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Short communication

# Pharmacological characterisation of a cloned dog 5-$HT_{1B}$ receptor cell line

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**Abstract**

In this study, the binding of [$^3$H]5-HT to the cloned dog 5-hydroxytryptamine$_{1B}$ (dog 5-$HT_{1B}$) receptor, stably expressed in Chinese hamster ovary cells (ATCC CCL 61) (CHO-K1), was characterised and its pharmacology compared with that of the cloned human and rat 5-$HT_{1B}$ receptors. [$^3$H]5-HT specifically labeled, with high affinity, an apparently homogeneous population of binding sites in the dog 5-$HT_{1B}$ receptor cell line yielding a p$K_d$ of 8.1. [$^3$H]5-HT inhibition and agonist-induced [$^{35}$S] guanosine 5′[γ-thio] triphosphate ([$^{35}$S]GTPγS) binding studies revealed comparable results with the human but not the rat 5-$HT_{1B}$ receptor. In all three recombinant receptor cell lines, methiothepin displayed inverse agonism and GR127935 (*N*-[4-methoxy-3-(4-methyl-1-piperizinyl)phenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazole-3-yl)[1,1′-biphenyl]-carboxamide) weak partial agonism. 

*Keywords:* 5-$HT_{1B}$ receptor, cloned; (Dog); (Human); (Rat)

## 1. Introduction

5-$HT_{1D}$ and 5-$HT_{1B}$ receptors are the most closely related members of the large and diverse 5-HT receptor family sharing an amino acid identity of 61% (human receptors). Despite this similarity, these receptors display an unexpectedly disparate pharmacology in rat and mouse. This is due to the substitution of a threonine in transmembrane domain VII of the human 5-$HT_{1B}$ receptor to an asparagine in the rat 5-$HT_{1B}$ receptor (Oksenberg et al., 1992).

In this study the dog 5-$HT_{1B}$ receptor, stably expressed in Chinese hamster ovary cells (ATCC CCL 61) (CHO-K1), was characterised using [$^3$H]5-HT radioligand and agonist-induced [$^{35}$S]GTPγS binding studies. The profile generated resembled a human-like pharmacology as would be anticipated from its amino acid sequence. This work confirms and extends that reported by Branchek et al. (1995) and Zgombick et al. (1997).

## 2. Materials and methods

### 2.1. Cloning of the dog and human 5-$HT_{1B}$ receptors

The dog 5-$HT_{1B}$ receptor was cloned by Euroscreen (Bruxelles, Belgium) using standard techniques. The resulting amino acid sequence corresponds to that published by Zgombick et al. (1997) for the dog 5-$HT_{1B}$ receptor.

DNA encoding the human 5-$HT_{1B}$ receptor was obtained by polymerase chain reaction techniques using human genomic DNA as a template and primers based on published sequences (Weinshank et al., 1992). Following sequencing, the DNA was subcloned into the mammalian expression vector pcDNA1neo (Invitrogen) for transfection studies.

### 2.2. Stable cell lines

CHO-K1 cells were stably transfected with either dog or human 5-$HT_{1B}$ receptor DNA using a standard calcium phosphate precipitation technique (Cullen, 1987) and selected by resistance to neomycin. Confluent cells were induced in medium containing 2.5 mM sodium butyrate 24 h prior to harvesting in phosphate buffered saline, and stored at −70°C. Human cervix epitheloid carcinoma cells

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(ATCC CCL 2) (HeLa), stably expressing the rat 5-$HT_{1B}$ receptor, obtained from Dr. M.W. Hamblin, University of Washington, USA, were grown to confluence, harvested in phosphate buffered saline and stored at −70°C.

### 2.3. [$^3$H]5-HT radioligand binding studies

[$^3$H]5-HT binding was carried out as previously described by Stanton and Beer (1997). In brief, membranes prepared from transfected and untransfected cells (approximately 100–140 μg protein/tube for the dog and rat receptor cell lines and approximately 180–220 μg protein/tube for the untransfected CHO-K1 cells and the human receptor cell line) were incubated with test drug or assay buffer (50 mM Tris, 0.1% ascorbate, 10 μM pargyline and 4 mM $CaCl_2$, pH 7.7 at room temperature), 2.0 nM [$^3$H]5-HT (inhibition studies) or 0.3–80 nM [$^3$H]5-HT (saturation studies) in a final assay volume of 1 ml, at 37°C in a shaking water bath. 5-HT (10 μM) was used to define nonspecific binding. The reaction was terminated, after 30 min, by rapid filtration over GF/B filters using a Brandel cell harvester.

### 2.4. Agonist-induced [$^{35}$S]GTPγS binding

Agonist-induced [$^{35}$S]GTPγS binding was carried out as previously described by Stanton and Beer (1997). Essentially membranes prepared from transfected and untransfected cells (30–80 μg protein/tube) were incubated with 30 μM GDP and test drug or assay buffer (20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, 0.1% ascorbate and 10 μM pargyline, pH 7.4 at room temperature) for 20 min at 30°C before being transferred to ice for 15 min. [$^{35}$S]GTPγS (100 pM) was added to all tubes (1 ml final assay volume) and incubated for a further 30 min at 30°C before being filtered over GF/B filters using a Brandel cell harvester.

### 2.5. Data analysis

All curves were analysed by nonlinear, least squares regression analysis using an iterative curve fitting routine (Marquardt–Levenberg method) provided by the data manipulation software RS/1 (Software Products, Cambridge MA, USA). Data are expressed as mean ± S.E.M. from at least three experiments.

### 2.6. Materials

5-hydroxy[$^3$H]tryptamine creatinine sulphate (80–120 Ci/mmol) and [$^{35}$S] guanosine 5′[γ-thio] triphosphate ([$^{35}$S]GTPγS) (1000–1500 Ci/mmol) were purchased from Amersham International. Cyproheptadine, 3-tropanyl-3,5,-dihydrochlorobenzoate (MDL 72222), ketanserin, methiothepin, (−)-pindolol, *S*(−)-propranolol, rauwolscine, 1-(2-methoxyphenyl)-4-[4-(2-phthalimido) butyl] piperazine (NAN 190), (±)-8-hydroxy-dipropylamino-tetralin (8-OH-DPAT), mesulergine, spiperone and 5-methoxytryptamine were purchased from RBI. GDP, GTP, ATP, noradrenaline, 5-HT and dopamine were purchased from Sigma. 5-methoxy-3-(1,2,3,6-tetrahydropyridin-4-yl)-1*H*-indole (RU24969), metergoline, cyanopindolol and 3-[3-(2-dimethylaminoethyl)-1*H*-indol-5-yl]-*N*-(4-methoxybenzyl)acrylamide (GR46611) were purchased from Tocris. Anpirtoline was purchased from ICN. Guanosine 5′-O (3-thiotriphosphate) (GTPγS) was purchased from Boehringer Mannheim. 5-carboxamidotryptamine (5-CT), 3-1,2,5,6-tetrahydropyrid-4-yl)-pyrrolo[3,2-b]pyrid-5-one (CP 93,129), *N*-methyl-4[5-(1,2,4-triazol-4-yl)-1*H*-indol-3-yl] piperidine (L-741,519), *N*,*N*-dimethyl-2-[5-(1,2,4-triazol-4-yl)-1*H*-indol-3-yl] ethylamine (L-741,604), 3-(*S*-[*N*-benzyl)aminomethyl]-1-[2-(5-(1,2,4-triazol-4-yl)-1*H*-

Table 1
A comparison of [$^3$H]5-HT binding affinities ($pK_i \pm$ S.E.M.) for compounds at the cloned dog, human and rat 5-$HT_{1B}$ receptors

| Compound | Dog 5-$HT_{1B}$ | Human 5-$HT_{1B}$ | Rat 5-$HT_{1B}$ |
|---|---|---|---|
| L-694,247 | 9.71 ± 0.14 | 9.50 ± 0.17 | 10.02 ± 0.11 |
| GR46611 | 9.33 ± 0.07 | 9.12 ± 0.05 | 9.64 ± 0.09 |
| 5-CT | 8.90 ± 0.08 | 8.81 ± 0.03 | 8.94 ± 0.13 |
| L-741,604 | 8.87 ± 0.06 | 9.02 ± 0.06 | 7.91 ± 0.06 |
| L-741,519 | 8.83 ± 0.06 | 8.67 ± 0.13 | 7.78 ± 0.04 |
| GR127935 | 8.71 ± 0.06 | 8.95 ± 0.09 | 8.78 ± 0.11 |
| Zolmitriptan | 8.62 ± 0.03 | 8.38 ± 0.09 | 7.74 ± 0.16 |
| Metergoline | 8.46 ± 0.08 | 8.58 ± 0.07 | 8.33 ± 0.14 |
| 5-HT | 8.42 ± 0.03 | 8.45 ± 0.08 | 8.56 ± 0.08 |
| 5-Methoxytryptamine | 8.20 ± 0.05 | 8.15 ± 0.11 | 8.24 ± 0.12 |
| CP 122,288 | 8.13 ± 0.14 | 8.31 ± 0.14 | 6.81 ± 0.09 |
| Sumatriptan | 8.02 ± 0.03 | 8.13 ± 0.07 | 7.29 ± 0.12 |
| L-760,790 | 7.95 ± 0.05 | 7.47 ± 0.01 | 7.53 ± 0.12 |
| L-762,183 | 7.94 ± 0.15 | 7.70 ± 0.15 | 6.69 ± 0.05 |
| RU24969 | 7.79 ± 0.13 | 7.81 ± 0.05 | 9.38 ± 0.03 |
| Methiothepin | 7.67 ± 0.09 | 7.55 ± 0.16 | 7.21 ± 0.12 |
| Anpirtoline | 7.45 ± 0.07 | 7.47 ± 0.02 | 7.74 ± 0.04 |
| L-775,606 | 7.30 ± 0.02 | 7.29 ± 0.07 | 6.10 ± 0.08 |
| Rauwolscine | 7.28 ± 0.03 | 7.32 ± 0.10 | 5.86 ± 0.13 |
| Cyanopindolol | 6.91 ± 0.05 | 6.98 ± 0.05 | 9.00 ± 0.06 |
| 8-OH-DPAT | 6.42 ± 0.09 | 6.63 ± 0.08 | 5.12 ± 0.04 |
| Cyproheptadine | 6.34 ± 0.11 | 6.45 ± 0.13 | 5.16 ± 0.06 |
| CP 93,129 | 6.09 ± 0.03 | 6.35 ± 0.04 | 8.12 ± 0.03 |
| NAN 190 | 6.06 ± 0.10 | 6.05 ± 0.05 | 5.91 ± 0.12 |
| Mesulergine | 5.66 ± 0.05 | 5.85 ± 0.11 | 5.43 ± 0.13 |
| (−)-Propranolol | 5.36 ± 0.05 | 5.64 ± 0.06 | 7.71 ± 0.08 |
| (−)-Pindolol | 5.18 ± 0.04 | < 5.1 | 7.57 ± 0.05 |
| Dopamine | < 5.1 | < 5.1 | 5.15 ± 0.01 |
| MDL 72222 | < 5.1 | < 5.1 | < 5.1 |
| Spiperone | < 5.1 | < 5.1 | < 5.1 |
| Ketanserin | < 5.1 | < 5.1 | < 5.1 |
| Noradrenaline | < 5.1 | < 5.1 | < 5.1 |

Affinity ($pK_i$) values for compounds at the [$^3$H]5-HT recognition site using cloned dog, human and rat 5-$HT_{1B}$ receptor expressing cell lines. Results are arithmetic means ± S.E.M. of ≥ 3 experiments. Data yielded inhibition curves which were fitted best by a one site model ($P > 0.05$, partial *F*-test).

indol-3-yl)ethyl] pyrrolidine (L-760,790), 1-{3-[5-(1,2,4-triazol-4-yl)-1*H*-indol-3-yl]propyl}-4-(hydroxy)-4-(benzyl) piperidine (L-762,183), 2-[5-[3-(4-methylsulphonylamino)-benzyl-1,2,4-oxadiazol-5-yl]-1*H*-indole-3-yl]ethylamine (L-694,247), 1-(3-[5-(1,2,4-Triazol-4-yl)-1*H*-indol-3-yl]propyl)-4-(2-(3-fluorophenyl)ethyl) piperazine (L-775,606), 5-methylaminosulphonylmethyl-3-(*N*-methoxy-pyrrolidin-2*R*-yl-methyl)-1*H*-indole (CP 122,288), *N*-[4-methoxy-3-(4-methyl-1-piperizinyl)phenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazole-3-yl)[1,1′-biphenyl]-carboxamide (GR127935), *S*-4[[3-[2-(dimethylamino)ethyl]-1*H*-indol-5-yl]methyl]-2-oxazolidinone (zolmitriptan) and sumatriptan were synthesised at MSD.

## 3. Results

### 3.1. [$^3$H]5-HT radioligand binding studies

Approximately 990, 650 and 700 fmol of [$^3$H]5-HT were bound, per mg protein, in inhibition, and 120–5400, 80–5000 and 150–3600 fmol in saturation studies using the dog, human and rat 5-HT$_{1B}$ receptor expressing cell line membranes, respectively. No specific [$^3$H]5-HT binding was detected in untransfected CHO-K1 cells ($n = 3$, data not shown).

Binding with [$^3$H]5-HT was saturable and labeled, with high affinity, a homogeneous population of binding sites in all three cell lines. CHO-K1 cells stably expressing dog 5-HT$_{1B}$ receptors yielded a p$K_d$ of $8.09 \pm 0.11$ and a $B_{max}$ of $4400 \pm 700$ fmol/mg protein (mean $\pm$ S.E.M., $n = 4$). This compared to p$K_d$ values of $8.31 \pm 0.05$ and $8.13 \pm 0.05$ and $B_{max}$ values of $3700 \pm 1500$ and $2800 \pm 500$ fmol/mg protein (mean $\pm$ S.E.M., $n = 3$) for the human and rat 5-HT$_{1B}$ receptor expressing cell lines, respectively.

The p$K_i$ values for 26 compounds are given in Table 1.

The p$K_i$ values for compounds at the dog 5-HT$_{1B}$ receptor are in excellent agreement with those generated for the human 5-HT$_{1B}$ receptor (correlation coefficient ($r$) = 0.99, $P = 0.0001$) but not for those obtained with the rat 5-HT$_{1B}$ receptor ($r = 0.59$, $P = 0.0013$). As expected there was a poor correlation between p$K_i$ values generated for the human vs. the rat 5-HT$_{1B}$ receptor ($r = 0.65$, $P = 0.0004$).

GDP, GTP and GTPγS, but not ATP, inhibited [$^3$H]5-HT specific binding from all three cell lines with the following rank order of potency: GTPγS > GTP ≥ GDP ($n = 3$, data not shown).

### 3.2. Agonist-induced [$^{35}$S]GTPγS binding studies

Basal [$^{35}$S]GTPγS binding levels were approximately 91, 52, 69 fmol/mg protein for the dog, human and rat 5-HT$_{1B}$ receptor cell lines, respectively. 5-HT caused a dose-dependent increase in [$^{35}$S]GTPγS binding yielding maximal responses of $255 \pm 33$, $295 \pm 34$ and $128 \pm 9$% over basal for the dog, human and rat 5-HT$_{1B}$ receptor cell lines, respectively (mean $\pm$ S.E.M., $n \geq 9$). Ten compounds, with potencies ranging over four orders of magnitude, displayed full agonism, with respect to 5-HT, in all three cell lines. The p$D_2$ values for these full agonists at the dog and human 5-HT$_{1B}$ receptors yielded a correlation coefficient of 0.99 ($P = 0.0001$) in contrast with the rat

Table 2
Potency and efficacy values for compounds using agonist-induced [$^{35}$S]GTPγS binding to cloned dog, human and rat 5-HT$_{1B}$ receptors

| Compound | Dog 5-HT$_{1B}$ | | Human 5-HT$_{1B}$ | | Rat 5-HT$_{1B}$ | |
|---|---|---|---|---|---|---|
| | p$D_2$ ± S.E.M. | $E_{max}$ ± S.E.M. | p$D_2$ ± S.E.M. | $E_{max}$ ± S.E.M. | p$D_2$ ± S.E.M. | $E_{max}$ ± S.E.M. |
| 5-HT | 7.90 ± 0.04 | 100 | 7.74 ± 0.02 | 100 | 8.01 ± 0.08 | 100 |
| L-694,247 | 9.28 ± 0.08 | 103 ± 3 | 9.12 ± 0.07 | 104 ± 1 | 9.74 ± 0.14 | 102 ± 2 |
| GR46611 | 9.12 ± 0.10 | 102 ± 1 | 9.11 ± 0.12 | 101 ± 2 | 8.77 ± 0.10 | 105 ± 4 |
| 5-CT | 8.33 ± 0.07 | 105 ± 2 | 8.28 ± 0.08 | 106 ± 2 | 8.37 ± 0.12 | 103 ± 6 |
| L-741,604 | 8.16 ± 0.03 | 106 ± 2 | 7.99 ± 0.12 | 102 ± 3 | 7.23 ± 0.08 | 102 ± 6 |
| L-741,519 | 7.95 ± 0.05 | 96 ± 2 | 7.79 ± 0.07 | 90 ± 1 | 7.05 ± 0.12 | 96 ± 4 |
| Zolmitriptan | 7.80 ± 0.08 | 101 ± 2 | 7.30 ± 0.06 | 106 ± 3 | 6.75 ± 0.05 | 111 ± 5 |
| Sumatriptan | 7.22 ± 0.06 | 106 ± 3 | 6.95 ± 0.05 | 105 ± 1 | 6.55 ± 0.21 | 112 ± 5 |
| RU24969 | 7.20 ± 0.07 | 87 ± 2 | 7.18 ± 0.18 | 82 ± 5 | 8.64 ± 0.07 | 105 ± 10 |
| CP 122,288 | 7.10 ± 0.05 | 96 ± 5 | 6.94 ± 0.21 | 90 ± 3 | 5.85 ± 0.09 | 92 ± 5 |
| L-760,790 | 6.65 ± 0.08 | 107 ± 4 | 6.37 ± 0.13 | 109 ± 5 | 6.69 ± 0.09 | 105 ± 4 |
| L-762,183 | 7.09 ± 0.06 | 81 ± 4 | 7.11 ± 0.19 | 75 ± 5 | 6.15 ± 0.06 | 70 ± 5 |
| L-775,606 | 6.44 ± 0.09 | 78 ± 3 | 6.37 ± 0.09 | 71 ± 4 | 5.13 ± 0.28 | 57 ± 10 |
| GR127935 | 8.82 ± 0.07 | 29 ± 3 | 9.08 ± 0.23 | 23 ± 4 | 9.48 ± 0.04 | 34 ± 1 |
| Methiothepin | – | 22 ± 6[a] | – | 13 ± 2[a] | – | 29 ± 7[a] |

Potency (p$D_2$) and relative efficacy values ($E_{max}$) for compounds in the agonist-induced [$^{35}$S]GTPγS binding assay using cloned dog, human and rat 5-HT$_{1B}$ receptor expressing cell lines. Efficacy values ($E_{max}$) are the maximal stimulation achieved expressed as a percentage of the maximal 5-HT response. Results are arithmetic means ± S.E.M. of ≥ 3 experiments.

[a] Percent inhibition of basal response.

receptor ($r = 0.74$ and 0.78, $P = 0.0094$ and 0.0046 with dog and human, respectively).

L-762,183, L-775,606 and GR127935 had lower efficacies than 5-HT. Methiothepin displayed inverse agonism. Potency ($pD_2$) and efficacy ($E_{max}$) values are given in Table 2. The $pK_i$ values generated are generally higher than the corresponding $pD_2$ values. This is probably due to the use of an agonist radioligand which measures binding at the high affinity recognition site whereas the functional measure, carried out in the presence of GDP, monitors activity at the lower affinity state.

No 5-HT-induced [$^{35}$S]GTPγS binding was seen in untransfected CHO-K1 cells ($n = 3$, data not shown).

## 4. Discussion

In this study the dog 5-HT$_{1B}$ receptor, stably expressed in CHO-K1 cells, was characterised and compared with the human and rat 5-HT$_{1B}$ receptors using radioligand binding and functional studies. [$^3$H]5-HT labeled, with high affinity, a homogenous population of sites at the dog ($pK_d$ = 8.09), human ($pK_d = 8.31$) and rat ($pK_d = 8.13$) 5-HT$_{1B}$ receptors.

[$^3$H]5-HT binding in all three recombinant receptor cell lines was guanine nucleotide sensitive (GTPγS > GTP ≥ GDP) and ATP insensitive indicating inhibition of radioligand from the high affinity state of the receptor.

Endogenous 5-HT$_{1B}$ receptors have been demonstrated in CHO-K1 cells using inhibitory adenylyl cyclase assays (Giles et al., 1996). However, both ourselves (this study), and others (Giles et al., 1996), have been unable to detect their presence either by radioligand binding or agonist-induced [$^{35}$S]GTPγS binding presumably due to their low abundance.

The affinities of 26 compounds were determined at all three cell lines. The dog vs. human 5-HT$_{1B}$ receptor $pK_i$ values yielded a correlation coefficient of 0.99. When either the dog or human $pK_i$ values were compared with those of the rat, however, lower correlation coefficients were obtained (0.59 and 0.65, respectively). CP 122,288, sumatriptan, rauwolscine and cyproheptadine as well as the triazol substituted indoles L-741,604, L-741,519, L-762,183 and L-775,606 displayed higher affinities for the dog and human compared with the rat 5-HT$_{1B}$ receptor. Conversely, CP 93,129, cyanopindolol, (−)-pindolol, (−)-propranolol and RU24969 yielded higher affinities at the rat 5-HT$_{1B}$ receptor. Indeed, it is now well established that the rat receptor displays its unexpected pharmacology due to the substitution of a threonine in transmembrane domain VII to an asparagine (Oksenberg et al., 1992), which is similarly located in the 5-HT$_{1A}$ receptor, and which may hydrogen bond with the oxygen atom directly linked to the aromatic ring of aryloxyalkylamines (Kobilka et al., 1988).

The functionality of 15 compounds was investigated in all three cell lines utilising the agonist-induced [$^{35}$S]GTPγS binding assay. The rank order of potencies obtained were the same for the dog and human but not the rat 5-HT$_{1B}$ receptor reflecting the radioligand binding studies. Ten compounds, with potencies ranging over four orders of magnitude, displayed full agonism, with respect to 5-HT, in all three recombinant receptor cell lines. The piperidine L-762,183 and piperazine L-775,606 were less efficacious in all three cell lines with $E_{max}$ values ranging from 57 to 81%. Since the potency: affinity ratios for these compounds are constant in all three cell lines these results reflect differences in compound efficacy rather than in receptor reserve. The 5-HT$_{1B/1D}$ receptor 'antagonist' GR127935 was a weak partial agonist yielding $E_{max}$ values of 29, 23 and 34% at the dog, human and rat 5-HT$_{1B}$ receptor cell lines, respectively, again indicating similar levels of receptor reserve, with respect to this compound. Pauwels et al. (1997) have also reported weak partial agonism with this compound at human 5-HT$_{1B}$ receptors. Conversely the 5-HT$_{1/2}$ receptor 'antagonist' methiothepin displayed inverse agonism, inhibiting basal [$^{35}$S]GTPγS binding in all three species. This seems to be a characteristic of this compound in several 5-HT recombinant receptor cell lines, e.g., human 5-HT$_{1A}$ (Stanton and Beer, 1997) and human 5-HT$_{1B/1D}$ (Pauwels et al., 1997). The enhanced resolution seen with recombinant receptor cell lines might result in a reclassification of compounds previously thought of as lacking intrinsic activity.

This study, has demonstrated that the dog 5-HT$_{1B}$ receptor displays a similar pharmacology to that of the human 5-HT$_{1B}$ receptor. The improved resolution of the agonist-induced [$^{35}$S]GTPγS binding assay may lead to a reassessment of agonist intrinsic activities.

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