEM of four to six experiments.

<table>
<thead>
<tr>
<th>R</th>
<th>h5-HT₂A WT</th>
<th>h5-HT₂A S→A</th>
<th>r5-HT₂A WT</th>
<th>r5-HT₂A A→S</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Å</td>
<td>568±63</td>
<td>88±8</td>
<td>52±9</td>
<td>540±33</td>
</tr>
<tr>
<td>3-Å</td>
<td>853±78</td>
<td>332±56</td>
<td>303±12</td>
<td>761±31</td>
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<td>3-Å</td>
<td>375±29</td>
<td>102±10</td>
<td>97±7</td>
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<tr>
<td>3-Å</td>
<td>1921±161</td>
<td>883±43</td>
<td>744±68</td>
<td>1124±140</td>
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<td>3-Å</td>
<td>1211±93</td>
<td>543±54</td>
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<td>3-Å</td>
<td>2708±320</td>
<td>563±16</td>
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<td>1504±117</td>
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<td>3-Å</td>
<td>782±43</td>
<td>114±10</td>
<td>116±11</td>
<td>618±60</td>
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<tr>
<td>3-Å</td>
<td>336±36</td>
<td>181±25</td>
<td>194±19</td>
<td>350±88</td>
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<tr>
<td>3-Å</td>
<td>1773±131</td>
<td>1428±41</td>
<td>1433±166</td>
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<tr>
<td>3-Å</td>
<td>900±60</td>
<td>350±14</td>
<td>319±11</td>
<td>877±37</td>
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MOL #59204

<table>
<thead>
<tr>
<th></th>
<th>Value 1 ± Error 1</th>
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<tr>
<td>Compound 1</td>
<td>1218 ± 95</td>
<td>456 ± 15</td>
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<td>Compound 2</td>
<td>990 ± 128</td>
<td>739 ± 53</td>
<td>676 ± 53</td>
<td>1017 ± 58</td>
</tr>
</tbody>
</table>

All values expressed in nM
Table 6.

The impact 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$_{\text{max}}$) values were determined by fluorescent light polarization reader (FLIPR) assays utilizing Fluo-4 as the indicator dye for intracellular calcium. Percent efficacies (in parentheses) were calculated based on the maximal elevation of fluorescent light units produced by serotonin.

<table>
<thead>
<tr>
<th>R</th>
<th>h5-HT$_{2A}$ WT</th>
<th>h5-HT$_{2A}$ S→A</th>
<th>r5-HT$_{2A}$ WT</th>
<th>r5-HT$_{2A}$ A→S</th>
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</thead>
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<tr>
<td></td>
<td>2565±70 (34±1)</td>
<td>76±41 (64±8)</td>
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<td>1228±532 (75±1)</td>
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<td>394±4 (89±5)</td>
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<td>40±7 (44±2)</td>
<td>1801±590 (65±10)</td>
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<td>&gt;10000 (0)</td>
<td>&gt;10000 (0)</td>
</tr>
</tbody>
</table>

All data expressed as nM
Figure 1
Figure 2

% Bound

\[ \log [\text{drug}] \]

- Human WT
- Human S242A
- Rat WT
- Rat A242S

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 3
Figure 4

% Change from Baseline

- Vehicle
- Comp-1
- Ket 1.5mpk
- Comp-1+Ket 1.5mpk
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

Figure 5
Figure 6
Figure 7

% Bound

log [drug]

-11 -10 -9 -8 -7 -6 -5

Dog Cortex
Rat Cortex
Cyno Cortex

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Figure 8