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SELECTIVITY OF SEROTONERGIC DRUGS FOR MULTIPLE BRAIN SEROTONIN RECEPTORS

ROLE OF [^{3}H]-4-BROMO-2,5-DIMETHOXYPHENYLISOPROPYLAMINE ([^{3}H]DOB), A 5-HT$_2$ AGONIST RADIOLIGAND

MILT TITELER,*† ROBERT A. LYON,* KATHARINE H. DAVIS* and RICHARD A. GLENNON‡

*Department of Pharmacology and Toxicology, Albany Medical College, Albany, NY 12208; and ‡Department of Medical Chemistry, Virginia Commonwealth University, Richmond, VA 23298, U.S.A.

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Abstract—The affinities of putative serotonin receptor agonists and antagonists for 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1C}$, and 5-HT$_2$ receptors were assayed using radioligand binding assays. The 5-HT$_1$ sites were labeled with the agonist radioligands [^{3}H]-8-hydroxy-2-(di-*n*-propylamino)-tetralin [^{3}H]-8-OH-DPAT, [^{3}H]-5-HT, and [^{3}H]mesulergine. The 5-HT$_2$ receptor was labeled with the antagonist radioligand [^{3}H]ketanserin or the agonist radioligand [^{3}H]-4-bromo-2,5-dimethoxyphenylisopropylamine ([^{3}H]DOB). The apparent 5-HT$_1$ receptor selectivity of agonist compounds was found to be 50- to 100-fold higher when the 5-HT$_2$ receptor affinity was determined using the antagonist radioligand [^{3}H]ketanserin than when the agonist radioligand [^{3}H]DOB was used. Quipazine, a putative specific 5-HT$_2$ agonist, appeared to be only 3-fold more potent at 5-HT$_2$ than at 5-HT$_{1A}$ receptors when [^{3}H]ketanserin was used as the 5-HT$_2$ radioligand. When [^{3}H]DOB was used as the 5-HT$_2$ radioligand, quipazine was determined to be 100-fold more potent at 5-HT$_2$ receptors than at 5-HT$_{1A}$ receptors. 1-(3-trifluoromethylphenyl)piperazine (TFMPP), a putative specific 5-HT$_{1B}$ receptor agonist was apparently 10-fold more potent at 5-HT$_{1B}$ receptors than at 5-HT$_2$ receptors when [^{3}H]ketanserin was used as the 5-HT$_2$ radioligand. When [^{3}H]DOB was used as the 5-HT$_2$ radioligand, TFMPP was found to be equipotent at 5-HT$_{1B}$ and 5-HT$_2$ receptors. Using the 5-HT$_2$ antagonist radioligand [^{3}H]ketanserin, a similar pattern of underestimating 5-HT$_2$ receptor selectivity and/or overestimating 5-HT$_{1A}$ or 5-HT$_{1B}$ receptor selectivity was observed for a series of serotonin receptor agonists. Antagonist receptor selectivity was not affected significantly by the nature of the 5-HT$_2$ receptor assay used. These data indicate that, by using an antagonist radioligand to label 5-HT$_2$ receptors and agonist radioligands to label 5-HT$_1$ receptors, the 5-HT$_1$ receptor selectivity may be overestimated. This may be an especially severe problem in serotonin drug development as drugs that interact potently with 5-HT$_2$ receptors have been reported to be psychoactive and/or hallucinogenic.

Evidence for the existence of multiple functional serotonin receptors in the brain and periphery has been accumulating rapidly [1, 2]. 5-HT$_2$ receptors have been labeled with [^{3}H]spiperone [3] and [^{3}H]ketanserin [4, 5] which are antagonist radioligands. [^{3}H]Serotonin-radiolabeling studies have revealed three distinct sites, each possessing characteristics indicating that they may be functional receptors [6–8]. To date, the majority of studies have used the agonist radioligand [^{3}H]-8-OH-DPAT§ to label 5-HT$_{1A}$ sites [6], [^{3}H]-5-HT to label the 5-HT$_{1A}$, 5-HT$_{1B}$, and 5-HT$_{1C}$ sites and [^{3}H]mesulergine to label 5-HT$_{1C}$ sites [2, 8–10]. In studies aimed at assessing the relative affinities of serotonergic compounds for 5-HT$_2$, 5-HT$_{1A}$, 5-HT$_{1B}$, and 5-HT$_{1C}$ receptors, laboratories have been using antagonist radioligands to label the 5-HT$_2$ receptors and agonist radioligands to label the multiple 5-HT$_1$ receptors [8–10].

Studies from our laboratory have demonstrated that the 5-HT$_2$ receptor apparently exists in two states, both labeled with equal affinity by the antagonist radioligand [^{3}H]ketanserin [5, 11, 12]. Apparent agonist affinities for the receptor appear to be a combination of their affinities for the two states of the receptor for which the agonists have very different affinities. The resultant apparent affinity is predominantly influenced by the low agonist affinity state of the receptor as this state appears to exist in excess of the high agonist affinity state of the receptor [5, 11]. Assays labeling 5-HT$_{1A}$, 5-HT$_{1B}$, and 5-HT$_{1C}$ receptors do not involve the low affinity state of these receptors as agonist radioligands selectively label the agonist high affinity state of the receptor as revealed by the high affinity serotonin displays in radioligand binding assays of these sites. Therefore, an agonist radioligand that would selectively label the agonist high affinity state of the 5-HT$_2$ receptor would be a useful tool in determining true relative affinities of drugs for the multiple serotonin receptors (i.e. 5-HT$_{1A}$ vs 5-HT$_2$). [^{3}H]DOB, a putative potent and specific 5-HT$_2$ agonist radioligand, has been developed [13–15]. We report herein results of studies using [^{3}H]DOB, in conjunction with other radio-

† To whom all correspondence should be sent.

§ Abbreviations: 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)-tetralin; DOB, 4-bromo-2,5-dimethoxyphenylisopropylamine; TFMPP, 1-(3-trifluoromethylphenyl)piperazine; and GppNHp, guanyl-5′-imidodiphosphate.

ligand assays, to screen serotonergic drugs for their selectivities for agonist radiolabeled 5-HT_2, 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1C} receptors.

MATERIALS AND METHODS

Tissue preparation. Following decapitation, the brains of male Sprague–Dawley rats were removed and placed in 0.9% ice-cold saline and then dissected over ice until the tissue was prepared. Tissues were stored in ice-cold saline for no longer than 1 hr and, following blot drying and weighing, they were prepared and frozen at −30° until used. Freshly dissected tissue was homogenized (polytron setting 6 for 20 sec) in 30 vol. of ice-cold buffer containing 50 mM Tris–HCl (pH 7.4 at 37°; pH 8.0 at 4°), 0.5 mM Na_2EDTA and 10 mM $MgSO_4$ and centrifuged at 30,000 *g* for 15 min. The supernatant fraction was discarded, and the pellet was resuspended and pre-incubated for 15 min at 37°. The homogenate membranes were washed twice by centrifugation and resuspension. The final suspension buffer contained 10 μM pargyline and 0.1% ascorbate and was added last to the assay incubation medium. Protein determinations were made by the method of Lowry *et al.* [16].

Radioligand binding assays. The agonist high affinity state of the 5-HT_2 receptor was labeled with 0.4 nM [^{3}H]DOB (40 Ci/mmol, New England Nuclear) using 20 mg of membrane suspension prepared from rat frontal cortical tissue [13,14]. Cinanserin (10^{-6} M) was used to define non-specific binding. The agonist low affinity state of the 5-HT_2 receptor was assayed with 0.4 nM [^{3}H]ketanserin (76 Ci/mmol, New England Nuclear) and 3 mg of homogenized rat frontal cortical tissue in the presence of 10^{-5} M GppNHp [5]. In addition, the results of agonist competition for [^{3}H]ketanserin-labeled 5-HT_2 receptors in the absence of GppNHp were computer-analyzed (see below) to determine the agonist affinity for the agonist low affinity state. The 5-HT_{1A} receptor was labeled with 0.1 nM [^{3}H]-8-OH-DPAT (120 Ci/mmol, New England Nuclear) using 6 mg of rat hippocampal homogenates. 8-OH-DPAT (10^{-6} M) was used to define nonspecific binding. The 5-HT_{1B} receptor was labeled with 2.0 nM [^{3}H]serotonin (23 Ci/mmol, New England Nuclear) using 8 mg of rat striatal membrane homogenates. 5-HT (10^{-6} M) was used to define non-specific binding, and 10^{-7} M 8-OH-DPAT and mesulergine [6, 8] were included to block 5-HT_{1A} and 5-HT_{1C} receptors respectively. The 5-HT_{1C} receptor was labeled with 1.0 nM [^{3}H]mesulergine (85 Ci/mmol, Amersham) using 20 mg of pig cortical homogenate in the presence of 20 nM spiperone. Non-specific binding was determined with 10 μM serotonin. Eleven concentrations of non-radioactive competing drugs were made fresh daily in assay buffer. Following incubation with membranes and radioligand at 37° for 15 min (for 5-HT_2 assays) or 30 min (for 5-HT_1 assays), samples were rapidly filtered over glass fiber filters and washed with 10 ml of ice-cold 50 mM Tris–HCl buffer. Individual filters were inserted into vials and equilibrated with 5 ml of scintillation fluid for 6 hr before counting at 50% efficiency. Results were analyzed using an updated version of the program EBDA [17] or RS1 (BBN software). EBDA is a program for the IBM PC that uses non-linear regression to best-fit the competition curve data to the Hill equation [17]. RS1 is a graphics program for the IBM AT that was used to plot the competition curves according to the parameters determined using EBDA.

RESULTS

The results presented in Figs. 1–3 demonstrate that the apparent affinity of serotonin and two putative

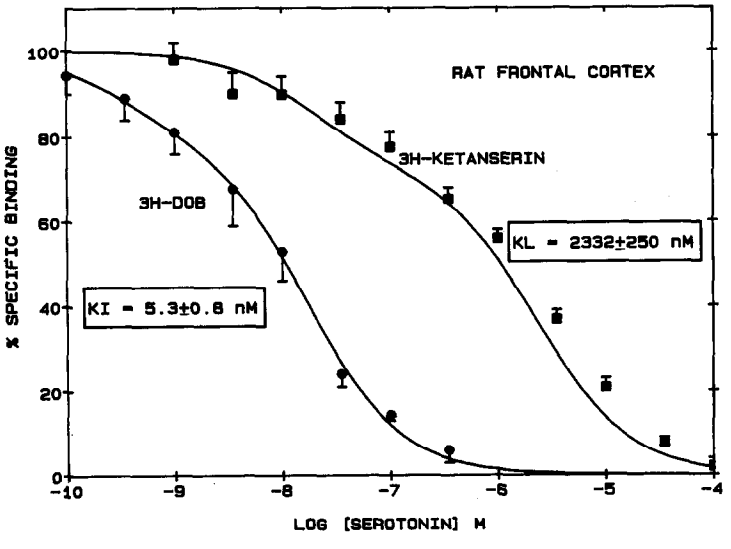

Fig. 1. Serotonin competition for 5-HT_2 receptors labeled with 0.4 nM [^{3}H]DOB or 0.4 nM [^{3}H]ketanserin. Total dpm for [^{3}H]DOB was 1180 ± 37 of which 54% was specific. Total dpm for [^{3}H]ketanserin was 4100 ± 380 of which 84% was specific. The K_L values refer to agonist affinities for the low agonist affinity state of the [^{3}H]ketanserin labeled 5-HT_2 receptor and were determined using computer-assisted analysis of a two-site model (see Material and Methods). Error bars are the SEM from three independent experiments, each point determined in triplicate.

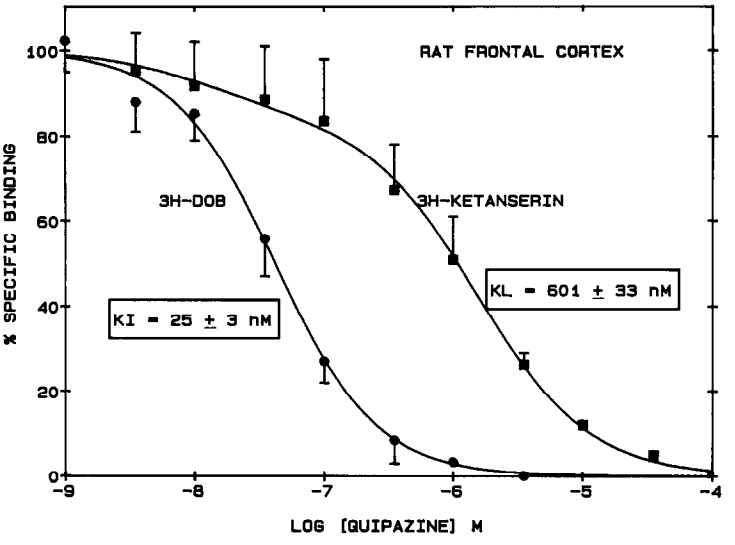

Fig. 2. Quipazine competition for 5-HT_2 receptors labeled with 0.4 nM [^{3}H]DOB or 0.4 nM [^{3}H]ketanserin (see legend of Fig. 1). K_L was determined by computer-assisted analysis of a two-site model.

serotonin receptor agonists, quipazine and TFMPP, for the radiolabeled 5-HT_2 receptor is dependent on the agonist/antagonist nature of the radioligand used. In assays using [^{3}H]DOB, an agonist radioligand for 5-HT_2 receptors [13, 14], serotonin, quipazine, and TFMPP produced potent competition curves, far to the left of the competition curve produced by these agonists in competing for the 5-HT_2 antagonist radioligand [^{3}H]ketanserin. The high affinities of serotonin and DOB for the [^{3}H]DOB-labeled 5-HT_2 receptor were similar to the high affinities of these substances previously reported using computer-assisted two-site analysis of [^{3}H]ketanserin binding [5, 12]. However, the competition curves produced by the antagonists ketanserin and propranolol appeared to be independent of the radioligand used to label the 5-HT_2 receptor (Figs. 4 and 5). Chlorpromazine demonstrated a marginally higher affinity (4- to 5-fold) for the [^{3}H]ketanserin-labeled 5-HT_2 receptor than for the [^{3}H]DOB-labeled 5-HT_2 receptor. The exact meaning of this anomalous observation is not clear.

The K_I values determined for a series of agonists at the two states of the 5-HT_2 receptor and three 5-HT_1 receptor sub-types are listed in Table 1. Table 2 lists the affinity ratios for the two states of the 5-HT_2 receptor and illustrates that, using the K_I values determined with the 5-HT_2 antagonist radioligand, the potency ratios of agonists for the 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1C} receptor sub-types were dra-

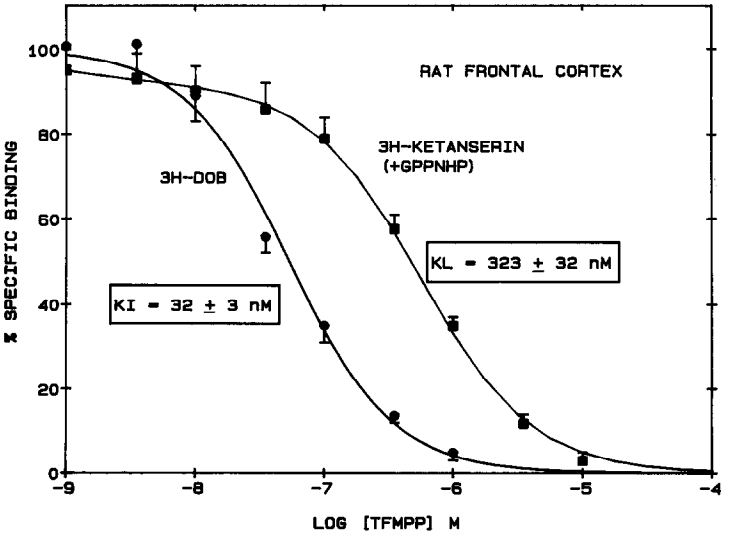

Fig. 3. TFMPP competition for 5-HT_2 receptors labeled with 0.4 nM [^{3}H]DOB or 0.4 nM [^{3}H]ketanserin in the presence of 10^{-5} M GppNHp (see legend of Fig. 1). The [^{3}H]ketanserin K_L value represents the affinity of TFMPP for the agonist low affinity state of the 5-HT_2 receptor (see Materials and Methods).

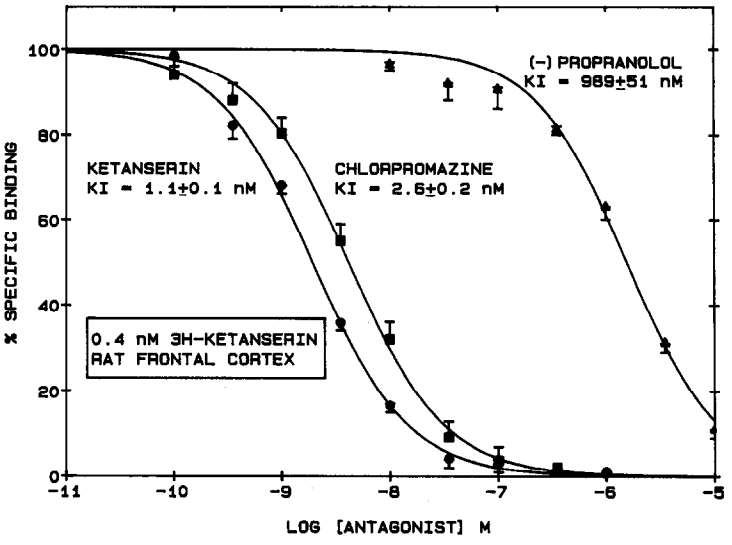

Fig. 4. Antagonist competition for 5-HT_2 receptors labeled with 0.4 nM [^{3}H]ketanserin (see legend of Fig. 1).

matically different than the potency ratios calculated using the K_I values determined using the 5-HT_2 agonist radioligand. Antagonist potency ratios appear to be relatively independent of the nature of the 5-HT_2 radioligand used.

DISCUSSION

There is a great deal of interest in developing drugs that are specific agonists at various serotonin receptors, using radioligand binding assays as screening tools [9, 18]. The data displayed herein indicate that the apparent affinity of putative serotonergic agents for the 5-HT_2 receptor was dependent on the nature (intrinsic activity) of the radioligand. Agonists appeared to have 50- to 100-fold higher affinity when the agonist radioligand [^{3}H]DOB was used to label the receptor than when the antagonist radioligand [^{3}H]ketanserin was used. This observation is strongly supportive of, and consistent with, previous work from our laboratory, indicating the presence of agonist high and low affinity states for the 5-HT_2 receptor due to 5-HT_2 receptor/GTP-binding protein interactions [5, 11, 12, 14]. The 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1C} receptor sub-types are typically labeled with radioactive ligands that appear to label the agonist high affinity states of these receptors. As a result, when affinities of putative agonists are compared at ^{3}H-antagonist-labeled 5-HT_2 receptors and ^{3}H-agonist-labeled 5-HT_1 receptor sub-types, the 5-HT_1 selectivity may be greatly overestimated.

Recent evidence has indicated the distinct possi-

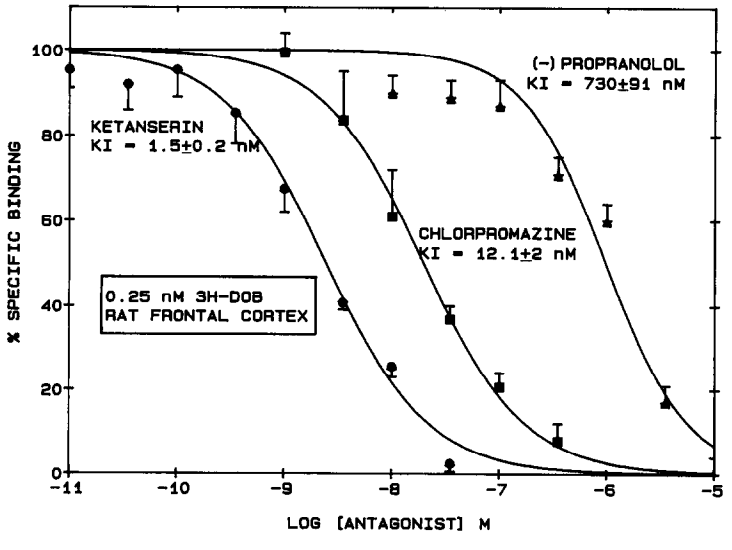

Fig. 5. Antagonist competition for 5-HT_2 receptors labeled with 0.4 nM [^{3}H]DOB (see legend of Fig. 1).

Table 1. Affinities of selected agonists and antagonists for radiolabeled 5-HT_2 and 5-HT_1 receptors

	K_i (nM)				
	5-HT_{2H}	5-HT_{2L}	5-HT_{1A}	5-HT_{1B}	5-HT_{1C}
Agonists					
Serotonin	5.3 ± 0.8	2,332 ± 250	1.7 ± 0.3	5.4 ± 0.5	9.5 ± 1.5
Quipazine	25 ± 2	601 ± 33	2,038 ± 84	191 ± 36	624 ± 87
TFMPP	32 ± 3	323 ± 32	173 ± 12	27 ± 4	120 ± 12
(±)DOB	0.44 ± 0.05	59 ± 4	3,770 ± 118	831 ± 37	69 ± 16
Buspirone	289 ± 38	576 ± 20	14 ± 1.0	>10,000	1,160 ± 81
Ipsapirone	1,047 ± 55	1,305 ± 31	5.5 ± 0.3	>10,000	3,306 ± 325
RU 24969	41 ± 5	1,860 ± 464	3.8 ± 0.3	0.91 ± 0.15	560 ± 70
8-OH-DPAT	696 ± 112	5,350 ± 374	1.2 ± 0.12	>10,000	5,500 ± 100
Antagonists					
Spiperone	0.8 ± 0.04	0.42 ± 0.04	101 ± 9	>10,000	62 ± 6
Ketanserin	1.5 ± 0.2	1.1 ± 0.1	1,933 ± 219	>10,000	49 ± 4
Chlorpromazine	12.1 ± 2	2.6 ± 0.2	737 ± 72	1,489 ± 101	30 ± 3
(−)Propanolol	730 ± 91	989 ± 51	55 ± 4	17 ± 4	1,292 ± 78
(±)Pindolol	4,542 ± 570	16,851 ± 2,600	31 ± 4	34 ± 3	>10,000

The agonist high affinity state (5-HT_{2H}) of the 5-HT_2 receptor was labeled with the agonist radioligand [^{3}H]DOB; the agonist low affinity state (5-HT_{2L}) with [^{3}H]ketanserin, the 5-HT_{1A} receptor with [^{3}H]-8-OH-DPAT, the 5-HT_{1B} receptor with [^{3}H]-5-HT, and the 5-HT_{1C} receptor with [^{3}H]mesulergine. Values are the means ± SEM of three independent experiments.

bility that brain 5-HT_2 receptor activation may be the causative factor in the hallucinogenic (psychoactive) effects of many drugs of abuse, including LSD [12, 19]. Therefore, it seems especially important in serotonin receptor drug development to not underestimate the 5-HT_2 receptor affinity of novel compounds. The agonist radioligand [^{3}H]DOB appears to be a screening tool that will avoid this complication.

One important implication of the data presented in Tables 1 and 2 is that the early pharmacological definition of 5-HT_2 receptors as possessing micromolar affinity for serotonin was an over-simplification [1]. Serotonin displays a K_i value of 5.3 nM for [^{3}H]DOB-labeled 5-HT_2 receptors (Table 1). A question raised by these results is why [^{3}H]serotonin does not label 5-HT_2 receptors. Studies in our laboratory (M. Titeler and G. Battaglia, unpublished observations) demonstrated that approximately 5–10% of specific [^{3}H]serotonin binding was potently inhibited by the 5-HT_2 receptor antagonists spiperone, cinanserin, and ketanserin. The apparent reason for such a low level of 5-HT_2 receptor binding is that, relative to the total amounts

Table 2. 5-HT_2:5-HT_1 serotonin receptor selectivities of serotonergic drugs

	Ratio of K_i values						
	5-HT_{2L} / 5-HT_{2H}	5-HT_{2H} / 5-HT_{1A}	5-HT_{2L} / 5-HT_{1A}	5-HT_{2H} / 5-HT_{1B}	5-HT_{2L} / 5-HT_{1B}	5-HT_{2H} / 5-HT_{1C}	5-HT_{2L} / 5-HT_{1C}
Agonists							
Serotonin	440	3.1	1372	0.98	432	0.56	245
Quipazine	24	0.012	0.29	0.13	3.1	0.04	0.96
TFMPP	10	0.18	1.87	1.19	12	0.27	2.69
(±)DOB	134	1.2×10^{-4}	0.016	5.3×10^{-4}	0.07	0.006	0.86
Buspirone	2.0	21	41	21	0.058	0.25	0.50
Ipsapirone	1.2	190	237	190	0.13	0.32	0.39
RU 24969	45	10.8	489	10.8	2044	0.07	3.32
8-OH-DPAT	7.7	580	4458	580	0.54	0.13	0.97
Antagonists							
Spiperone	0.5	7.9×10^{-3}	4.2×10^{-3}	8×10^{-5}	4.2×10^{-5}	0.13	0.01
Ketanserin	0.7	7.8×10^{-4}	5.7×10^{-4}	1.1×10^{-4}	1.1×10^{-4}	0.03	0.02
Chlorpromazine	0.2	9.5×10^{-3}	3.5×10^{-3}	4.7×10^{-3}	1.7×10^{-3}	0.40	0.09
(−)Propranolol	1.4	13.3	18	43	58	0.59	0.77
(±)Pindolol	3.7	147	544	134	496	0.45	1.69

The selectivity was quantified by determining the ratio of affinities (K_i values) of drugs for the two states of the 5-HT_2 receptor and 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1C} receptors (from Table 1). Note the influence of [^{3}H]DOB versus [^{3}H]ketanserin on the apparent selectivity. In column 1, the higher the ratio the more selective for the 5-HT_{2H} versus the 5-HT_{2L} state the drug appears to be. In columns 2–7, the larger the ratio the more selective for the 5-HT_{1A}, 5-HT_{1B}, or 5-HT_{1C} receptor the drug appears to be. The smaller the ratio the more selective for the 5-HT_2 receptor the drug appears to be.

of available receptor sites with high affinity for serotonin, the amount of agonist high affinity state of the 5-HT_2 receptor is very low [13, 14].

The results with quipazine help to explain the observed selectivity of this drug as a 5-HT_2 agonist *in vivo*. When [^{3}H]ketanserin was used as the radioligand, quipazine appeared to have higher affinity for 5-HT_{1B} sites than for 5-HT_2 and similar affinities for 5-HT_{1A} and 5-HT_2 receptors. However, quipazine has been found to be a potent and specific 5-HT_2 receptor agonist *in vivo* [18, 20]. When [^{3}H]DOB was used as the 5-HT_2 radioligand, quipazine appeared to be 10- to 100-fold less potent at the 5-HT_{1A} and 5-HT_{1B} sites than at the 5-HT_2 receptor. Thus, the use of [^{3}H]DOB as the radioligand apparently resulted in the determination of an affinity constant for quipazine which more accurately predicts its receptor specificity *in vivo*. Several interesting speculations may be made as a result of the data in Tables 1 and 2. TFMPP is a putative specific 5-HT_{1B} agonist [9]. However, when [^{3}H]DOB was used as the radioligand, the affinities of TFMPP for the 5-HT_{1B} and 5-HT_2 receptors were essentially identical. It would be reasonable to predict that the biological actions of TFMPP may be a result of stimulation of both 5-HT_{1B} and 5-HT_2 receptors, if indeed TFMPP is an agonist at the 5-HT_2 receptor (see below). Buspirone and ipsapirone (formerly TVX Q 7821) are putative 5-HT_{1A} agonists with anxiolytic effects [21, 22]. The data in Table 1 indicate that at 5-HT_2 receptors these drugs have similar apparent affinities whether [^{3}H]DOB or [^{3}H]ketanserin is used as the radioligand. This may indicate that these drugs are antagonists at the 5-HT_2 receptor as this pattern of binding is observed with well-characterized 5-HT_2 antagonists (Table 1). However, it is unlikely that the effects of buspirone or ipsapirone *in vivo* involve 5-HT_2 interactions as the potencies of these drugs at the 5-HT_2 receptor were much lower than their potencies at the 5-HT_{1A} receptor (Table 2).

It should be noted that the division of drugs into agonists and antagonists (Tables 1 and 2) is based on various criteria. For instance, quipazine [20], TFMPP [23], DOB [24], 8-OH-DPAT [25], and RU 24969 [26] have been shown to produce behavioural responses and/or neurochemical effects on brain serotonergic systems consistent with the direct stimulation of some class or classes of brain serotonin receptors. Buspirone [27] and ipsapirone [28] have been shown to potently inhibit firing of brain serotonergic neurons through stimulation of 5-HT_{1A} receptors on cell bodies in the dorsal raphe nucleus. The fact that a drug stimulates one serotonin receptor does not preclude the possibility that it may act as an antagonist at another serotonin receptor. For instance, while TFMPP clearly produces serotonin receptor agonist mediated effects, presumably through 5-HT_{1B} receptors [9], there is evidence that it may act as an antagonist at 5-HT_2 receptors [29]. As mentioned above, the data presented herein indicate the possibility that ipsapirone and buspirone may act as 5-HT_2 receptor antagonists as well as 5-HT_{1A} agonists. The classification of spiperone, ketanserin, and chlorpromazine as antagonists is based on reports demonstrating the blockade of well-studied serotonergic behaviors in rats [3]. Propranolol and pindolol are classical beta-adrenergic receptor antagonists [30] that have been shown to potently interact with brain serotonin systems [31–33]. These studies indicate that propranolol and/or pindolol have antagonist properties at 5-HT_{1A} receptors although some agonist-like effects have been noted. The classification scheme used in Tables 1 and 2 is meant to indicate the major pharmacological effect of the drug reported by serotonin receptor pharmacologists.

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