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Activities of novel aryloxyalkylimidazolines on rat 5-HT_{2A} and 5-HT_{2C} receptors

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Abstract

Using transfected NIH 3T3 mouse fibroblast cell lines expressing the rat 5-HT_{2A} and rat 5-HT_{2C} receptor subtypes, and techniques of 2-[^{125}I](+)-iodolysergic acid diethylamide ([^{125}I]LSD) binding and serotonin (5-hydroxytryptamine, 5-HT)-stimulated phosphoinositide hydrolysis, we have characterized a new structural class of 5-HT receptor ligands, the aryloxyalkylimidazolines. These compounds were found to be potent competitors of [^{125}I]LSD binding at both receptor subtypes ($K_i \sim 5$–200 nM) and to have efficacy ranging from potent competitive antagonists ($IC_{50} \sim 25$ nM) to moderately potent full agonists ($EC_{50} \sim 200$ nM). Some of these compounds are agonists at both receptor subtypes, while others are 5-HT_{2C} receptor agonists with 5-HT_{2A} receptor antagonist activity. None of the aryloxyalkylimidazolines reported here have 5-HT_{2A} or 5-HT_{2C} receptor selective antagonist activity. Since these compounds are novel structures, we compared them with a variety of reference 5-HT receptor ligands selected from other chemical classes that have previously been studied at 5-HT_{2A} and 5-HT_{2C} receptors in native tissues.

Keywords: 5-HT (5-hydroxytryptamine, serotonin); 5-HT_{2A} receptor; 5-HT_{2C} receptor; Phosphoinositide hydrolysis; 5-HT receptor antagonist; Aryloxyalkylimidazoline

1. Introduction

The biogenic amine serotonin (5-hydroxytryptamine, 5-HT), is a neurotransmitter that mediates a diverse array of physiological responses in the nervous system by binding to cell surface receptors. These receptors are of two major structural/functional types; G protein-coupled proteins which modulate distinct intracellular signaling systems, and ligand-gated non-selective cation channel proteins (Julius, 1991; Spiegel, 1992; Zifa and Fillion, 1992; Peroutka, 1993). Recent cloning and sequencing of the known G protein-coupled serotonergic receptor genes has revealed the evolution of a large superfamily of these receptors. The 5-HT_{2A} and 5-HT_{2C} receptors are members of one family within this receptor superfamily (Hartig et al., 1990a, b; Sanders-Bush et al., 1990; Julius, 1991; see dendrogram in Erlander et al., 1993).

All G protein-coupled receptors share a putative amphipathic transmembrane domain structure believed to consist of seven highly conserved hydrophobic sequences arranged as antiparallel α helices that span the lipid bilayer of the plasma membrane (Dohlman et al., 1991). 5-HT_{2A} and 5-HT_{2C} receptors are 51% homologous overall and show 78% amino acid identity and 91% similarity in their putative transmembrane domain (Julius et al., 1988, 1990). This highly conserved transmembrane homology accounts for the very similar pharmacological profile of these two receptor subtypes (Hoyer, 1988a, b; Julius, 1991; Zifa and Fillion, 1992). 3-Dimensional molecular modeling and site-directed mutagenesis studies (Trumpp-Kallmeyer et al., 1992; Choudhary et al., 1993) indicate that 5-HT binds within a narrow central, dihedral cleft formed by the seven transmembrane helices. The molecular model of Hibert et al. (1991) suggests 5-HT_{2A} and 5-HT_{2C} receptors bind 5-HT in very similar agonist binding pockets, albeit the 5-HT_{2C} receptor has a higher affin-

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ity for 5-HT in radioligand binding and functional studies than does the 5-HT_{2A} receptor.

Functionally, both 5-HT_{2A} and 5-HT_{2C} receptors are coupled to phospholipase C activation and thus modulate intracellular Ca^{2+} levels via 1,4,5-inositol trisphosphate production and protein kinase C activation via concommitant diacylglycerol formation (Sanders-Bush et al., 1990).

Recently, several highly selective 5-HT_{2A} receptor antagonists have been developed (Carr et al., 1991; Rinaldi-Carmona et al., 1992; Doble et al., 1992; Bjork et al., 1994) which may prove to be useful therapeutic agents for the treatment of various nervous system disorders including schizophrenia. Selective 5-HT_{2C} receptor antagonists may prove to be uniquely useful agents for the treatment of migraine headache (Fozard and Gray, 1989), CSF overproduction (Lindvall-Axelsson et al., 1989), anxiety (Kennet et al., 1989), abnormal mood and affect (Van der Heyden et al., 1990).

In the course of examining a series of novel aryloxyalkylimidazolines for binding at a variety of receptors, some of the compounds were found to possess potent 5-HT_{2A} and 5-HT_{2C} receptor antagonism. We have utilized two newly available stable cell lines expressing the rat 5-HT_{2A} and 5-HT_{2C} receptors to investigate the relative efficacy and affinity of these novel aryloxyalkylimidazolines as ligands at these two 5-HT receptor subtypes. The resulting structure/activity relationship (Freedman et al., in preparation) has provided a new chemical class of 5-HT receptor ligands which have antagonist activities at the 5-HT_{2A} and 5-HT_{2C} receptors that are low nanomolar in potency. Interestingly, some of these compounds have 5-HT_{2A} and/or 5-HT_{2C} receptor agonist properties.

Since the aryloxyalkylimidazolines are novel structures, we compared them with a variety of reference 5-HT receptor ligands of other structural classes which have been previously characterized at 5-HT_{2A} and 5-HT_{2C} receptors in native tissues. These ligands include: the ergolines, (+)LSD ((+)lysergic acid diethylamide) and mesulergine; the phenylalkylamine, (±)DOI, ((±)-1-(2,5-dimethoxy-4-iodophenyl-2-aminopropane HCl); the benzazepine, SKF83566, ((±)-7-bromo-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine HCl); the dibenzodiazepine, clozapine; the benzoylpiperidinyl compound, ketanserin; the diphenylmethylenepiperidine, ritanserin; the butyrophenone, spiperone; the arylpiperazine, trazodone and its metabolite *m*-chlorophenylpiperazine (m-CPP); the α-phenyl-4-piperidinemethanol, MDL100907, ((*R*)-(+)-4-[1-hydroxy-1-(2,3-dimethoxyphenyl)methyl]-*N*-2-(4-fluorophenylethyl)piperidine), a selective 5-HT_{2A} receptor antagonist (Carr et al., 1991); and fluoxetine, a selective 5-HT uptake inhibitor (Fuller et al., 1991) which has been reported to be slightly selective for the 5-HT_{2C} receptor (Wong et al., 1990).

2. Materials and methods

2.1. Cell culture

Stable cell lines of transfected NIH 3T3 mouse fibroblasts expressing either the rat 5-HT_{2C} receptor (P_o cell line) or the rat 5-HT_{2A} receptor (GF_6 cell line) were gifts from David Julius, Stanford University. Both cell lines were subcloned by limiting dilution, and subclones were selected on the basis of [^{125}I]LSD binding. Cells were grown in Dulbecco's Modified Eagle Media (GIBCO) containing 0.45% glucose, 10% newborn calf serum (Whittaker), 1% glutamine, 1% penicillin/streptomycin, and 300 μg/ml geneticin. Cells were split with Versene (GIBCO) once a week, such that they were confluent the following week. Media was changed 3 days after each split.

2.2. Membrane preparation

Confluent cells grown in T175 flasks (Falcon) were harvested with 10 ml Versene, centrifuged at 500 × *g* for 5 min at 4°C and resuspended into 5 ml ice-cold 0.32 M sucrose per flask. The cells were then homogenized on ice using a Brinkman Polytron PT10/35 (setting 5.5, 10 s) and centrifuged at 40000 × g for 20 min at 4°C. The pellets were resuspended into 5 ml 50 mM Tris-HCl pH 7.6 (buffer A) per original flask, homogenized as before and aliquoted for storage at −80°C. Protein determination was performed according to the dye method of Bradford (1976). On the day of a binding assay, an aliquot of membranes was thawed, diluted with freshly prepared buffer A to an appropriate dilution and homogenized with the Polytron just before use.

2.3. Radioligand binding assays

2.3.1. [^{125}I]LSD binding assay

2-[^{125}I](+)-Iodolysergic acid diethylamide, (+)LSD, 2200 Ci/mmol, was obtained from Dupont, New England Nuclear, as a stock solution in ethanol. Immediately before use, the required volume of the radioligand was withdrawn, evaporated in a 12 × 75mm polystyrene tube to dryness under a stream of nitrogen gas at room temperature, or under vacuum in a Savant SpeedVac, and resuspended as a 5 × stock in buffer A. Low light conditions prevailed whenever (+)LSD or 5-HT were used in these studies. Assays were performed with a Beckman Biomek robot using polystyrene 1 ml minitube trays. Each assay tube with a final volume of 100 μl contained 20 μl buffer A, 20 μl radioligand, 20 μl test compound, and 40 μl membranes (~ 6 μg protein). Ascorbic acid was present at a final concentration in the assay of 0.02% to protect 5-HT and (+)LSD from oxidation. Non-specific bind-

ing was defined using 10 μM mesulergine. Assays were initiated by membrane addition and then transfered to a 37°C water bath. In equilibrium binding experiments, the incubation time was 60 min. Assays were terminated by the addition of 4 ml ice-cold buffer A, followed by filtration thru GF/B filter strips presoaked in 0.1% polyethyleneimine. Filters were then washed 2 times with 4 ml ice-cold buffer A and transferred to 12 × 75 mm polystyrene tubes for quantification of radioactivity using a Packard Cobra Auto-Gamma counter.

2.3.2. [^{3}H]5-HT binding assay

[^{3}H]5-HT (28 Ci/mmol) was obtained from Dupont, New England Nuclear. Assays were performed with Beckman 6 ml plastic mini scintillation vials at room temperature. Each assay tube with a final volume of 500 μl contained 350 μl buffer B (1 mM EGTA, 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5), 50 μl test compound in buffer B, 50 μl 200 nM [^{3}H]5-HT in buffer B containing 0.2% ascorbic acid, and 50 μl membranes (15 μg protein). Non-specific binding was defined using 10 μM mesulergine. Assays were initiated by membrane addition, and incubated at room temperature for 30 min. Assays were terminated by centrifugation at 40000 × *g* for 10 min at 4°C. The resulting supernatant was decanted and the pellets gently washed 3 times with 2 ml ice-cold buffer B. After drying in an inverted position on absorbent towels, 4 ml Beckman ReadiProtein was added to each vial and the vial then capped and vigorously vortexed. After sitting at room temperature for 4 h, the radioactivity was quantified using a Beckman LS5000TD scintillation counter.

2.4. Phospholipase C-mediated phosphoinositide hydrolysis

Cells for these studies were grown in 12 well plates (Costar 3512). 2 days prior to the experiment, the growth media was removed from the wells and replaced with 0.8 ml fresh growth media containing 4 μCi/ml [^{3}H]*myo*-inositol (45 Ci/mmol, Dupont, New England Nuclear). On the day of the experiment, when cells had not yet reached confluency, the growth media was removed from each well and replaced with 1 ml 5 mM unlabeled inositol in Krebs-Ringer bicarbonate solution (buffer C) containing 120 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1 mM KH_2PO_4, 20 mM $NaHCO_3$, 11.1 mM glucose, 0.001% phenol red, 10 mM $LiCl_2$, pH 7.4, equilbrated with 95%O_2-5%CO_2. The 5 mM inositol solution was then aspirated and replaced with a second 1 ml of the same, followed by replacement with 400 μl buffer C containing no inositol. The plates were returned to a 37°C incubator for 20 min. For studies of antagonist activity, 50 μl test compounds in buffer C were then added and incubated for 10 min prior to addition of the agonist. 5-HT and (+)LSD were made up as 10 × stock solutions in buffer C containing 0.2% ascorbic acid, pH 7.4, just prior to use. After addition of the agonists, the wells were incubated at 37°C for 20 min. The reaction was terminated by placing the plates on crushed ice and adding 0.5 ml ice-cold 10% perchloric acid to each well. The resulting 1ml of solution in each well was triturated 7 times with a 1 ml pipette and transfered to 1.5 ml Eppendorf centrifuge tubes on ice. The samples were centrifuged at 4°C in a Beckman Microfuge 12 for 8 min at a setting of 11. An aliquot of 0.8 ml of the resulting supernatant was transfered to 1.5 ml Eppendorf centrifuge tubes on ice containing 0.4 ml 2 M KOH/10 mM Na_2EDTA/0.1 M MES (2-[*N*-morpholino]ethanesulfonic acid) buffer, a solution previously shown to neutralize the 10% perchloric acid in the samples. The tubes were capped, shaken by hand vigorously and centrifuged as before. One ml of the supernatant was then transferred to 12 × 75 mm polypropylene tubes and stored at 4°C until used. Just prior to being placed on the ion exchange columns, 1 ml distilled water was added to each sample.

Phosphoinositide metabolites were quantified by the method of Berridge et al. (1982). 2 ml of a 1/1 (v/v) slurry of BioRad AG1-X8 resin, 100–200 mesh, formate form, in water was added to capped glass columns (0.7 mm i.d. × 140 mm) containing several ml water. The columns were then filled with distilled water and the resin allowed to settle. A small quantity of sea sand previously washed with 1 M formic acid and thoroughly rinsed with distilled water, was added to each column to prevent bed disruption during sample and wash additions. The resin was then washed with 20 ml water and the samples added, followed by washes of 10 ml 5 mM inositol in water; 5 ml 5 mM sodium tetraborate/60 mM ammonium formate; and 10 ml 0.1 N formic acid/1 M ammonium formate. This last eluate was collected separately and a 1 ml aliquot was added to 10 ml Beckman Readigel for quantification of radioactivity by scintillation spectroscopy.

2.5. Sources of chemicals and drugs

Unlabeled drugs were obtained as follows: all MDL compounds (aryloxyalkylimidazoline structures are shown in Fig. 1) and trazodone were synthesized at Marion Merrell Dow Research Institute; MDL100907 is (*R*)-(+)-4-[1-hydroxy-1-(2,3-dimethoxyphenyl)methyl]*N*-2-(4-fluorophenylethyl)piperidine; clozapine and mesulergine, Sandoz Pharmaceuticals (East Hanover, NJ); (±)-1-(2,5-dimethoxy-4-iodophenyl-2-aminopropane HCl ((±)-DOI) and (±)-7-bromo-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine HCl (SKF83566), Research Biochemicals (Natick, MA);

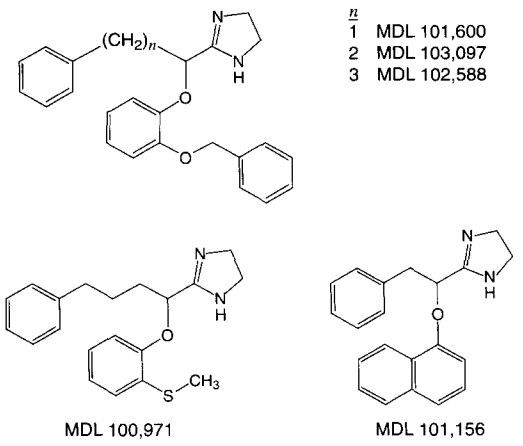

Fig. 1. Structural formula of the MDL aryloxyalkylimidazolines characterized in this study.

fluoxetine, Lilly Research Laboratories (Indianapolis, IN); 1-(3-chlorophenyl)piperazine (m-CPP), Aldrich Chemical Co. (Milwaukee, WI); ketanserin and ritanserin, Jansenn Pharmaceutica (Titusville, NJ); spiperone, McNeil Pharmaceuticals (Spring House, PA). All other drugs and reagents were from Sigma Chemical Co. (St. Louis, MO), except ammonium formate, formic acid, and perchloric acid from Fluka ChemikaBioChemika (Buchs, Switzerland), and Readi-gel and Readiprotein scintillation cocktails from Beckman Instruments (Fullerton, CA). Test compound stock solutions of 1 mM concentration were made up in 10% DMSO in H_2O or DMSO. The final concentration of DMSO in all assays was 0.1%, a concentration which had no effect on the various binding assays or studies of phosphoinositide hydrolysis.

2.6. Data analysis

Data analysis procedures have been previously described in Baron and Siegel (1990).

3. Results

3.1. [^{125}I]LSD binding characteristics

Saturable high affinity [^{125}I]LSD binding was observed with membranes prepared from both P_o and GF_6 cells which express the 5-HT_{2C} and 5-HT_{2A} receptors, respectively. Mesulergine at 10 μM was used routinely to define non-specific binding for both membrane preparations. Specific [^{125}I]LSD binding represented > 95% of the total binding. Scatchard analysis (not shown) indicated the P_o cell line had a $B_{max} = 19.0 \pm 5.4$ (mean ± S.E.M.) pmol/mg protein and a $K_d = 5.2 \pm 0.8$ nM ($n = 3$); the GF_6 cell line had a $B_{max} = 10.6 \pm 0.1$ pmol/mg protein and a $K_d = 0.72 \pm 0.03$ nM ($n = 3$).

Typical values in competition experiments, where the concentration of the radioligand was 1 nM, were: P_o cell line total dpm bound ~ 40 000 (8% of the dpm available) and non-specific dpm ~ 2000; GF_6 cell line total dpm bound ~ 20 000 and non-specific ~ 2000 dpm.

3.2. 5-HT-stimulated phosphoinositide hydrolysis

5-HT increased phosphoinositide hydrolysis in a concentration-dependent manner with an EC_{50} value of 1.8 ± 0.5 nM ($n = 5$) for the P_o cell line and an EC_{50} value of 108 ± 2 nM ($n = 5$) for the GF_6 cell line. Typically, maximal doses of 5-HT gave responses 2- to 8-fold above basal levels. The response was linear in both cell lines for at least 30 min.

3.3. Agonists

Table 1 shows [^{125}I]LSD binding and 5-HT-stimulated phosphoinositide hydrolysis data for various agonists. At the 5-HT_{2C} receptor, the rank order of the K_i

Table 1
The effects of various agonists on [^{125}I]LSD binding and phosphoinositide hydrolysis at rat 5-HT_{2C} and 5-HT_{2A} receptors expressed in NIH 3T3 mouse fibroblasts

Agonist	5-HT_{2C} LSD K_i (nM)	5-HT_{2C} EC_{50} (nM)	Relative efficacy [a]	5-HT_{2A} LSD K_i (nM)	5-HT_{2A} EC_{50} (nM)	Relative efficacy [a]
Serotonin	93 ± 13 (5) [b]	1.8 ± 0.5 (5)	1.00	1075 ± 275 (4)	108 ± 2 (5)	1.00
(+)LSD	2.7 ± 0.7 (3)	6 ± 2 (3)	0.60	1.3 ± 0.3 (3)	6 ± 0.5 (3)	0.40
MDL101156	29 ± 3 (3)	237 ± 36 (3)	1.00	144 ± 9 (3)	1157 ± 134 (3)	1.00
MDL100971	45 ± 10 (3)	323; 572	0.85	261; 185	2778; 1626	0.7
MDL102588	102 ± 13 (4)	6468 ± 779 (3)	0.70	211 ± 19 (3)	(4109; 3866) [c]	
(±)DOI	63; 46	54; 255	0.6	6; 35	45; 29	0.75
m-CPP	179; 112	165 ± 68 (3)	1.00	274; 107	475 ± 220 (4) (3050) [c]	0.60
SKF83566	25; 12	27 ± 2 (3)	1.00	13; 6	> 10 000 (3) (54; 392) [c]	0.30

[a] Relative efficacy is the maximum release of [^{3}H]phosphoinositides relative to the maximum effect of 5-HT, which is assigned the value of 1.00.
[b] Mean ± S.E.M. where (n) = number of experiments, if $n = 2$, both values are given. [c] Antagonist IC_{50} at the 5-HT_{2A} receptor.

values for [^{125}I]LSD binding was: (+)LSD > SKF83566 = MDL101156 > MDL100971 = (±)DOI > 5-HT > MDL102588 > mCPP. In contrast, the rank order potency of the EC_{50} values for the functional assay was: 5-HT > (+)LSD > SKF83566 > (±)DOI > mCPP > MDL101156 > MDL100971 > MDL102588 (see Fig. 2A,B). At the 5-HT_{2A} receptor, the rank order of the K_i values for [^{125}I]LSD binding was: (+)LSD > SKF83566 > (±)DOI > MDL101156 > mCPP > MDL102588 > MDL100971 > 5-HT. The corresponding order in the functional assay was: (+)LSD > (±)DOI > 5-HT > mCPP > MDL101156 > MDL-100971 > SKF83566 > MDL102588 (see Fig. 2C,D).

3.3.1. The natural agonist 5-HT

5-HT was 12-fold more potent at the rat 5-HT_{2C} receptor versus the 5-HT_{2A} receptor in competing for [^{125}I]LSD binding, and 60-fold more potent in stimulating phosphoinositide hydrolysis.

3.3.2. The aryloxyalkylimidazolines

MDL100971, MDL101156, and MDL102588 were relatively potent competitors for [^{125}I]LSD binding, displaying as much as 5-fold selectivity for the 5-HT_{2C} receptor subtype. In the functional assay, MDL101156 was a moderately potent full agonist at the 5-HT_{2C} receptor (EC_{50} = 237 nM), and a weaker agonist at the 5-HT_{2A} site (EC_{50} = 1157 nM). MDL100971 was slightly less efficacious at both receptor subtypes, while MDL102588 was an even weaker agonist at the 5-HT_{2C} receptor, and possessed no agonist activity and only weak antagonist activity at the 5-HT_{2A} receptor. MDL102588 possessed no antagonist activity at the 5-HT_{2C} receptor.

3.3.3. Reference 5-HT receptor agonists

(+)LSD was a potent partial agonist at both the 5-HT_{2C} and the 5-HT_{2A} receptors, being slightly more efficacious at the 5-HT_{2C} receptor. Consistent with a partial agonist effect, in the presence of 5-HT, this ergoline gave a maximum inhibitory response of 80% (IC_{50} = 5 nM) at both receptor subtypes. (−)LSD did not compete for [^{125}I]LSD binding and had no effect on 5-HT-stimulated phosphatidylinositol turnover at either of these two 5-HT receptor subtypes (data not shown).

(±)DOI, a hallucinogenic phenylalkylamine, was an equally potent competitor of [^{125}I]LSD binding at both receptor subtypes. This compound also displayed equal potency at both receptor subtypes in the functional assay.

m-CPP, a metabolite of the antidepressant drug trazodone, was a relatively potent full agonist at the 5-HT_{2C} receptor, and a partial agonist at the 5-HT_{2A} receptor. This arylpiperazine showed moderate affinity for the recognition site ([^{125}I]LSD binding), with equal potencies at the two 5-HT receptor subtypes.

SKF83566, a potent dopamine D_1 antagonist, was a good competitor for [^{125}I]LSD binding at both 5-HT receptor subtypes. This benzazepine was a potent full agonist at the 5-HT_{2C} receptor, and a weak partial agonist at the 5-HT_{2A} receptor.

3.3.4. Inhibition by mesulergine

Fig. 3 shows that 12 nM mesulergine inhibited phosphatidylinositol turnover ($P < 0.05$) elicted by each of the above agonists at the 5-HT_{2C} receptor, and had no effect on the basal response. In each case this concentration of the antagonist reduced the biochemical re-

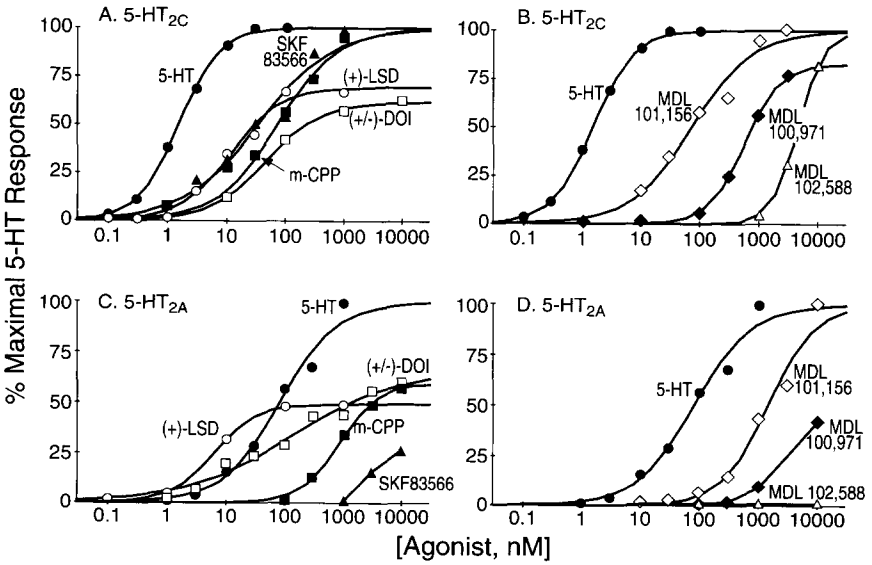

Fig. 2. Typical agonist concentration-response curves for phosphoinositide hydrolysis in P_o (5-HT_{2C} receptor) and GF_6 (5-HT_{2A} receptor) cells for known 5-HT receptor agonists at the 5-HT_{2C} (A) and 5-HT_{2A} (C) receptors, and various MDL compounds which are agonists at the 5-HT_{2C} (B) and 5-HT_{2A} (D) receptors.

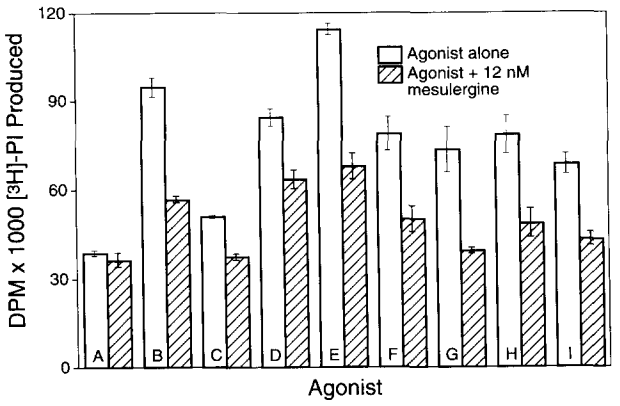

Fig. 3. The inhibitory effects of 12 nM mesulergine (hatched bars) on phosphoinositide hydrolysis in P_o cells (5-HT_{2C} receptor) stimulated by various agonists (open bars). (A, top) Basal, (B, bottom) 10 nM 5-HT, (C) 20 nM (+)LSD, (D) 300 nM MDL101156, (E) 300 nM MDL100971, (F) 8000 nM MDL102588, (G) 50 nM (±)DOI, (H) 120 nM m-CPP, (I) 27 nM SKF83566.

sponse by 45% or more, giving further evidence that all of these agonists are acting at the 5-HT_{2C} receptor.

3.4. Antagonists

Table 2 shows [^{125}I]LSD binding and 5-HT-stimulated phosphoinositide hydrolysis data for the various antagonists. An EC_{80} concentration of 5-HT added following a 10 min preincubation with the antagonist at 37°C was used in the functional assay.

3.4.1. The aryloxyalkylimidazolines

MDL101600 was an equally potent competitor for [^{125}I]LSD binding at both 5-HT receptor subtypes, and showed about a 3-fold selectivity for the 5-HT_{2A} site in antagonizing 5-HT-stimulated phosphoinositide hydrolysis. MDL103097 had intermediate binding properties between MDL101600 and MDL102588, and was a surprisingly weak antagonist at both 5-HT receptor subtypes. MDL103097 possesses a two carbon alkyl chain linking a phenyl group where the antagonist MDL101600 has a one carbon chain, and the agonist MDL102588 has a three carbon chain (see Fig. 1).

3.4.2. Reference 5-HT receptor antagonists

Mesulergine, an analog of LSD, was a potent 5-HT receptor antagonist which showed some selectivity for the 5-HT_{2C} receptor as evidenced by both [^{125}I]LSD binding and 5-HT-stimulated phosphoinositide hydrolysis. Ritanserin, a diphenylmethylenepiperidine, was equally potent at competing for [^{125}I]LSD binding at both 5-HT receptor subtypes, and showed about a 4-fold selectivity for the 5-HT_{2A} receptor in the functional assay. The structurally related molecule ketanserin exhibited 19-fold selectivity for the 5-HT_{2A} receptor in binding competition and a 38-fold selectivity in the functional assay.

MDL100907, a newly developed selective 5-HT_{2A} ligand, showed 55-fold selectivity at the 5-HT_{2A} site for [^{125}I]LSD binding competition, and about 700-fold selectivity for inhibiting 5-HT-stimulated phosphatidylinositol turnover.

Among all compounds tested in this report, spiperone, a butyrophenone with known affinity for the 5-HT_{2A} receptor, showed the greatest ability to differentiate the 5-HT_{2A} and 5-HT_{2C} receptors with 600-fold selectivity in [^{125}I]LSD binding and 2000-fold selectivity in the functional assay for the 5-HT_{2A} receptor.

The atypical antipsychotic clozapine was an equally potent competitor for [^{125}I]LSD binding at both 5-HT receptor subtypes. Surprisingly, in the functional assay, this tricyclic compound gave a large discrepancy for the 5-HT_{2C} receptor (200-fold) vs. the 5-HT_{2A} receptor (20-fold) relative to the respective K_i values obtained in the binding assays. This compound was 6-fold selective for the 5-HT_{2A} receptor in the functional assay.

Trazodone, the parent compound of m-CPP, was 25-fold selective for the 5-HT_{2A} receptor using

Table 2
Effects of various antagonists on [^{125}I]LSD and [^{3}H]5-HT binding, and 5-HT-stimulated phosphoinositide hydrolysis at rat 5-HT_{2C} and 5-HT_{2A} receptors expressed in NIH 3T3 mouse fibroblasts

Antagonist	5-HT_{2C} LSD K_i (nM)	5-HT_{2C} IC_{50} (nM)	5-HT_{2C} [^{3}H]5-HT IC_{50} (nM)	5-HT_{2A} LSD K_i (nM)	5-HT_{2A} IC_{50} (nM)
MDL101600	5 ± 1.4 (3) [a]	70 ± 16 (3)	2; 2	4 ± 1 (3)	25 ± 13 (3)
MDL103097	44 ± 13 (3)	1725; 1632		70 ± 12 (3)	1200; 953
Mesulergine	2; 2	21 ± 7 (3)	12; 9	6 ± 1 (3)	67; 38
Ritanserin	9 ± 2 (3)	229 ± 89 (3)	29 ± 14 (3)	6 ± 0.7 (3)	47; 76
Ketanserin	23; 35	856; 468	524 ± 43 (3)	1; 2	20; 15
MDL100907	33 ± 9 (3)	931; 608	4860 ± 1195 (3)	0.6 ± 0.2 (3)	0.8; 1.4
Spiperone	610; 801	> 10000		1; 1.5	5; 5
Clozapine	5 ± 0.3 (3)	1006 ± 134 (3)	62; 72	5; 5	51; 96
Trazodone	166; 205	8676; 8416	4527	7; 8	111; 45
Fluoxetine	81; 90	10000	664 ± 220 (3)	255; 32	> 10000

[a] Means ± S.E.M., (n) = number of experiments, if $n = 2$, both values are given.

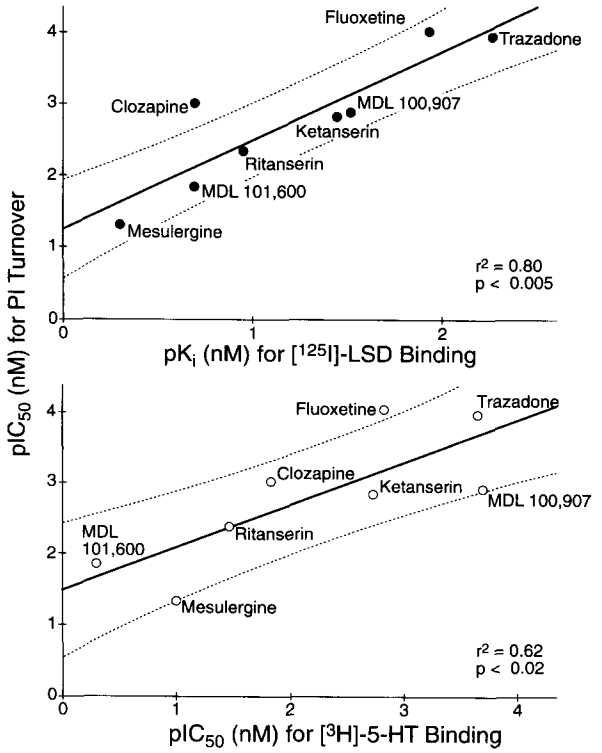

Fig. 4. Correlation plots for various 5-HT_{2C} receptor antagonists of (A, top) [^{125}I]LSD binding pK_i ($r^2 = 0.80$; $P < 0.01$) and (B, bottom) [^{3}H]5-HT binding pIC_{50} ($r^2 = 0.62$; $P < 0.02$) vs. pIC_{50} for inhibition of 5-HT-stimulated phosphoinositide hydrolysis with P_o cells (5-HT_{2C} receptor). Axis legends are given in nM such that a pIC_{50} (nM) value of 1 denotes an IC_{50} value of 10 nM. Confidence intervals (95%) are indicated by dashed lines.

[^{125}I]LSD binding and about 100-fold selective for the 5-HT_{2A} receptor in the functional assay.

Fluoxetine was relatively potent and non-selective for both the 5-HT_{2C} and 5-HT_{2A} receptors against [^{125}I]LSD binding, but was a very weak antagonist of 5-HT-stimulated phosphoinositide hydrolysis at both 5-HT receptor subtypes. This compound possessed no agonist activity at either 5-HT receptor subtype.

3.5. [^{3}H]5-HT binding

Fig. 4A demonstrates the good correlation between the pK_i of [^{125}I]LSD binding competition and the pIC_{50} for inhibition of 5-HT-stimulated phosphoinositide hydrolysis at the 5-HT_{2C} site ($r^2 = 0.80$; $P < 0.02$). However, since clozapine was an outlier, we decided to investigate the pharmacological profile of [^{3}H]5-HT binding to determine if [^{3}H]5-HT was a better predictor of functional potency than [^{125}I]LSD binding. [^{125}I]LSD competition experiments with unlabeled 5-HT ($n = 5$ experiments) indicated 5-HT interacts with two affinity states of the 5-HT_{2C} receptor. Curve fitting defined a high affinity site ($K_i = 23$ nM, 10% of total sites) and a low affinity site ($K_i = 660$ nM, 90% of total binding) (F statistic = 13.48; df(8,6); $P < 0.006$, data not shown). We performed [^{3}H]5-HT competition binding experiments with 20 nM radioligand, thus measuring ligand competition for the high affinity site. The data are presented in Table 2. As expected, K_i values for both [^{125}I]LSD and [^{3}H]5-HT binding were very similar for (+)LSD, MDL101600 and ritanserin. However, mesulergine, ketanserin, MDL100907, clozapine and trazodone were better competitors for [^{125}I]LSD binding than [^{3}H]5-HT binding. Surprisingly, the correlation plot of pIC_{50} [^{3}H]5-HT binding vs. the pIC_{50} for phosphoinositide hydrolysis inhibition shown in Fig. 4B had a lower correlation coefficient ($r^2 = 0.62$; $P < 0.05$) for these compounds than does the [^{125}I]LSD binding correlation plot, indicating that [^{125}I]LSD binding may be a better predictor of relative physiological potency than [^{3}H]5-HT. Clozapine was an outlier with [^{125}I]LSD binding and fluoxetine was an outlier with [^{3}H]5-HT binding. Neither of these radioligand binding assays could consistently predict the physiological potency of all the antagonists tested.

3.6. MDL101600 is a competitive inhibitor

MDL101600, the potent aryloxyalkylimidazoline antagonist, was studied in more detail to better characterize the kinetic basis for its inhibition in the functional assay. Concentration-response curves were obtained for the 5-HT-evoked response in the absence and presence of 100 nM MDL101600. As shown in Fig. 5, in the presence of the antagonist, there was a parallel dextral shift of the concentration-response curve to 5-HT. The natural agonist was still able to elicit a maximal response level in the presence of the antagonist. These results are consistent with MDL-101600 being a competitive antagonist of the 5-HT response. A Schild plot analysis of this single data

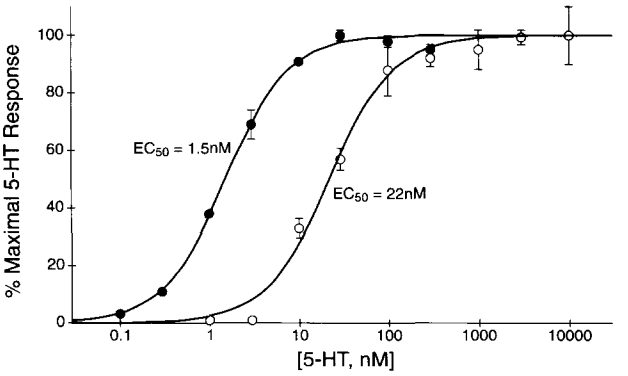

Fig. 5. Serotonin dose-response curves in the presence (○) and absence (●) of 100 nM MDL101600 at the 5-HT_{2C} receptor of P_o cells. The EC_{50} for 5-HT shifts dextrally from 1.5 nM to 22 nM in the presence of the competitive antagonist without the maximal attainable response being reduced.

point with a slope set at unity gave a K_i value of 3 nM, a value almost identical to the K_i value obtained for both [^{125}I]LSD and [^{3}H]5-HT binding with this compound.

4. Discussion

4.1. The natural agonist 5-HT

The cloning and stable expression of the rat 5-HT_{2C} and 5-HT_{2A} receptor genes in NIH 3T3 mouse fibroblasts has now made possible direct comparisons of these two 5-HT receptor subtypes with regard to ligand binding affinity, functional efficacy and potency, and elucidation of the antagonist properties of existing and novel chemical entities that interact with 5-HT receptors. In this study 5-HT was about 60-fold more potent at the rat 5-HT_{2C} receptor as an agonist of phosphoinositide hydrolysis. Using rat tissues, Conn and Sanders-Bush (1987) noted a 28-fold greater potency for 5-HT-stimulated phosphoinositide hydrolysis at 5-HT_{2C} relative to 5-HT_{2A} receptor preparations (EC_{50} = 50 nM and 1400 nM, respectively). It should be noted that the potency of 5-HT at both native receptors was less than one-tenth that seen in this study using the two receptor subtypes expressed in NIH 3T3 fibroblasts. This difference probably reflects the large receptor reserve present in the cell lines (Grotewiel et al., 1994).

The reason for this 5-HT_{2C} receptor selectivity by the natural agonist is currently unknown. The molecular model of Hibert et al. (1991) suggests that the binding pocket for 5-HT is essentially identical in both the 5-HT_{2C} and 5-HT_{2A} receptor subtypes (M. Hibert, personal communication). Site-directed mutagenesis studies (e.g., Choudhary et al., 1993) may be instrumental in elucidating the various proposed helical amino acid side chain interactions of these two 5-HT receptor subtypes with 5-HT and other 5-HT receptor ligands. One additional topological domain that may be responsible for the different levels of physiological response of these two 5-HT receptor subtypes is intracellular loop 3. Here, these two receptors possess little amino acid sequence homology (Julius, 1991), indicating possibly that these receptors interact differently with a common G-protein linked to phospholipase C, or that they are coupled to different G-proteins mediating a common transduction event.

4.2. The aryloxyalkylimidazolines

The aryloxyalkylimidazolines MDL101156 and MDL100971 are full and partial agonists, respectively, at both the rat 5-HT_{2C} and 5-HT_{2A} receptors, showing ~ 5-fold selectivity for the 5-HT_{2C} receptor. MDL102588 possesses weak agonist activity at the rat 5-HT_{2C} site and only weak antagonist activity at the 5-HT_{2A} site. In contrast, all three compounds exhibited moderate affinity for the recognition site ([^{125}I]LSD binding K_i = 29–261 nM), a property seen by 5-HT receptor agonists in general. The rank order potency of these agonists was qualitatively similar at both 5-HT receptor subtypes (Fig. 2B,D), though quantitatively less potent at the 5-HT_{2A} site, as was the natural agonist, 5-HT. These agonists have low potency compared to 5-HT, being two orders of magnitude less potent at the rat 5-HT_{2C} receptor and one order of magnitude less potent at the rat 5-HT_{2A} receptor.

It is interesting that extending an alkyl chain one and two additional carbon atoms turns a potent aryloxyalkylimidazoline antagonist (MDL101600) into a much weaker antagonist (MDL103097), and a weak agonist (MDL102588), respectively. This class of compounds possesses a nitrogen which would be protonated at physiological pH, and may ion pair with an aspartate within the receptors' central cleft. However, most interactions of these molecules with the receptor would be of a hydrophobic nature. Since MDL102588 is an agonist and is only two carbons longer on one side chain than the potent antagonist MDL101600, possibly 'pushing' the phenyl group deeper into a specific hydrophobic pocket can elicit a receptor conformational change that gives an agonist response. MDL100971 also possesses this longer alkyl linker to a phenyl group and is also an agonist at the 5-HT_{2C} receptor, one of greater potency than MDL102588. An interesting difference between these two aryloxyalkylimidazolines is that MDL100971 is an agonist at the 5-HT_{2A} receptor, while MDL102588 possesses no agonist activity at the 5-HT_{2A} receptor.

MDL101156 possesses a similar one carbon side chain linking the phenyl group as is present in the antagonist MDL101600. Therefore, the agonist activity of MDL101156 is probably due to the naphthyl group. These interesting observations may indicate that the 5-HT receptor can acquire an agonist conformation by being perturbed via different hydrophobic pockets within the binding cleft and/or several different agonist conformations of the receptor can exist to produce the same physiological response. Consistent with our results, none of the aryloxyalkylimidazolines discussed in this publication possess the structural characteristics suggested by Pierce et al. (1992) to be requisite for 5-HT_{2A} receptor selectivity.

4.3. Reference 5-HT receptor agonists

In agreement with results obtained using native rat tissue (Sanders-Bush et al., 1990; Burris et al., 1991), (+)LSD was a potent partial agonist at both the cloned rat 5-HT_{2C} and 5-HT_{2A} receptors. It is interesting that

(+)LSD has equal potencies in both the binding and the functional assays at both 5-HT receptor subtypes.

(±)DOI, a hallucinogenic phenylalkylamine, is an equally potent partial agonist at both the cloned rat 5-HT_{2C} and 5-HT_{2A} receptors. Like (+)LSD, this compound has very similar potencies at both 5-HT receptor subtypes for both binding affinity and functional potency. A similar functional response has been reported for the rat choroid plexus 5-HT_{2C} receptor (Sanders-Bush and Breeding, 1991). Seggel et al. (1990) have demonstrated that alkyl substitution at the 4 position can convert DOI analogs from agonists to 5-HT_{2A} receptor antagonists, another example of small chemical changes inducing major changes in the response of the 5-HT receptor subtypes like that seen in the aryloxyalkylimidazoline noted in the previous section.

m-CPP, a metabolite of the antidepressant trazodone, is a 5-HT mimetic agent used extensively in human clinical studies to elucidate the role of 5-HT in neuronal physiology (Curzon and Kennet, 1990). Our results with the cloned receptor subtypes indicate that m-CPP exhibits similar binding affinity for 5-HT_{2C} and 5-HT_{2A} receptors. The present study shows that m-CPP was a full agonist at the 5-HT_{2C} receptor and a partial agonist at the 5-HT_{2A} receptor. Conn and Sanders-Bush (1987), working with rat choroid plexus 5-HT_{2C} and rat cerebral cortex 5-HT_{2A} receptors, noted 5-HT_{2C} receptor agonism and 5-HT_{2A} receptor antagonism with a 5-HT_{2A} receptor agonist effect only at concentrations $> 100\ \mu M$, which was not inhibited by ketanserin. We therefore looked at the agonist activity of m-CPP (10 μM) with GF_6 cells in the presence of 10 nM MDL100907, the highly selective 5-HT_{2A} receptor antagonist, and noted complete blockade of the agonist effect (data not shown). This result gives further evidence that m-CPP is causing its agonist effect via the transfected 5-HT_{2A} receptor as opposed to another receptor on the mouse NIH 3T3 fibroblast host cell. Recently, Grotewiel et al. (1994) have provided an extensive investigation of this m-CPP agonist activity in GF_6 cells ($EC_{50} = 510$ nM) and comment on the clinical implications. Agonist activity of m-CPP has not been demonstrated for the 5-HT_{2A} receptor in native tissue. Recombinant systems such as GF_6 cells expressing the 5-HT_{2A} receptor have a much higher receptor density than native tissues and thus may respond differently to this arylpiperazine. Usefulness of m-CPP as a pharmacological tool to differentiate these receptor subtypes in vivo could depend on fortuitous differences in receptor reserve.

Trazodone, the parent compound of m-CPP, showed a 25-fold selectivity for the 5-HT_{2A} receptor in competing for [^{125}I]LSD binding and a 100-fold selectivity at the 5-HT_{2A} receptor in antagonizing the 5-HT functional response. Trazodone had no agonist activity at the 5-HT_{2C} receptor and was a very weak antagonist.

SKF83566 is a potent antagonist at the dopamine D_1 receptor. At the cloned rat 5-HT_{2C} receptor, this benzazepine is a potent agonist, whereas at the 5-HT_{2A} receptor it is a weak partial agonist. Hoyer et al. (1989) first demonstrated this potent 5-HT_{2C} receptor agonist effect in native tissue of a different species, pig choroid plexus. In our study, it is interesting that SKF83566 competes with equal high potency for [^{125}I]LSD binding at both receptors but the agonist potencies are very different. The Cl analog of SKF83566 (SCH23390) has been reported to be potent ($K_i \sim 15$ nM) at the rat 5-HT_{2C} receptor in competing for [^{125}I]LSD binding (Roth et al., 1992), and also has potent 5-HT_{2C} receptor agonist activity.

4.4. Antagonists

4.4.1. The aryloxyalkylimidazolines

MDL101600 is a potent competitor for [^{125}I]LSD binding and a potent competitive antagonist for both 5-HT_{2C} and 5-HT_{2A} receptor-mediated phosphatidylinositol turnover, showing a 2–3-fold selectivity for the 5-HT_{2A} receptor in the functional assay. This aryloxyalkylimidazoline is only two carbon atoms shorter on one side chain than MDL102588 which is a weak 5-HT_{2C} receptor agonist and 5-HT_{2A} receptor antagonist. MDL103097, the analog intermediate between these two compounds that possesses only one carbon atom in the given side chain, was a slightly less potent competitor of [^{125}I]LSD binding than MDL101600, yet surprisingly was a much weaker antagonist in the functional assay at both 5-HT receptor subtypes. These small differences in chemical structure which elicit such large changes in functional efficacy and potency may assist in the elucidation of the mechanisms whereby some 5-HT receptor ligands are agonists and others are antagonists.

4.4.2. Reference 5-HT receptor antagonists

All of these compounds are recognized 5-HT receptor ligands because they have been previously tested in native tissue prior to the availability of the cloned 5-HT receptors. Our data provide a direct side by side comparison of the pharmacology of rat 5-HT_{2A} and 5-HT_{2C} receptors cloned into the same cell line, where they can be studied in isolation from each other and other receptors. In general, there are no differences in the relative potencies and selectivities of these various 5-HT receptor antagonists in the native tissue vs. the cloned receptors.

Mesulergine had the greatest selectivity for the 5-HT_{2C} receptor (2.5-fold) among the antagonists examined in this publication. Like mesulergine, ritanserin had potent binding affinities for both 5-HT receptor subtypes but was 4-fold selective for the 5-HT_{2A} receptor in the functional assay. Our data indicate that

ketanserin has a 10-fold greater selectivity for the 5-HT_{2A} receptor than ritanserin. Similar rank order potencies for mesulergine, ritanserin and ketanserin have been noted using native tissue (Hoyer, 1988a, b; Hoyer et al., 1989; Sanders-Bush and Breeding, 1988; Sahin-Erdemli et al., 1991; Apud et al., 1992), and the cloned rat 5-HT_{2C} receptor transiently expressed in COS-7 cells (Roth et al., 1992).

MDL100907, an α-phenyl-4-piperidinemethanol, a selective competitive 5-HT_{2A} receptor antagonist with negligible dopaminergic activity (Carr et al., 1991; Sorensen et al., 1993; Palfreyman et al., 1993), was about 55-fold selective for binding to the cloned rat 5-HT_{2A} receptor relative to the 5-HT_{2C} receptor. This compound was 700-fold selective for the 5-HT_{2A} receptor in antagonizing the 5-HT-mediated functional response. MDL100907 has the molecular structural basis for 5-HT_{2A} receptor selectivity as defined by Pierce et al. (1992); a carbonyl or carbinol interposed spatially between an aromatic ring and nitrogen atom. This compound has been investigated by Schmidt et al. (1992) to explore the permissive role of 5-HT in the stimulation of dopamine function produced by the amphetamine analog 3,4-methylenedioxymethamphetamine (MDMA).

Spiperone, a dopamine D_2 receptor selective antagonist was also a potent, selective 5-HT_{2A} receptor antagonist. With the cloned receptors, this compound was $>$ 600-fold selective in competing for [^{125}I]LSD binding for the rat 5-HT_{2A} receptor over the rat 5-HT_{2C} receptor, and was $>$ 2000-fold selective in antagonizing 5-HT-stimulated phosphoinositide hydrolysis at the 5-HT_{2A} receptor. Conn et al. (1986) demonstrated a $>$ 1400-fold selective antagonist effect on 5-HT_{2A} receptors over 5-HT_{2C} receptors in native rat tissue. A similar potency for [^{125}I]LSD binding at the rat 5-HT_{2C} receptor transiently expressed in COS-7 cells has been reported by Roth et al. (1992).

The atypical antipsychotic agent clozapine has highest affinity for the dopamine D_4 receptor among the five dopamine receptor subtypes (Seeman, 1992). This tricyclic compound bound with equal affinity to both the rat 5-HT_{2C} and 5-HT_{2A} receptors expressed in NIH 3T3 fibroblasts, however it was more than 10-fold selective for the 5-HT_{2A} receptor in antagonizing 5-HT-stimulated phosphoinositide hydrolysis. This large discrepancy between the binding affinity (K_i = 5 nM) and antagonist potency ($IC_{50} \sim$ 1000 nM) at the 5-HT_{2C} receptor in the functional assay is presently unexplained. Kuoppamaki et al. (1993) noted an IC_{50} = 110 nM for clozapine as an antagonist at the rat choroid plexus 5-HT_{2C} receptor. Possibly the 10-fold difference between the native vs. the cloned rat 5-HT_{2C} receptor reflects a difference in G protein-coupling, or a difference in receptor reserve altering the stoichiometry between receptors and G-proteins (Boddeke et al., 1992). Competition for [^{3}H]5-HT binding at the 5-HT_{2C} receptor by clozapine was less potent than competition for [^{125}I]LSD binding, and correlated better with its functional potency than did [^{125}I]LSD binding data. Roth et al. (1992) also noted clozapine binds potently to the rat 5-HT_{2C} receptor transiently expressed in COS-7 cells.

Burris and Sanders-Bush (1992) have shown the off-rate of [^{3}H]LSD to be slow (20 min) at the rat 5-HT_{2A} receptor and increasing concentrations of 5-HT could not overcome the blocking effect of (+)LSD on phosphoinositide hydrolysis of NIH 3T3 fibroblasts transfected with the rat 5-HT_{2A} receptor, reflecting either a prolonged occupancy of the receptor by a slowly reversible antagonist, or a non-competitive mode of antagonism by the ergot. It is possible therefore that [^{125}I]LSD binding may reflect occupancy at an allosteric site rather than at the primary binding site.

Fluoxetine, a potent, selective 5-HT uptake inhibitor had about the same affinity for both the 5-HT_{2C} and 5-HT_{2A} receptors in competing for [^{125}I]LSD binding. Wong et al. (1990) reported the *R*- enantiomer and racemic fluoxetine inhibited [^{3}H]mesulergine binding to bovine choroid plexus 5-HT_{2C} receptors with IC_{50} values of 200 and 450 nM, respectively, while the IC_{50} of *S*-fluoxetine was 17 000 nM. At the cloned rat 5-HT_{2C} and 5-HT_{2A} receptors, we detected only very weak antagonist activity for a racemic mixture of fluoxetine. Inhibition of [^{3}H]5-HT uptake by rat brain synaptosomes (Fuller et al., 1991) gave K_i = 33 nM for the *R* enantiomer and K_i = 21 nM for the *S* enantiomer. The role, if any, of this 5-HT_{2C} antagonism in the therapeutic effect or side effects of fluoxetine remains to be elucidated. In our studies, fluoxetine possessed no agonist activity at either the 5-HT_{2C} or 5-HT_{2A} receptor subtype.

4.5. [^{3}H]5-HT binding

[^{125}I]LSD had about one order of magnitude higher affinity for the rat 5-HT_{2C} receptor than did [^{3}H]5-HT (5 nM vs. 23 nM). This is possibly a reflection that (+)LSD possesses a second nitrogen atom which can enhance binding at an additional site by hydrogen bonding. Also, (+)LSD is a larger, more hydrophobic molecule than 5-HT, and therefore more hydrophobic interactions with the receptor's helical side chains are possible. Probably the recognition sites for the two ligands are composed of different but overlapping molecular elements with one or several shared by both ligands. Unfortunately, [^{3}H]5-HT binding cannot be measured directly with the GF_6 cell line since affinity for 5-HT is about 50-fold less than that of the P_o cell line.

Our results demonstrate that both [^{125}I]LSD and [^{3}H]5-HT binding data have about equal value in pre-

dicting antagonist potency in the functional assay. Competition of these various ligands for [^{125}I]LSD binding may be different than that for [^{3}H]5-HT because of differences in the molecular features of the radioligand recognition sites.

4.6. Summary for the aryloxyalkylimidazolines

To date, the aryloxyalkylimidazolines investigated are, in general, equipotent as antagonists at the 5-HT$_{2C}$ and 5-HT$_{2A}$ receptor subtypes. When these compounds are selective for the 5-HT$_{2C}$ receptor, they are functionally agonists.

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