Biochemical Pharmacology, Vol. 38, No. 23, pp. 4237–4244, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

5-HYDROXYTRYPTAMINE$_2$ (5-HT$_2$) STRUCTURE–FUNCTION RELATIONSHIPS OF THE NITRO AND AMINO PHENYLPIPERAZINES ON INTACT HUMAN PLATELETS

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(*Received* 17 *October* 1988; *accepted* 10 *May* 1989)

Abstract—Nitro and amino phenylpiperazines were synthesized to study the agonist and antagonist activities of the phenylpiperazines at the human platelet 5-hydroxytryptamine$_2$ (5-HT$_2$) receptor. Amplification of ADP-induced aggregation and binding competition experiments with [^{3}H]ketanserin were used to evaluate receptor interactions in this system. All the monosubstituted phenylpiperazines were antagonists despite the wide variation in electronic and hydrophobic properties. The parent compound, unsubstituted phenylpiperazine (PP), had the lowest affinity for the [^{3}H]ketanserin binding site. The intensely electron-withdrawing NO_2 substituent increased binding affinity at all ring positions and this activity correlated with antagonist potency in platelet aggregation studies (rank order: 4-NO_2-PP > 3-NO_2-PP > 2-NO_2-PP). NH_2 substitution decreased binding affinity at the 4- and 2-positions compared with the analogous NO_2 substituted compounds; however, evaluation of NH_2-PP antagonist potency in aggregation studies was complicated due to slow association with the receptor. To compare the activities of the phenylpiperazines at other 5-HT sites, binding competition experiments were performed using [^{3}H]5-HT in bovine brain membranes. The rank order of the affinities for the NO_2 substituted compounds was distinctly different from that determined with platelets, reflecting the heterogeneous composition of 5-HT receptor subtypes in the brain membrane preparations. The platelet aggregation experiments demonstrated that marked alteration of the electronic and hydrophobic properties of phenylpiperazine by ring substitution did not impart 5-HT$_2$ agonist activity. By contrast, 5-HT$_2$ antagonist activity appeared to be enhanced markedly by electron-withdrawing resonance effects which decreased the electron density at the 1′-piperazine nitrogen. This enhancement appeared to be specific for the 5-HT$_2$ receptor subtype.

5-Hydroxytryptamine (5-HT) is an endogenous amplifier of platelet aggregation [1]. In humans, abnormally increased platelet responsiveness to 5-HT may be observed in cardiovascular disease, thrombolytic therapy and in the prethrombotic state [2–6]. Thus, pharmacologic antagonism of 5-HT$_2$ activity may be beneficial in a variety of clinical settings. While numerous 5-HT$_2$ antagonists have been described [7–11], structure–function relationships of antagonists at the human platelet 5-HT receptor are not well understood.

Recent studies from this laboratory indicate that heteroaromatic nitrogens in either fused or single ring arylpiperazines confer agonist activity at the platelet 5-HT$_2$ receptor [12]. In contrast, monosubstituted phenylpiperazines, such as 1-(3-chlorophenyl)piperazine HCl (mCPP) and 1-(3-trifluoromethylphenyl)piperazine (TFMPP), which lack heteroaromatic nitrogens, are pure antagonsits. Experiments performed with platelets [12] correlate well with studies of rat CNS tissue [13]; however, mCPP and TFMPP do no differ significantly in physiochemical parameters which might affect agonist or antagonist interactions. In the present study, nitro- and aminophenylpiperazines were synthesized and studied with intact platelets. These compounds embody a wide variation in electronic (σ^- substituent constant) and hydrophobic (π^- substituent constant) properties [14] not previously studied at 5-HT receptors. Platelet aggregation experiments and [^{3}H]ketanserin displacement studies were performed in parallel in order to compare receptor binding at the antagonist site with physiologic response. For comparison of binding affinity, the same compounds were studied at non 5-HT$_2$ binding sites in bovine brain. Substitution with nitro or amino groups at all ring positions affected 5-HT$_2$ antagonist activity and receptor binding in platelets significantly. None of the substitutions conferred agonist activity.

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METHODS

Reagents. 1-(4-Nitrophenyl)piperazine HCl (4-NO_2-PP), phenylpiperazine HCl (PP), 1-(3-chlorophenyl)piperazine HCl (mCPP), 2-fluoronitrobenzene, 3-nitroaniline, bis(chloroethyl)amine HCl, 2,4-dinitrofluorobenzene, 4-fluoro-3-nitroaniline and 2-fluoro-4-nitroaniline were obtained from the Aldrich Chemical Co. (Milwaukee, WI). Tetrabenazine was obtained from the Fluka Chemical Corp. (Ronkonkoma, NY). Phentolamine HCl was obtained from Ciba-Geigy (Summit, NJ). 5-HT–creatinine sulfate was obtained from the Regis

Table 1. Synthesis of amino and nitro phenylpiperazine compounds

Compound	Synthetic method*	Yield (%)	Melting point (°C)	Formula
2-NO_2-PP	I	47.0	159–161	$C_{10}H_{13}N_3O_2 \cdot HCl$
3-NO_2-PP	II	12.6	246 dec	$C_{10}H_{13}N_3O_2 \cdot (HCl)_2$
2-NH_2-PP	III	78.0	265 dec	$C_{10}H_{15}N_3 \cdot (HCl)_2$
3-NH_2-PP	III	83.4	298 dec	$C_{10}H_{15}N_3 \cdot (HCl)_2 \cdot (CH_3OH)_4$
4-NH_2-PP	III	49.8	288 dec	$C_{10}H_{15}N_3 \cdot (HCl)_3 \cdot (CH_3OH)$
2,4-$(NO_2)_2$-PP	I	17.1	262 dec	$C_{10}H_{12}N_4O_4 \cdot (HCl)$
4-NH_2,2-NO_2-PP	I	54.4	239 dec	$C_{10}H_{14}N_4O_2 \cdot (HCl)_2 \cdot (H_2O)_4$
2-NH_2,4-NO_2-PP	I	86.1	258 dec	$C_{10}H_{14}N_4O_2 \cdot (HCl)_2 \cdot (H_2O)$
2,4-$(NH_2)_2$-PP	III	55.9	235 dec	$C_{10}H_{19}N_4 \cdot (HCl)_3 \cdot (H_2O)_4 \cdot (CH_3OH)_4$

* Synthetic method I refers to nucleophilic substitution using piperazine as the nucleophile and 2-fluoronitrobenzene; 2,4-dinitrofluorobenzene; 4-fluoro-3-nitroaniline and 2-fluoro-4-nitroaniline as substrates to yield 2-NO_2-PP; 2,4-$(NO_2)_2$-PP; 4-NH_2,2-NO_2-PP; and 2-NH_2,4-NO_2-PP respectively. Synthetic method II refers to the amino-dehalogenation of bis(chloroethyl)amine HCL with 3-nitroaniline. Synthetic method III is catalytic hydrogenation of 2-NO_2-PP; 3-NO_2-PP; 4-NO_2-PP and 4-NH_2,2-NO_2-PP to yield 2-NH_2-PP; 3-NH_2-PP; 4-NH_2-PP and 2,4$(NH_2)_2$-PP respectively.

Chemical Co. (Morton Grove, IL) and 1-(3-trifluoromethylphenyl)piperazine (TFMPP) from Research Biochemicals, Inc. (Natick, MA). [*Ethylene*-^{3}H]ketanserin (61.0 to 61.5 Ci/mmol) and [^{3}H]5-HT (24.0 to 30.0 Ci/mmol) were purchased from New England Nuclear (Boston, MA). All other chemicals were reagent grade; apyrase, Grade I from potato, was obtained from the Sigma Chemical Co. (St Louis, MO). Donated drugs were citalopram-HCl (Pfizer, New York, NY), LY-53857 (Eli Lilly, Indianpolis, IN), *d*-LSD (National Institute of Drug Abuse, MD) and ketanserin tartrate (Janssen Pharmaceutica, Piscataway, NJ).

Drug synthesis. 1-(3-Nitrophenyl)piperazine (3-NO_2-PP) was prepared by amino-dehalogenation of bis(chloroethyl)amine with 3-nitroaniline as previously described for this class of compounds [15–17], while the other nitro substituted phenylpiperazines were prepared by nucleophilic substitution using piperazine as the nucleophile and various substituted fluoronitrobenzenes or anilines as the substrates as outlined in Table 1. The nitro phenylpiperazines were reduced to the corresponding amines by catalytic hydrogenation over 65 mg of 10% Pd-C at 50 psi H_2 for 4 hr at room temperature. Purity and structural confirmation were determined by TLC with silica gel G sorbant ($CHCl_3/CH_3OH$, 2:1), ^{1}H-NMR spectroscopy (Varian EM-390 or General Electric QE-300 spectrometer) using Me_4Si as an internal standard, and mass spectroscopy (Finnigan MAT 8230) using perfluorokersene as an internal standard. All spectral data were obtained using free bases prepared from the isolated salts. In each case, the spectral data were consistent with the assigned structure. Formula weights were determined by elemental analyses performed by Atlantic Microlab, Inc. (Atlanta, GA). Determined values were within 0.4% of theoretical. Uncorrected melting points were obtained on a Laboratory Devices Mel-Temp apparatus. The relationship between the parameters associated with hydrophobicity (π^- substituent constant) and polarity (σ^- substituent constant) of the nitro and amino phenylpiperazines described in Table 1 are shown in Fig. 1.

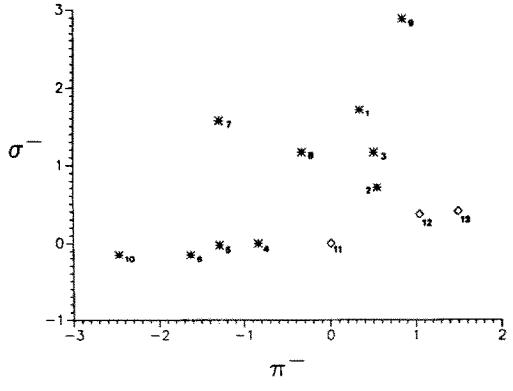

Fig. 1. Two-dimensional plot of σ^- versus π^- for the amino and nitro phenylpiperazines (*), and for PP, TFMPP and mCPP (◇). The numbers beside the symbols refer to the following substituents: (1) 2-NO_2-; (2) 3-NO_2-; (3) 4-NO_2-; (4) 2-NH_2-; (5) 3-NH_2-; (6) 4-NH_2-; (7) 4-NH_2,2-NO_2-; (8) 2-NH_2,4-NO_2-; (9) 2,4-$(NO_2)_2$-; (10) 2,4-$(NH_2)_2$-; (11) H-; (12) 3-Cl-; and (13) 3-CF_3-.

Human platelets. Platelet-rich plasma (PRP) was prepared from venous blood drawn from four healthy volunteers with no history of recent aspirin usage or caffeine consumption and no chronic medication. The first 5 ml was discarded; the remainder was anticoagulated with sodium citrate (3.8%, w/v) and centrifuged at 200 *g* for 5 min at 20°. Platelet-poor plasma (PPP) was prepared from the remaining cells by high speed centrifugation. For aggregation studies, the PRP was supplemented with 1.0 mg/ml glucose, the cell count was adjusted to 250,000 platelets/μl, and the platelets were rotated at 25° in completely filled and sealed containers 30 min prior to use.

For preparation of gel-filtered platelets, PRP was diluted 1:1 with wash buffer (137 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 22 mM citric acid/trisodium citrate, pH 6.3) and centrifuged (570 *g*) at room temperature for 15 min. The packed platelets were resuspended in wash buffer and chro-

matographed on Sepharose 2B-CL (2.8 × 40 cm) equilibrated with 137 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 11 mM glucose, 0.35% (w/v) bovine serum albumin (BSA), 5 units/ml heparin and 0.68 units/ml ATPase (from apyrase), pH 7.0.

Platelet aggregation. Aggregation studies on PRP were performed in silanized glass cuvettes at 37° using a Dual Channel Aggregometer (Payton Scientific Inc., Buffalo, NY). The instrument was calibrated to measure turbidity as percent transmission (% T) (PPP = 100% T, PRP = 0% T). ADP at a concentration of 10 μM caused irreversible aggregation. For each donor on a given day, the concentration of ADP was titrated (0.25 to 5.0 μM) to evoke only a minimal change in turbidity (< 5% of the 10 μM ADP response). 5-HT, in the absence of ADP, had no signicifant effect on aggregation. In the presence of subthreshold concentrations of ADP, 5-HT or a serotonergic agonist amplified the aggregation response. The maximum slope of the change in turbidity (aggregation response) was used to quantitate the amplification. The maximum amplification of ADP-induced aggregation was demonstrated at 1–15 μM 5-HT and 13 μM was used in antagonist studies.

5-HT receptor antagonist studies were performed by preincubating 450 μl PRP with 25 μl antagonist for 4 min at 37° with stirring, followed by the addition of 25 μl of 5-HT/ADP in isotonic saline to give 13 μM 5-HT/0.25 to 1 μM ADP (final concentration). For each antagonist drug study, complete inhibition was defined as the platelet response to the subthreshold concentration of ADP (0.25 to 1.0 μM) without 5-HT. Absence of inhibition was defined by the response to 13 μM 5-HT/0.25 to 1 μM ADP without antagonist. Platelet responses to the various aggregation agents was monitored continuously during the period of experimentation to control for any changes in platelet responsiveness.

All aggregation inhibition studies were performed with a minimum of eight drug concentrations. These studies were repeated on different days for a total of at least three complete concentration curves for each drug studied. Log IC_{50} values for individual experiments were determined by plotting the maximum slope of aggregation versus drug concentration using iterative non-linear regression. Each curve was fit to the equation:

$$Y = A + \frac{B - A}{1 + \frac{(10^X)^D}{(10^C)^D}}$$

where X was the log of the ligand concentration, A was the bottom plateau log concentration, B was the top plateau log concentration, C was the IC_{50} concentration, and D was the slope factor (Hill coefficient). The percent error of the fitted concentration–response curve for individual experiments averaged 2.9%.

[^{3}H]*Ketanserin binding to intact platelets.* Binding assays were performed with 95,000–100,000 gel-filtered platelets/μl in the buffer used for chromatography (volume of 1 ml). Each ligand concentration was studied in triplicate. The platelets were preincubated with citalopram–HCl (2 μM), tetrabenazine (0.7 μM) and phentolamine–HCl (0.5 μM) for 10 min at room temperature to block 5-HT uptake [18] and non-serotonergic ketanserin binding sites [19, 20]. The platelets were then cooled to 0°. [^{3}H]Ketanserin with or without competing drugs was added and the mixture incubated with agitation for 2 hr. This time period was determined to be sufficient for ketanserin binding to reach steady state. Platelets were rapidly rinsed with ice-cold binding buffer (15 ml) on Toyo glass membranes pretreated with 0.5% polyethylenimine to reduce radioligand filter binding [21]. Glass filters were transferred to scintillation vials, solubilized with 10 ml of Cytoscint (ICN Radiochemicals), and analyzed in a Beckman LS-9800 liquid scintillation spectrometer. Saturable binding to platelets was measured using 0.5 to 28 nM [^{3}H]ketanserin. The concentration of ketanserin used in drug competition studies was 2 nM. Either 5 μM ketanserin or 30 μM LY 53857 was used to determine non-specific binding which was approximately 50%. All binding was corrected for ligand binding to the glass filters.

[^{3}H]*5-HT binding to bovine brain membranes.* Bovine cerebral cortex was obtained from freshly slaughtered cows. The frontal cerebral grey matter was carefully dissected from the pial membranes and large blood vessels and homogenized, The membranes were prepared from the homogenate as previously described [22] and stored over liquid N_2. The protein content in the membrane preparations was determined by the amidoschwartz technique [23] using bovine serum alumbin and Serachem (Fisher Scientific, Orangeburg, NY) as standards. Binding assays used 125 μg membrane protein per replicate in 50 mM Tris/HCl, 6.56 mM $CaCl_2$, pH 7.4, containing 10 μM pargyline, 1.0 mM ascorbic acid, and 200 μM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX). [^{3}H]5-HT competition studies were performed using 2.0 nM radioligand in bovine cortical membranes as previously described [22]. Briefly, membranes in binding buffer were incubated with agitation for 15 min at 30°. [^{3}H]5-HT and competing/blanking drug or an equivalent buffer volume were added, and the membranes were incubated for another 20 min at 30° with agitation. The membranes were then washed with 15 ml of ice-cold buffer on pre-rinsed Toyo glass filters. The filters were processed as described for the platelet binding assays above. *d*-LSD (5 μM) was used to define non-specific binding which was approximately 25–30%. Individual compounds were tested in triplicate at sixteen concentrations, a minimum of three times, using at least two separate membrane preparations. The three isomers of NO_2-PP were evaluated in [^{3}H]5-HT competition assays, in the presence and absence of 200 nM ketanserin which blocked 5-HT$_2$ sites.

Binding data analysis. Iterative nonlinear regression (LUNDON 1, Lundon Software Inc., Cleveland, OH) was used for fitting the binding isotherms and deriving the K_D [24] for [^{3}H]ketanserin with intact platelets and for [^{3}H]5-HT with brain membranes. Competition experiments were analyzed using both the LIGAND program [25] adapted to the PC by G. A. McPherson [26] (Ver. 3.0)

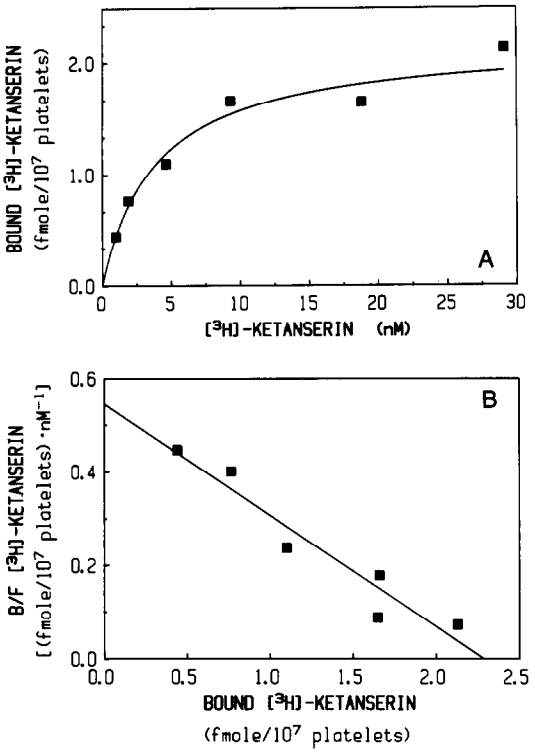

Fig. 2. Representative binding saturation curve (A) and Rosenthal plot (B) of [^{3}H]ketanserin binding to gel-washed, viable human platelets in the presence of 2 μM citalopram, 0.7 μM tetrabenazine and 0.5 μM phentolamine. LY-53857 (30 μM) was used to determine specific binding which was 50%.

and LUNDON 2 (Ver. 2.50). Statistical comparisons between K_i values were performed by Student's unpaired, two-tailed t-tests.

RESULTS

Drug competition studies on intact platelets. Steady-state binding studies with [^{3}H]ketanserin demonstrated saturable binding with a linear Rosenthal plot [27] (Fig. 2). Replicate experiments demonstrated a K_D of 3.4 ± 0.8 nM and a B_{max} of 222 ± 28 fmol/10^9 platelets. [^{3}H]Ketanserin competition assays with LY-53857, mCPP and TFMPP demonstrated log K_i values of -5.28, -4.95 and -4.87 respectively.

In [^{3}H]ketanserin binding competition assays, unsubstituted phenylpiperazine (PP) had the lowest affinity for the platelet 5-HT_2 site while 4-NO_2-PP had the highest affinity (Fig. 3 and Table 2). Substitution of phenylpiperazine with the electron-withdrawing NO_2 group significantly increased affinity at all ring positions in the rank order: 4-NO_2-PP > 3-NO_2-PP > 2-NO_2-PP. The effect of substituent position was most significant when the 3- and 4-positions were compared ($P = 0.02$). In contrast to the nitro group, substitution with the amino group increased affinity only when the amino group was in the 3-position ($P = 0.03$). Substitution at the 2- and 4-positions on the phenyl ring did not have a significant effect. Overall, NO_2 substitution produced greater affinity for the 5-HT_2 site. This effect was most evident when the NO_2 and NH_2 substitutions at the 4-position were compared. The affinities of the 2-NO_2 and 2-NH_2 phenylpiperazines varied less. The nature of the substituent at the 3-position caused only minor variation in affinity.

Platelet aggregation studies. The nitro phenylpiperazine isomers blocked 5-HT amplification of ADP aggregation (Table 2). None of the nitro or amino phenylpiperazines stimulated or inhibited the response to ADP alone. The rank order of 5-HT_2 antagonist potency for the NO_2 substituted compounds was 4-NO_2-PP > 3-NO_2-PP > 2-NO_2-PP > PP. This rank order correlated with the K_i values determined with [^{3}H]ketanserin above.

The NH_2 phenylpiperazines were poor antagonists of 5-HT amplified, ADP-induced platelet aggregation. None of the NH_2 substituted compounds were as effective as the parent compound. These results did not correlate well with the [^{3}H]ketanserin binding competition studies. To address this problem, the effects of incubation time and drug concentration were examined in the aggregation system. As shown in Table 3, the inhibitory potency of each drug at the K_i concentration ([^{3}H]ketanserin displacement) was augmented by increasing the incubation time beyond the usual 4 min. A comparable effect was obtained by increasing the drug concentration. These data demonstrate that the interactions of the NH_2 substituted compounds with the platelet 5-HT receptor during the aggregation assay are governed by complex kinetic parameters under non-steady-state conditions. The standard aggregation assay did not reflect accurately the receptor interactions of the NH_2 phenylpiperazines; however, the apparent rank order of inhibitory potencies after a 10-min incubation was the same as for the K_i values derived from the [^{3}H]ketanserin displacement experiments.

Drug competition studies on cerebral cortex membranes. [^{3}H]5-HT competition studies performed in the presence and absence of 200 nM ketanserin showed that less than 14% of the specific binding in the membrane preparations was attributable to 5-HT_2 sites and that the rank order of the drug affinities was not affected significantly by the presence of ketanserin. All competition data were best fit to a single site model when analyzed by nonlinear regression techniques. In contrast to the 5-HT_2 platelet site, amino substitution on the phenyl ring of the parent piperazine always lowered affinity for the brain 5-HT sites, while nitro substitution raised or lowered affinity according to substituent position (Table 4). The rank order of affinity for the NO_2-isomers was 2-NO_2 > 3-NO_2 > 4-NO_2. The rank order for the NH_2 substituted compounds was 2-NH_2 ≈ 3-NH_2 > 4-NH_2. Compared with the NO_2 phenylpiperazines, the NH_2 substituted compounds bound with significantly lower affinity when the substituent was at the 2-position (7.4-fold decrease) or 4-position (10-fold decrease). The nature of the substituent at the 3-position affected the affinity of the compounds less markedly.

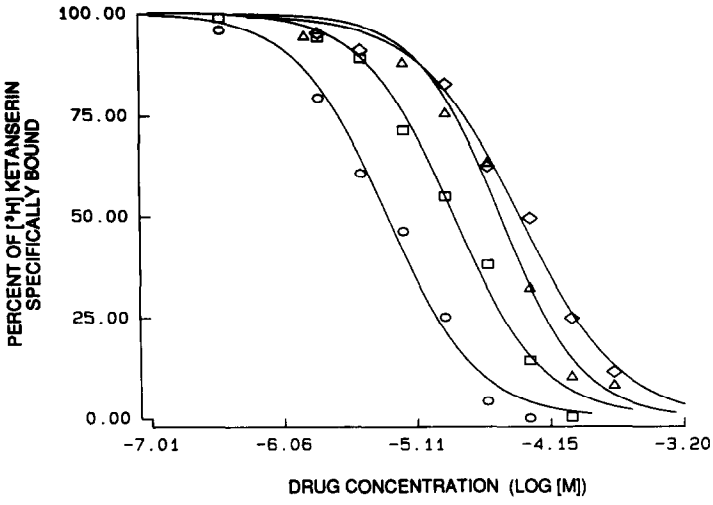

Fig. 3. Displacement of 2 nM [^{3}H]ketanserin from gel-washed viable platelets by 2-NO_2-PP (□), 4-NO_2-PP (○), 4-NH_2-PP (△) and PP (◇). All competition studies were performed in the presence of 2 μM citalopram, 0.7 μM tetrabenazine and 0.5 μM phentolamine. Non-specific binding was defined by 5 μM ketanserin. The displacement curves were generated by the LIGAND program (see Methods).

Table 2. Antagonist activity of the nitro and amino phenylpiperazines on 5-HT amplification of ADP-induced platelet aggregation and on [^{3}H]ketanserin binding to intact platelets

Compound	Aggregation assay IC_{50} [μM]	[^{3}H]Ketanserin binding site K_i [μM]
2-NO_2-PP	4.2 ± 1.5	8.3 ± 2.5
2-NH_2-PP	>100	14.1 ± 0.6
3-NO_2-PP	3.1 ± 2.6	7.2 ± 0.8
3-NH_2-PP	>100	8.1 ± 4.7
4-NO_2-PP	0.22 ± 0.16	3.6 ± 0.5
4-NH_2-PP	>100	18.6 ± 1.8
PP	66 ± 43*	24.5 ± 3.0

The aggregation studies were performed in platelet-rich plasma. Drug competition studies with ≈2 nM [^{3}H]-ketanserin were performed on gel-filtered platelets using LY-53857 (30 μM) to define non-specific binding. All aggregation studies and drug competition experiments were repeated at least three times on different days using three healthy donors. The data are means ± SE.

* Data from Ref. 12.

Table 3. Effects of incubation time and concentration on NH_2 phenylpiperazine inhibition of 5-HT amplified platelet aggregation in platelet-rich plasma

Compound	Percent inhibition at 2 min	Percent inhibition at 10 min
2-NH_2-PP		
14.1 μM	13	30
141 μM	18	55
3-NH_2-PP		
8.1 μM	17	51
81 μM	39	77
4-NH_2-PP		
18.6 μM	2	25
186 μM	38	58

The aggregation response was measured as the maximum slope of the change in turbidity induced by 13 μM 5-HT/5 μM ADP without inhibitors. The platelet-rich plasma was incubated with the inhibitors for 2 min and 10 min instead of the standard 4 min used for the determination of aggregation K_i values.

DISCUSSION

Intact blood platelets represent a unique system to study human 5-HT_2 structure–function relationships since drug binding may be compared with a physiologic response, aggregation, in viable cells. 5-HT causes a pronounced amplification of the aggregation response to subthreshold concentrations of ADP and, under well-controlled conditions, is a consistent way to assess 5-HT_2 receptor activation [1, 12]. In the present investigation, serotonergic drugs were compared in aggregation experiments and in [^{3}H]ketanserin binding competition assays using gel-filtered platelets in a defined buffer system. In contrast to membrane preparations, the gel-filtered platelets have the advantage of intact cells for ensuring ligand interaction with only plasma membrane receptors. Gel-filtration quite effectively removes, with minimal cell damage, sources of potential binding artifacts contributed by the plasma or by soluble, surface associated-proteins, such as fibrinogen. Specific and saturable binding of ketanserin to the washed platelets was demonstrated. The K_D was 3.4 nM and the B_{max} was 222 fmol/10^9 platelets. These values are similar to those reported previously for platelet membrane preparations (K_D of 1.02 nM with a B_{max} of 86 fmol/10^9 platelets) [11]. The slightly higher B_{max} reported here may reflect loss of some serotonin binding activity in the membrane preparation procedure used previously.

A number of studies with platelets, vascular tissue and brain tissue have implicated the existence of

Table 4. Binding affinity constants and comparative affinities of the nitro and amino phenylpiperazines for [^{3}H]5-HT binding sites in bovine cerebral cortex

Compound	K_i (μM)	Relative affinity
2-NO_2-PP	0.53 ± 0.01	3.7
2-NH_2-PP	4.19 ± 0.19	0.5
3-NO_2-PP	1.88 ± 0.08	1.1
3-NH_2-PP	5.37 ± 0.31	0.4
4-NO_2-PP	25.3 ± 0.10	0.1
4-NH_2-PP	145 ± 16	0.01
4-NH_2,2-NO_2-PP	7.3 ± 0.37	0.3
2-NH_2,4-NO_2-PP	102 ± 6	0.02
2,4-$(NO_2)_2$-PP	151 ± 2	0.01
2,4-$(NH_2)_2$-PP	185 ± 7	0.01
PP	2.0 ± 0.2	1.0

The data are the means of computer-fitted K_i values for [^{3}H]5-HT binding sites ± SEM from at least three experiments using different membrane preparations from bovine frontal cortex. [^{3}H]5-HT (2.0 nM) was the radioligand and *d*-LSD (5 μM) was used to define non-specific binding. The relative affinity values were based upon the K_i of phenylpiperazine as 1.0.

ketanserin binding sites distinct from the 5-HT_2 receptor [19, 20, 28, 29]. In the present study, these sites were blocked since phentolamine and tetrabenazine were included in each binding solution. In addition, incubations were performed at 4° to eliminate non-specific trapping of the [^{3}H]ketanserin by the platelet canalicular system. As in all ligand binding studies, the fundamental assumption of this investigation is that binding affinities determined at 4° in a defined buffer system adequately reflect 5-HT_2 receptor interactions of the same compounds at 37° in human plasma. This assumption permits intriguing correlations between receptor binding and physiologic response as determined by platelet aggregation.

In our intact platelet binding studies, the K_i of 4-NO_2-PP (3.6 μM) was comparable to that of the 5-HT_2 antagonist LY-53857 (5.2 μM). Previous studies with rat brain and vascular smooth muscle have demonstrated a higher affinity for LY-53857 at 5-HT_2 receptors [7, 8]. This difference may reflect the dissimilarities of the tissue and/or the assay system. The present studies used washed, intact human platelets at 4° with tetrabenazine and phentolamine as receptor blockers in a modified Tyrode solution containing BSA, apyrase and heparin. Previous binding studies used crude membrane preparations in hypotonic Tris–HCl buffer at 37°, whereas smooth muscle bioassays were performed in modified Krebs' solutions at 37°. The differences in affinity also raise the possibility that the human platelet 5-HT_2 receptor may have pharmacologic properties distinct from 5-HT_2 receptors in other tissues. This, however, cannot be addressed by the present data.

The nitro and amino substituted phenylpiperazines demonstrated affinities for the platelet 5-HT_2 site that were comparable to or greater than that of the parent compound. Compared with 4-NO_2-PP, the affinities of the remaining nitro phenylpiperazines, the amino phenylpiperazines, TFMPP and mCPP were somewhat lower. The reduction in affinity demonstrated by each compound correlated with the distance of the σ^- and π^- coordinates from those of 4-NO_2 in Fig. 1 ($\pi^- = 0.5$, $\sigma^- = 1.17$).

The activities of the nitro phenylpiperazines, phenylpiperazine, TFMPP and mCPP in the platelet aggregation studies were considered with reference to the electronic and hydrophobic constants in Fig. 1. The most potent antagonist was 4-NO_2-PP, consistent with the binding studies described above. As was the case for the binding data, drug potency in the aggregation system decreased (> 400-fold) as a function of distance from the coordinates of 4-NO_2-PP.

For the NO_2-PP isomers, the platelet binding studies at 4° and the aggregation experiments at 37° in plasma permit correlation of the phenylpiperazine structure with both physiologic activity and plasma membrane interactions. For the amino phenylpiperazines, the data from the binding experiments at 4° did not correlate with the results obtained by standard platelet aggregation studies. This discrepancy most likely reflects the different ways in which the two assays measure drug–receptor interactions. Platelet aggregation in response to 5-HT receptor activation is measured by the maximum slope of the turbidity curve and the entire response is completed over several minutes. Therefore, the aggregation assay is strongly dependent on the rate of drug association with receptors, whereas the 4° binding studies reflect drug–receptor interactions under steady-state conditions. The data in Table 3 confirm the importance of kinetic parameters in platelet aggregation studies with the NH_2-PP isomers by demonstrating significant increases in inhibition when the incubation period with the drugs was increased. Thus, the standard aggregation assay may not have reflected accurately receptor interactions with the NH_2 phenylpiperazines because of a relatively slow association rate. An alternative explanation is that the ketanserin binding site in the drug competition assay is not identical with the 5-HT receptor site modulating aggregation; however, the aggregation data in Table 3 make this less likely. Despite the limitations of the standard aggregation studies, the rank order of antagonist potencies in the aggregation assays with 10-min incubations was consistent with the 4° binding data.

Analysis of binding and aggregation data by the use of the σ^- and π^- constants for each compound may be incomplete since these parameters do not account for all electronic and steric effects produced by substitution at the various ring positions. Since structural isomers in the phenylpiperazine series vary so significantly in σ^- or π^- constants, the importance of steric effects on receptor binding and activity could not, with the current series of isomers, be determined independently.

The importance of the 1′-piperazine nitrogen for CNS 5-HT receptor interactions has been postulated previously [30]. In the bioactive coplanar orientation of the phenylpiperazines [31], nitro substitution significantly decreases electron density at the 1′-piperazine nitrogen by both resonance and induction, depending on substituent position. Theoretically, the

inductive effects act at all ring positions; however, resonance interactions occur only at the 4- and 2-positions. Steric hindrance can also play an important role at the 2-position by preventing resonance effects. Therefore, only the 4-position NO_2 will withdraw mesomeric electrons from the 1'-piperazine nitrogen. Our results concerning the potency of 4-NO_2-PP support the idea that a decrease in electron density at the 1'-piperazine nitrogen is an important determinant of phenylpiperazine antagonist activity at the platelet 5-HT$_2$ receptor. Depending on the degree of solvation and protonation, the 4-NH_2 group would either not participate in any resonance effects at the 1'-phenylpiperazine nitrogen or would increase density by electron-donating effects. This is consistent with the reduced binding affinity of 4-NH_2-PP compared with 4-NO_2-PP. The principal inductive effects of the NH_2 group at the 3-position are electron withdrawing [32], and this is most likely why the binding affinity of 3-NH_2-PP is higher than that of phenylpiperazine but lower than that of 4-NO_2-PP or 3-NO_2-PP. Thus, the results of our studies support models of 5-HT$_2$ receptor interactions that stress the importance of the 1'-piperazine nitrogen.

The bovine brain membrane preparations used in this investigation contain serotonin binding sites which are primarily not of the 5-HT$_2$ type. These preparations, therefore, provided an important contrast to the platelet 5-HT$_2$ site. Human frontal cortex was not used since results obtained with this tissue are subject to error introduced by pre-agonal anoxia/ischemia, age and post-mortem storage of tissue. Bovine frontal cortex membrane preparations have less variation in the deterioration of receptor binding than the human preparations (VandenBerg and Gonias, unpublished data). The present data do not, however, provide any definitive information on structure–activity relationships at the pharmacologically distinct subtypes of CNS 5-HT$_1$ and 5-HT$_3$ sites.

Nitro and amino substitution affected the binding of phenylpiperazine to the distinct 5-HT receptor types in the brain membranes and platelets differently. Nitro substitution generally increased affinity at the 5-HT receptors in the brain membranes; however, the rank order was reversed from that at the 5-HT$_2$ receptors in platelets. Thus, mesomeric electron withdrawal effects on the 1'-phenylpiperazine nitrogen were not as significant for the brain 5-HT receptor subtypes. Substitution with NH_2 groups uniformly resulted in decreased binding affinity compared with phenylpiperazine in the brain membranes, a second contrast with the platelet system. These differences emphasize the distinct physicochemical requirements for bioactive antagonist interactions at the 5-HT$_2$ receptor.

Acknowledgements—This work was supported by Grants NS 22455 from the National Institutes of Health, J-80 from the Jeffress Memorial Trust and the Pew Scholars Program in the Biomedical Sciences (S. L. G.). S. R. V. was supported in part by Teacher Investigator Development Award NS 854 from the National Institute of Neurological and Communicative Disorders and Stroke. S. G. B. was supported in part by Graduate Neuropathology Training Grant TS NS 7236 from the National Institute of Neurological and Communicative Disorders and Stroke. The helpful assistance of Dr Michael Kinter (Department of Pathology, University of Virginia Health Sciences Center) with the mass spectroscopy is gratefully acknowledged.

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