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The human serotonin 5-HT2B receptor: pharmacological link between 5-HT2 and 5-HT1D receptors

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Abstract The human serotonin 5-HT2B receptor, isolated from a human liver cDNA library, was transfected in COS-1 cells. Its pharmacological profile shows divergence with serotonin 5-HT2B receptors of other species. In particular, although strong correlation is observed between the human and the rat 5-HT2B receptor pharmacology, the correlation is almost as significant for the mouse 5-HT2B and the human 5-HT1D receptor agonists. The major sites of expression of its mRNA are in the human liver and kidney, with detectable expression in lung and heart. Therefore, this human 5-HT2B receptor could account for functions attributed to the peripheral 5-HT1D/5-HT2-like receptors, especially in the cardiovascular system. Thus, its detailed original pharmacology is of prime importance for therapeutic drug development.

Key words: G protein-coupled receptor; cDNA cloning; Polymerase chain reaction; Cardiovascular system; DOI binding

1. Introduction

The large diversity of functions of the monoamine serotonin (5-hydroxytryptamine, 5-HT) [1] is paralleled by the pharmacological complexity of serotonin receptors. At least four classes have been distinguished pharmacologically: 5-HT1, 5-HT2, 5-HT3, and 5-HT4. These categories are defined by binding and effector coupling properties of the receptors [2,3]. Molecular biological data have confirmed the existence of multiple serotonin receptors, including both ligand-gated receptors (5-HT3) and G protein-coupled receptors (GPRs) [4]. This later class can be split into three classes [5,6], reflecting the second messenger system to which the receptor is coupled: the 5-HT2 subfamily which is coupled to the activation of the phospholipase C, the 5-HT1 family which interacts negatively with the adenylyl cyclase, and the family including 5-HT4, 5-HT6 and 5-HT7 receptor subtypes which interacts positively with the adenylyl cyclase.

5-HT2 receptors mediate many of the central and peripheral physiological functions of serotonin. Cardiovascular effects include contraction of blood vessels and shape change in platelets; central nervous system effects include neuronal sensitisation to tactile stimuli and mediation of hallucinogenic effects of lysergic acid diethylamide and related phenylisopropylamine hallucinogens. Many investigators have observed that 5-HT2A and 5-HT2C receptors do not reflect all the properties attributed to them. For example, some 5-HT2-like effects of serotonin on peripheral smooth muscles are classified as 'atypical', leading to the hypothesis that other 5-HT2 receptor subtypes might exist and differ in different species [7]. We reported previously the cloning of a new 5-HT2 receptor, the mouse 5-HT2B receptor [8]. A similar receptor but with distinct expression and pharmacology has been described in the rat fundus [9,10].

We report here the detailed pharmacological and molecular characterisation of the human 5-HT2B receptor and show that, pharmacologically, it is distantly related to the rat and mouse 5-HT2B receptors, and that its tissue distribution is species specific. Therefore, this new human 5-HT2B receptor may account for atypical peripheral 5-HT1D/5-HT2-like receptor subtypes.

2. Materials and methods

2.1. Drugs and chemicals

Restriction endonucleases, AMV reverse transcriptase, DNA polymerase I, T4 polynucleotide kinase, T4 DNA ligase, T3 or T7 RNA polymerase, were purchased from Bethesda Research Laboratories, New England Biolabs, Boerhinger-Mannheim, and Stratagene. *Taq* polymerase for PCR was purchased from Perkin Elmer-Cetus. Ketanserin, ritanserin and setoperone were kindly provided by Janssen (Beerse, Belgium). ICS 205-930, LY 53857 and MDL 72 222 were gifts from Sandoz (Basel, Switzerland), Lilly (Indianapolis, USA) and Merell-Dow (Strasbourg, France), respectively. Other neurochemicals were from the RBI, or Sigma. ^{32}P- and ^{35}S-labelled nucleotides as well as [^{125}I]2,5-dimethoxy-4-iodophenyl-2-aminopropane ([^{125}I]DOI) [11] (2200 Ci/mmol) were from New England Nuclear.

2.2. Standard molecular biology techniques

Classical published procedures were used for library screening, plasmid subcloning, and COS cell transfections [8]. An hexamer ^{32}P-labelled *Pst*I fragment was used to probe a Northern blot containing human RNA samples (Clontech), treated as recommended by the manufacturer, and exposed for 2 weeks.

2.3. PCR experiments

We synthesised degenerate oligonucleotides coding for the mouse 5-HT2B IIIrd and VIIth transmembrane (TM) domains sequences. 1 μg of human genomic DNA was PCR amplified for 20 cycles with a first two sets of primers. 1/10th of the reaction was re-amplified with a second set of primers for 20 more cycles. Products were used as probes to screen a human lambda GEM genomic library. From the sequence we designed oligonucleotides to PCR-screen several human cDNA libraries.

2.4. [^{125}I]DOI binding assays

[^{125}I]DOI was used as the radioligand to detect expression of the 5-HT2B gene product in membrane fractions isolated from COS-1 cells 48 h after DNA transfection. Briefly, cells were resuspended in cold 4 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 10 mM imidazole buffer pH 7.3 and centrifuged for 10 min at 5,000 × *g*. The resulting superna-

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tant was poured onto a 20% sucrose cushion, and centrifuged 90 min at 100,000 × g. The pellet containing membranes was resuspended in 75 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM imidazole buffer, pH 7.3, and used for binding assays. The incubation medium (200 μl) contained 50 μl of radioligand (0.1–10 nM of [^{125}I]DOI), 50 μl of buffer (50 mM Tris buffer, pH 7.40) alone or containing competing drug, and 100 μl of membrane suspension (protein concentration, 50 μg/ml). The mixture was incubated at 30°C for 30 min. The assay was terminated by addition of ice-cold iso-osmotic solution, and rapid filtration through GF/B filters, followed by 4 washes of 5 ml of ice-cold buffer. Filters were dried rapidly and their radioactivity determined by γ-radiation counting. Non-specific binding, determined in the presence of 10 μM unlabelled DOI, represented about 30% of total binding. Competition studies for [^{125}I]DOI binding were performed by adding increasing concentrations of test drug to the reaction. Up to ten different concentrations of each competing drug were used. Each value, expressed as pK_D (− log mol/l) is the mean of at least 3 independent trial runs in triplicate. Data were analysed using the iterative non-linear regression fitting program LIGAND (Version 3.0; Mc Pershon 1985) and RS1 (Release 4.0).

3. Results

3.1. Sequence comparison of the human 5-HT2B receptor

We used G protein-coupled receptor (GPR) homology at the amino acid level, especially within the TM regions, to generate 5-HT2B-specific probes to screen a human liver cDNA library. The complete cDNA contains an open reading frame of 1443 bp encoding a protein of 481 amino acids with a predicted molecular weight of 54,258 Da. This deduced protein has extensive homology with other members of the GPR family. When compared with other 5-HT2 receptors, the human sequence displays only 79 and 82% identity to the rat and mouse 5-HT2B proteins, respectively. By comparison, the homologies between the 5-HT2A or the 5-HT2C receptors from different species have homologies over 95 and 85%, respectively, and the rat and mouse 5-HT2B have 88% homology over the TM domains. Alignment of the TM region of 5-HT2 receptors places this receptor within the 5-HT2B subfamily [12], being slightly more divergent from 5-HT2A than from 5-HT2C; Homologies reach 71% with the human 5-HT2A receptor and 73% with the human 5-HT2C [13] and are around 70% for the 5-HT2A and 5-HT2C receptors of the same species. In addition, the human 5-HT2B genomic structure includes two introns (Fig. 1), which are present at identical positions to the introns in the 5-HT2A gene [14], and lack the first intron of the 5-HT2C gene [15]. This confirms a probable common evolutionary origin for the 5-HT2 subclass, different from the 5-HT1 family which is devoid of introns.

3.2. Pharmacology of the human 5-HT2B receptor

After introduction of the cDNA coding sequence into the eukaryotic vector pSG5 [16] and transfection of the monkey kidney COS-1 cells, the pharmacological profile of the 5-HT2B receptor was analysed. Steady-state binding assays with [^{125}I]DOI demonstrated that this ligand interacts specifically with the transfected COS-1 membrane preparation (and not to non-transfected cells) with high affinity and in a saturable fashion. The resulting saturation data exhibits a best-fit to a single site mode with an apparent K_d of 11.8 ± 1.6 nM and a B_{max} of 4.6 ± 1.4 pmol of receptor/mg of protein. The K_d values of DOI for the mouse 5-HT2B receptor is 25 nM [8]. Competitive inhibition studies were performed in order to refine the pharmacological profile of this receptor. Inhibition of the [^{125}I]DOI binding by various drugs resulted in monophasic curves best fitted by a one-site binding model. We observed the following rank order of potencies for selected drugs (Table 1): *N*-acetyl 5-HT ≈ pizotifen > 5-HT > ritanserin ≈ 5-MeOH-tryptamine ≈ methiothepine ≈ 5-CT ≈ yohimbine ≈ mesulergine > cyproheptadine ≈ RU24 969 ≈ methysergide ≈ TFMPP > rauwolscine ≈ 2-Me-5-HT > setoperone ≈ α-Me-5-HT ≈ tryptamine > ketanserin ≫ 8-OH-DPAT. The high affinity of this receptor for serotonin would place it in the 5-HT1 family of receptors, despite the low affinity for the 5-HT1A-selective agonist 8-OH-DPAT. In addition the poor competition by the 5-HT3-selective compounds ICS 205-930, MDL 72222, and quipazine rule out the possibility that this belongs to the 5-HT3 family. The receptor affinity for ketanserin is too low for it to be a typical 5-HT2, although the high affinity that it has for the 5-HT2 antagonist pizotifen is a discriminative characteristic of this receptor. We noted the unexpectedly high affinity of compounds such as 5-carboxy-amido-tryptamine (5-CT), yohimbine, and RU 24969, which are typical 5-HT1D receptor ligands. The human 5-HT2B pK_D values were compared with those of other 5-HT receptors (Fig. 2). Using the non-parametric Spearman rank correlation test, we find that correlation with the mouse 5-HT2B ($r = 0.592$, $P < 0.01$, $n = 35$) receptor is significant, although the strongest correlation is obtained for the rat 5-HT2B receptor ($r = 0.864$, $P < 0.005$, $n = 7$; Fig. 2A,B; Table 2). Moreover a significant correlation is observed with the human brain 5-HT1D receptor pharmacology ($r = 0.433$, $P < 0.05$, $n = 24$; Fig. 2C; Table 2), as well as with the human saphenous vein pharmacology values ($r = 0.618$, $P < 0.05$, $n = 11$; Fig. 2D).

3.3. Pattern of 5-HT2B expression in human tissues

In order to have more complete information concerning the in vivo expression of this serotonin receptor, Northern blot was performed (Fig. 3). A single hybridizing mRNA of about 4.5 kb was detected in the human liver. Transcripts were also detectable in lung, heart and kidney mRNA. Fainter signals were observed in the brain and placenta (visible on the original autoradiography). We extended and confirmed these observations by PCR-screening of different human cDNA libraries: the identical 5-HT2B cDNA sequence is also detected in the human spinal cord, foetal brain, aorta, internal mammary artery, placenta, neuroblastoma cell line SHSY-5Y, and CSH carcinoid tumour libraries.

4. Discussion

4.1. The structure of the human 5-HT2B receptor is distantly related to the other 5-HT2B receptors

The comparison of the human, rat and mouse 5-HT2B receptor protein sequences shows less homology than that observed between either the 5-HT2A or 5-HT2C receptors of different species. In addition, conserved DNA sequences outside of the 5-HT2B coding sequence (not shown) may argue in favour of homologous subtypes. These observations are of interest since it is still debatable whether the differences observed between species represent new subtypes or not [17].

As for the other 5-HT2B receptors, consensus sequences for phosphorylation by protein kinases are found in the cytoplasmic regions of the human 5-HT2B, in addition to the presence of 19 Ser or Thr residues within the 96 carboxy-terminal residues (Fig. 1). We observed that, after deletion of the last

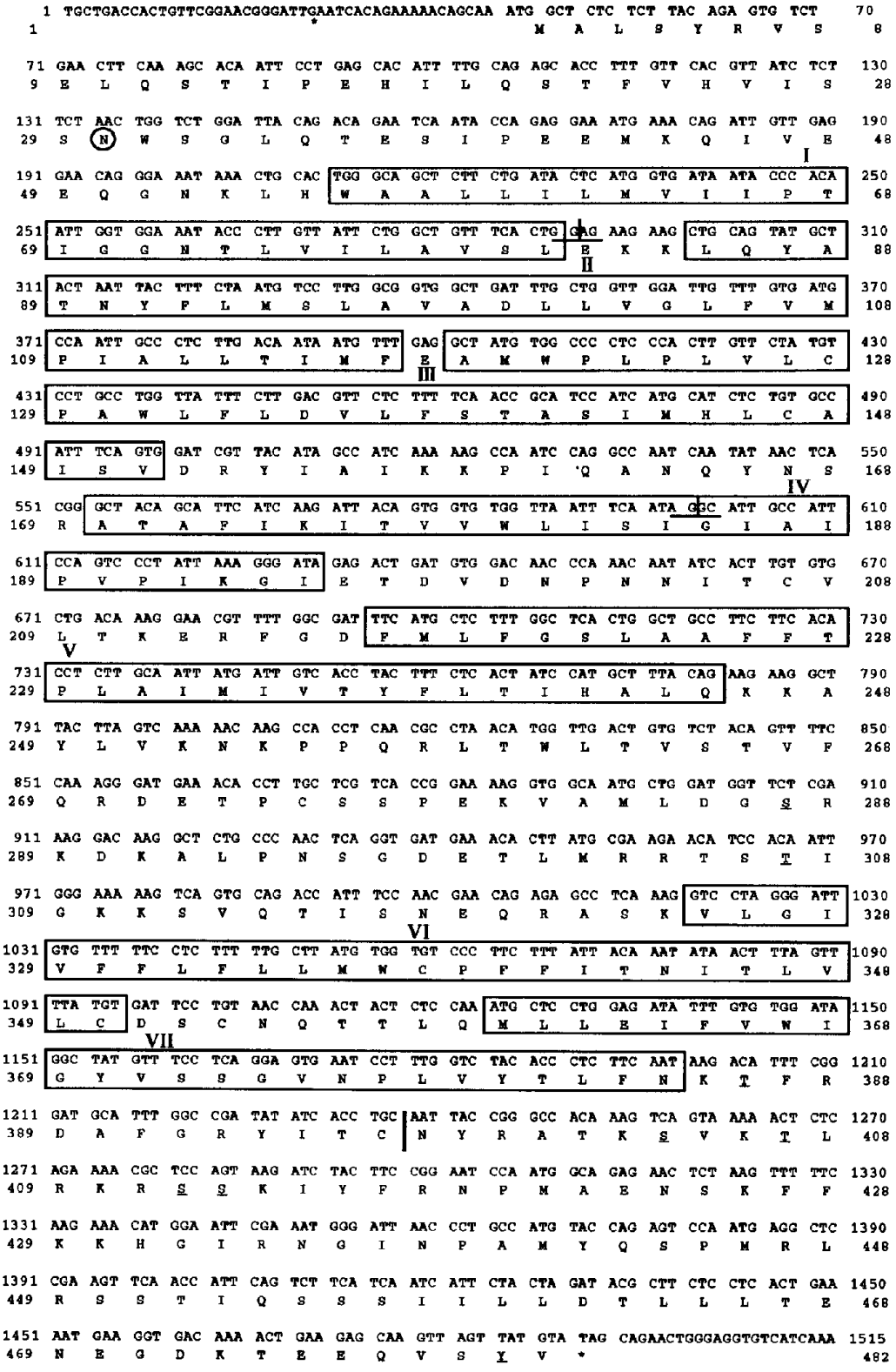


Fig. 1. Sequence of the human 5-HT2B cDNA. The human 5-HT2B cDNA and deduced protein sequences are displayed. Roman numbers over the boxes localise the TM domains. The left numbering is for the DNA sequence on top and for the protein at the bottom. In-frame stop codons are shown by *. The circled N indicates putative N-glycosylation sites. Underlined serines or threonines represent consensus for phosphorylation by protein kinase A and protein kinase C. Intron–exon boundaries are located by vertical and horizontal lines. The thick vertical line locates the C-terminal deletion site.

Table 1
Pharmacology of the human 5-HT2B receptor expressed in COS-1 cells as DOI competition

			pK_D	S.E.M.
Agonists	1	N-Acetyl-5-HT	8.15	0.15
	2	Tryptamine	6.78	0.11
	3	5-CT	7.46	0.14
	4	(±) α-Me-5-HT	6.80	0.10
	5	5-HT	7.89	0.14
	6	1-Me-5-HT	5.75	0.12
	7	5-MeOH-tryptamine	7.59	0.14
	8	8-OH-DPAT	5.46	0.09
	9	Quipazine	6.64	0.11
	10	2-Me-5-HT	7.03	0.06
	11	5-Me-tryptamine	4.70	0.11
	12	NNdiMe-5-MeOH-tryptamine	5.17	0.14
	28	RU 24969	7.25	0.11
Antagonists	13	Ritanserin	7.61	0.08
	14	Pizotifen	8.11	0.06
	15	Methysergide	7.19	0.10
	16	Mesulergine	7.38	0.12
	17	Methiothepine	7.54	0.13
	18	Cyproheptadine	7.26	0.16
	19	Ketanserin	6.42	0.14
	20	Spiperone	4.91	0.15
	21	Setoperone	6.87	0.13
	22	TFMPP	7.16	0.08
	23	Rauwolscine	7.04	0.10
	24	Buspirone	5.79	0.09
	25	Yohimbine	7.41	0.03
	26	Bufotenine	6.25	0.18
	27	ICS 205-930	4.82	0.08
	29	Iodocyanopindolol	4.31	0.22
	30	MDL 72 222	<4	0.17
	31	Mianserin	7.14	0.15
	32	Cis-flupenthixol	6.77	0.08
	33	Haloperidol	4.78	0.17
	34	Clozapine	6.33	0.09
	35	Sulpiride	4.30	0.13

Competition experiment for [^{125}I]DOI-labeled membrane from transiently transfected COS-1 cells. Each value, expressed as pK_D (−log mol/l) is the mean of at least 3 independent trial runs in triplicate. Ten different concentrations of each competing drug were used.

83 amino acids, the pharmacology was not modified (not shown), with a similar K_d for DOI (12.2 ± 1.8 vs. 11.8 ± 1.6 nM) although the B_{max} is raised (8.7 ± 1.7 vs. 4.6 ± 1.4 pmol/mg of protein). This may indicate that the C-terminal region is involved in the receptor desensitisation as demonstrated for the β-adrenergic receptor [18].

4.2. The pharmacology of the human 5-HT2B receptor is distantly related to the other 5-HT2B receptors

Confirming the sequence comparisons, the pharmacology of the human 5-HT2B is different from the pharmacology of other species' 5-HT2B receptor. The high affinity for serotonin discriminates the human 5-HT2B receptor from the mouse (Table 1). The receptor affinity for serotonin, spiperone, and haloperidol is close to the typical 5-HT2C values, although the high affinity that it has for the compounds yohimbine, RU 24969 and rauwolscine gives this human receptor a new original pharmacology (Fig. 2). This would relate this receptor to the 5-HT1B/D subfamily despite its low affinity for the agonist sumatriptan (pK_D = 4.38). As for the mouse 5-HT2B receptor, the LY53857 is non-competitive. Moreover, the pharmacology of the rat 5-HT2B receptor [10,19] does not correlate with that of the mouse (not shown), but is significantly correlated to that of the human 5-HT2B receptor (Fig. 2B). The correlations are even stronger if we separate the antagonists from the agonists (Table 2): the human 5-HT2B pharmacology correlates with the pig 5-HT2C [20] (r = 0.489, P < 0.01, n = 35) or with the antagonist values (r = 0.708, P < 0.01, n = 23) but are not significant for the agonist values (Table 2). The correlation with functional values of the rat fundus contractions [21] is significant (r = 0.723, P < 0.01, n = 14) as well as with those of agonists (r = 0.714, P < 0.05, n = 8) which indicates that the functionality of the 5-HT2B receptor is conserved. In addition, the recently reported human 5-HT2B receptor [22] has an identical sequence and its IP3 coupling values correlate strongly with our binding data (r = 0.964, P < 0.01, n = 7). The correlation with the pharmacology of the human 5-HT1D agonists is striking since the protein receptor sequence is structurally different, despite the possibility that the 5-HT1D receptor interacts with the same IP3 effector coupling [23].

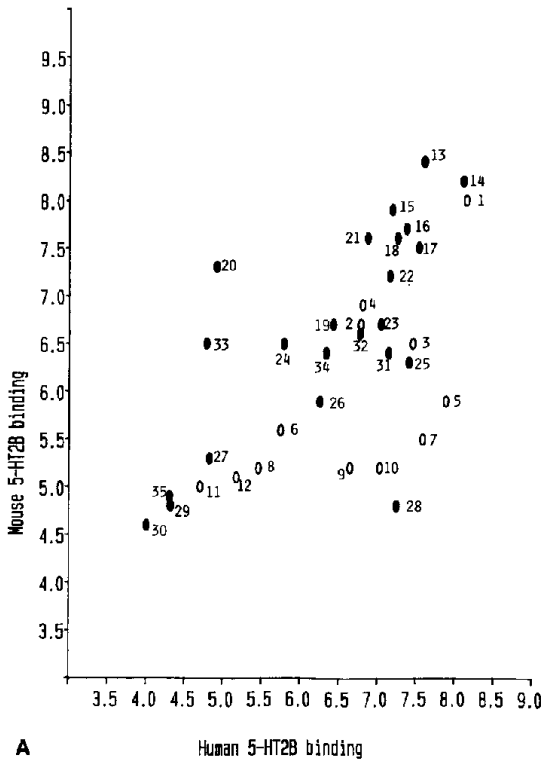
However, sequence comparison between 5-HT2B and 5-HT1B/1D receptors reveals few amino acid differences within the first TM domains, which may be involved in agonist interaction [24,25]. In particluar, in the second TM domain the valine at position 99 in human is present in rat 5-HT2B and in all 5-HT1B sequences but is changed to isoleucine in the mouse 5-HT2B receptor, two amino acids away from the aspartate residue which is believed to interact with the amine residue of serotonin [24,26]. In the first TM domain, the serine at position 80 in human and present in the rat sequence, is changed to alanine in mouse; as well the leucine at position 62 in the human and mouse 5-HT2B sequence is changed to phenylalanine in rat 5-HT2B. More strikingly, the fifth TM domain has five changes between mouse and human 5-HT2B sequence out of which three are common to human and rat and may account for some of the observed differences in pharmacology. Some pharmacological properties of antagonist compounds relate this receptor to the 5-HT1B-1D subfamily. This may correspond to the presence of conserved residues in the human 5-HT2B sequence such as the isoleucine 234 in TM domain V also found in rat 5-HT2B and in all 5-HT1D receptor but not in the mouse 5-HT2B. Moreover, the low affinity of the human 5-HT2B for sumatriptan could be explained by the presence in the VIIth TM domain of a valine 366 instead of the threonine known to interact with this compound in 5-HT1D receptors [27].

4.3. The human 5-HT2B receptor could account for 5-HT1D/5-HT2-like activity in the cardiovascular system

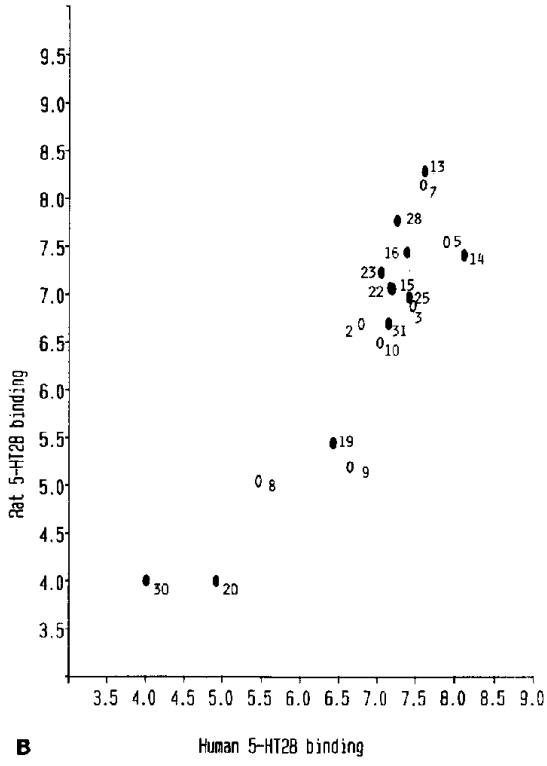
The discrepancy observed between the pharmacology of serotonin receptors in the cardiovascular system [7] may be, at least partially, due to the species divergence, since we have

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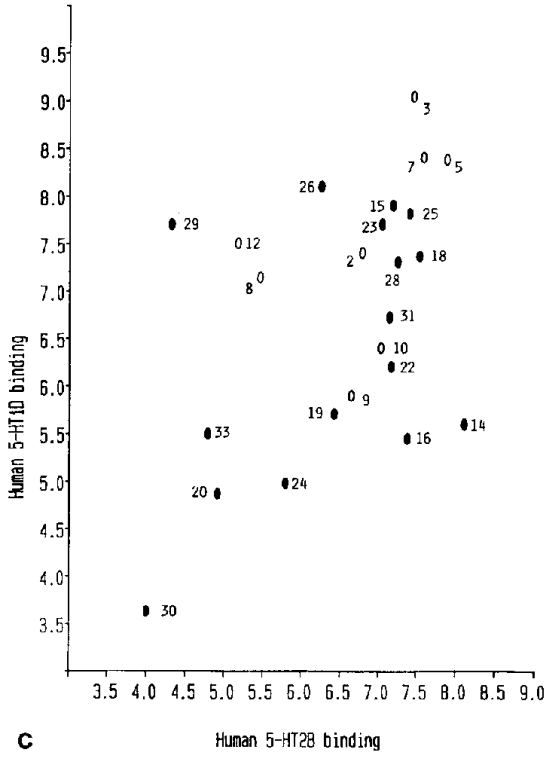
Fig. 2. Comparison of pharmacological properties of human 5-HT2B and various 5-HT receptors. Comparisons have been performed between values for the human 5-HT2B transfected membrane and published values for (A) mouse 5-HT2B [28], (B) rat 5-HT2B [10], (C) human 5-HT1B [32], and (D) human saphenous vein [31,33]. Agonist (open symbols), or antagonist (closed symbols) and numbers are as described in Table 1.


Mouse 5-HT2B binding
Human 5-HT2B binding

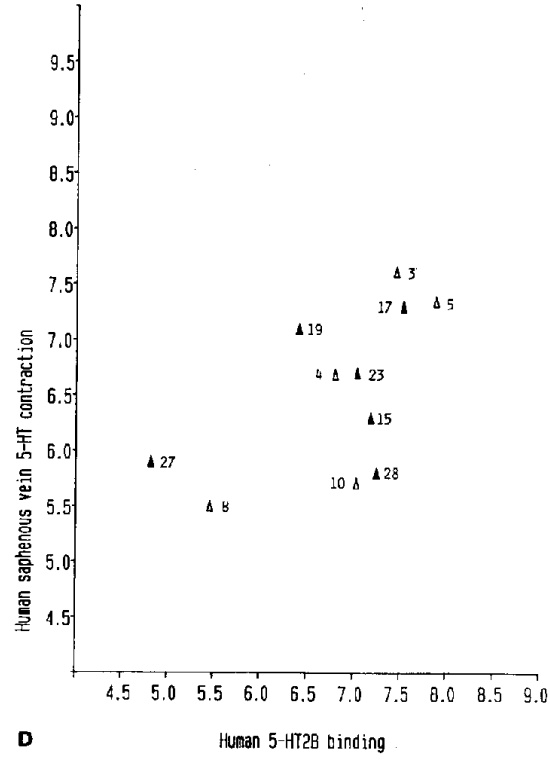
A


Rat 5-HT2B binding
Human 5-HT2B binding

B


Human 5-HT1D binding
Human 5-HT2B binding

C


Human saphenous vein 5-HT contraction
Human 5-HT2B binding

D

Table 2
Spearman correlations between the binding pK_D values of the human 5-HT2B and that of various 5-HT receptors (see text for references)

	Agonist + antagonist	Agonist	Antagonist
5-HT2B hu/5-HT2B:rat	0.864 ($P < 0.005$) ($n = 19$)	0.929 ($P < 0.005$) ($n = 7$)	0.774 ($P < 0.01$) ($n = 12$)
5-HT2B hu/5-HT2B:mouse	0.592 ($P < 0.01$) ($n = 35$)	0.704 ($P < 0.05$) ($n = 12$)	0.667 ($P < 0.01$) ($n = 23$)
5-HT2B hu/5-HT2A:rat	0.202 (ns) ($n = 35$)	0.141 (ns) ($n = 12$)	0.449 (ns) ($n = 23$)
5-HT2B hu/5-HT2C:pig	0.489 ($P < 0.01$) ($n = 35$)	0.147 (ns) ($n = 12$)	0.708 ($P < 0.01$) ($n = 23$)
5-HT2B hu/5-HT1D:human	0.433 ($P < 0.05$) ($n = 24$)	0.651 ($P < 0.05$) ($n = 8$)	0.381 (ns) ($n = 16$)

shown that the mouse 5-HT2B receptor is expressed in the cardiovascular system [8,28], and we also detected the human 5-HT2B mRNA in heart, aorta and internal mammary artery libraries. Several reports have provided evidence that the peripheral action of serotonin cannot be mediated by the known 5-HT2 receptors. Serotonin coronary vasoconstriction has been shown in several species to be mediated by a 5-HT2-like receptor [29], the effects of which are potentiated in coronary artery disease [7]. Serotonin has a direct vasoconstricting effect via 5-HT2-like receptors when the endothelium is damaged (as in coronary artery disease) [30]. More recently, the contractions of isolated human coronary artery have been described to be under the control of a 5-HT2 receptor and of a '5-HT1D-like' receptor [31]. Some of these effects could be mediated by the 5-HT2B receptor since we found it in heart as well as human arteries, and since its pharmacology correlates with that of human saphenous vein.

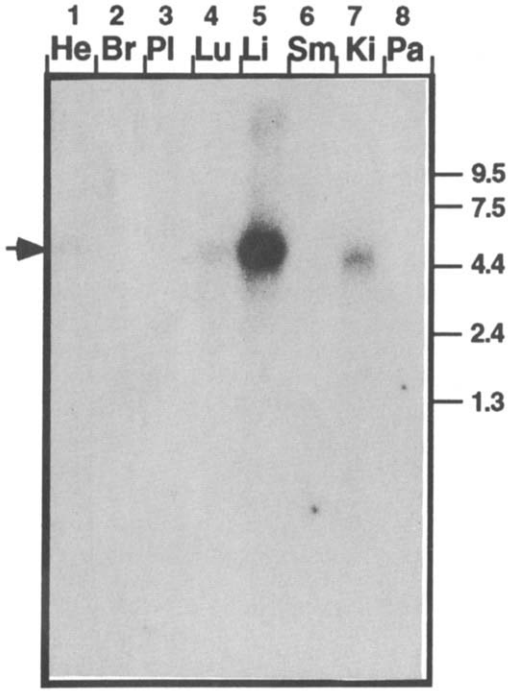


Fig. 3. Human 5-HT2B in vivo expression visualised by Northern blot hybridisation. This Northern blot contains 4 μg of poly(A)$^+$ mRNA from different human tissues (Clontech). After hybridisation with a random primed labelled cDNA probe, it has been exposed for two weeks. Each lane contains the same amount of RNA (not shown). Sizes are determined by RNA size marker (Clontech). Lane 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas.

The implications of this work for drug design are profound: the same receptor protein in various mammalian species differ so greatly in expression sites and in pharmacological properties that a particular non-human receptor may be of very little value in the design of human therapeutic agents.

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