BIOCHEMICAL PHARMACOLOGY 75 (2008) 2007–2019

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Molecular cloning and pharmacological characterization of rat melatonin MT_1 and MT_2 receptors

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ARTICLE INFO

Article history:
Received 9 January 2008
Accepted 14 February 2008

Keywords:
Melatonin
Melatonin receptors
Selective agonists
Selective antagonists
Rat

ABSTRACT

In order to interpret the effects of melatonin ligands in rats, we need to determine their activity at the receptor subtype level in the corresponding species. Thus, the rat melatonin rMT_1 receptor was cloned using DNA fragments for exon 1 and 2 amplified from rat genomic DNA followed by screening of a rat genomic library for the full length exon sequences. The rat rMT_2 receptor subtype was cloned in a similar manner with the exception of exon 1 which was identified by screening a rat genomic library with exon 1 of the human hMT_2 receptor. The coding region of these receptors translates proteins of 353 and 364 amino acids, respectively, for rMT_1 and rMT_2. A 55% homology was observed between both rat isoforms. The entire contiguous rat MT_1 and MT_2 receptor coding sequences were cloned, stably expressed in CHO cells and characterized in binding assay using 2-[^{125}I]-Iodomelatonin. The dissociation constants (K_d) for rMT_1 and rMT_2 were 42 and 130 pM, respectively. Chemically diverse compounds previously characterized at human MT_1 and MT_2 receptors were evaluated at rMT_1 and rMT_2 receptors, for their binding affinity and functionality in [^{35}S]-GTPγS binding assay. Some, but not all, compounds shared a similar binding affinity and functionality at both rat and human corresponding subtypes. A different pharmacological profile of the MT_1 subtype has also been observed previously between human and ovine species. These *in vitro* results obtained with the rat melatonin receptors are thus of importance to understand the physiological roles of each subtype in animal models.

1. Introduction

The indole hormone melatonin was first discovered in the bovine pineal gland [1], and was subsequently found in a large variety of vertebrates species [2]. Melatonin is synthesized and released in a circadian fashion by the pineal gland and has been shown to play a modulatory role in diverse physiological functions including circadian entrainment, reproduction, sleep and blood pressure [2–4]. Two mammalian receptors have been cloned. The MT_1 and MT_2 receptors are G-protein coupled receptors that exhibit sub-nanomolar affinity for melatonin [5–7]. A third melatonin binding site named MT_3 has

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0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.02.022

a 10 nM range affinity for melatonin and was described in hamster as the human homologue of the cytoplasmic quinone reductase 2 [8,9]. In order to assign specific roles for each subtype, pharmacological tools are needed [10]. There are only a few ligands selective for one melatonin receptor subtype: Luzindole, 4P-PDOT, S 24773 and S 24014 are selective for the MT_2 melatonin receptor subtype [11–16], whereas S 26131 and S 26284 are selective for MT_1 [17]. We have recently published the characterization of several different compounds distinguishing human MT_1 and MT_2 receptors expressed in CHO cells [17]. All these affinity data have been obtained on human receptors whereas *in vivo* experiments are usually performed on rats. We wondered whether the data found on human recombinant receptors could be predictive or not to that at the rat recombinant receptors. No systematic survey or characterization of melatonin binding at recombinant receptors from different species is available yet, apart from our work on human and ovine species [17,18]. Since there is yet no published data describing the rat melatonin receptors either at the molecular level or at the pharmacological level, we undertook this study in which we systematically compared the rat and human MT_1 and MT_2 receptors.

2. Methods

2.1. Reagents and ligands

The two radioligands (2-[^{125}I]-iodomelatonin (specific activity: 2000 Ci/mmol) and [^{35}S]-GTPγS (Guanosine-5′[γ-^{35}S]-triphosphate; specific activity: 1000 Ci/mmol) were purchased from Perkin-Elmer. Melatonin and 2-iodomelatonin were obtained from Sigma and 4P-PDOT (4-phenyl-2-propionamidotetraline), luzindole (2-benzyl-*N*-acetyltryptamine), 6-chloromelatonin from Tocris. Fourteen analogues of melatonin were evaluated (Fig. 1). Their synthesis were reported in the following reports, mostly patents: S 20098 (*N*-[2-(7-methoxynapht-1-yl)ethyl]acetamide) by Yous et al. [19] and Depreux et al. [20], S 20928 (*N*-[2-(1-naphtyl)ethyl]cyclobutanecarboxamide) by Leclerc et al. [21], S 22971 (*N*-[2-(5-ethyl-1-benzofuran-3-yl)ethyl]acetamide) by Lesieur et al. [22], S 25150 (*N*-[2-(8,9-dihydro-7H-furo[3,2-*f*]chromen-1-yl)ethyl]-2-iodoacetamide) by Lesieur et al. [23], S 24014 (*N*-[2-(2-(3-methoxybenzyl)5-methoxybenzo(*b*)furan-3-yl)ethyl]acetamide) by Wallez et al. [16], S 23219 (*N*-methyl-[4-(2,3-dihydro-1,4-benzodioxin-5-yl)]butanamide) by Charton et al. [24], S 24268 (*N*-[2-(7-{8-[2-(acetylamino)ethyl]-2-naphtyl}-1-naphtyl)ethyl]acetamide), S 26284, *N*-(2-{7-[4-({8-[2-acetylamino)ethyl]-2-naphtyl}oxy) butoxy]-1-naphtyl}ethyl)acetamide) by Descamps-François et al. [25], S 25567 ((*R*,*S*)*N*-[2-(6-hexyloxy-3,4 dihydro-2H-1-benzopyran-4-yl)ethyl]acetamide) by Guillaumet et al. [26], S 23950 (*N*-[2-(7-methoxy-3-phenyl-1-naphthyl)ethyl]acetamide), S 24601 (*N*-{2-[3-(3-iodophenyl)-7-methoxy-1-naphtyl]ethyl}acetamide) and S 24773 (*N*-{2-[3-(3-aminophenyl)-7-methoxy-1-naphthyl]ethyl}acetamide) by Lefoulon et al. [27], S 22701 (4-methoxy-2-propionamido-2,3-dihydro-1*H*-phenalene) by Mathé-Allainmat et al. [28]. All compound structures are presented in Fig. 1. Compounds were dissolved in DMSO at a stock concentration of 10 mM and stored at −20 °C. They were diluted extemporaneously in the assay buffer. Lipofectamine and pcDNA3.1 expression vector were purchased from Invitogen. All other reagents were from Sigma.

2.2. Cloning of rat melatonin receptors

2.2.1. Cloning rat MT_1 receptor sequence

Degenerate primers based on mouse and human MT_1 receptors were designed to amplify a 128 bp fragment of exon 1 using rat genomic DNA as template; forward primer 5′-GGRMGRVVRCGRCCSTCSTGG; reverse primer 5′-TTRTT-CTTCGAGTCCTTGMGT. PCR conditions were 94 °C 1 min, 50 °C 30 s and 72 °C for 60 s for 35 cycles using native Taq polymerase (Promega, UK). The amplified DNA fragment was cloned in to pBluescript (Stratagene, UK) and sequenced to confirm exon 1. The PCR fragment was used to screen a rat genomic DNA library in the vector λ DashII (Wistar, Male liver—Stratagene, UK) by Southern hybridization on plaque lifts. From one hybridizing clone a 3 kb EcoRI/XbaI fragment was identified as containing exon 1 plus 5′ upstream sequence. This 3 kb fragment was clone into bluescript and sequenced to confirm the presence of the entire exon 1. Exon 2 was cloned in a similar manner to exon 1. Primers were designed to amplify bases 30–466 of the partial rat DNA mt1 receptor sequence (Genebank Accession number U14409); forward primer 5′-TAGGATATACAGTAACAACAAT; reverse primer 5′-AGTAAC-TAGCCACGAAGAGC. PCR conditions for amplification were 35 cycles of 94 °C—60 s; 50 °C—90 s; 72 °C—60 s. The resultant DNA fragment was used to screen the Wistar genomic library by Southern hybridization on plaque lifts. From one hybridizing clone a 2 kb SpeI fragment was isolated, cloned into bluescript and sequence to verify the presence of the entire region of exon 2. With the sequence of the entire MT_1 receptor known, primers were designed to amplify the entire coding region from −57 to +1104 using RNA prepared from rat brain; forward primer 5′-GCGCGGGGCTACAGGATGAT; reverse primer 5′-ACCCCAACCAGCGAGCGTAAC. PCR amplification conditions were 94 °C—30 s; 52 °C—90 s; 72 °C—90 s for 40 cycles using native Taq DNA polymerase. The resultant PCR product was purified and cleaved with AatII. A 324 bp fragment containing exon 1 and 70 bp of exon 2 was isolated by agarose gel electrophoresis and ligated into SmaI/AatII linearised pBluescript containing exon 2 (Fig. 2). The ligated exon 1 was sequenced to check for sequence errors due to amplification but none were found. To add a FLAG epitope sequence (DYKDDDDK) was added to the C-terminal end of the MT_1 sequence, two complementary oligonucleotides were designed to provide the additional sequence plus suitable restriction enzyme sites for ligation; upper oligo 5′-ACTCTGTTGGTACCGACTACAAGGACGACGATGACAAGTAAT; lower complementary oligo 5′-CTAGATTACTTGTCATCG-TCGTCCTTGTAGTCGGTACCAACAG. Oligos were heated to 100 °C in 0.3 M NaCl, 10 mM Tris–HCl solution pH 7.6 and allowed to cool at 1 °C/min. A SalI/HinfI fragment containing the all but 9 bp at the 3′ end of the MT_1 sequence (stop codon inclusive) was ligated with Sal1/XbaI cut pBKCMV expression vector (Stratagene, UK) and annealed oligonucleotide linker in a ratio of 1:3:30 and transformed into XL-Blue bacterial cells (Stratagene, UK). One clone was identified as containing the full length sequence plus FLAG epitope and was subject to sequencing to verify no mutations had been introduced.

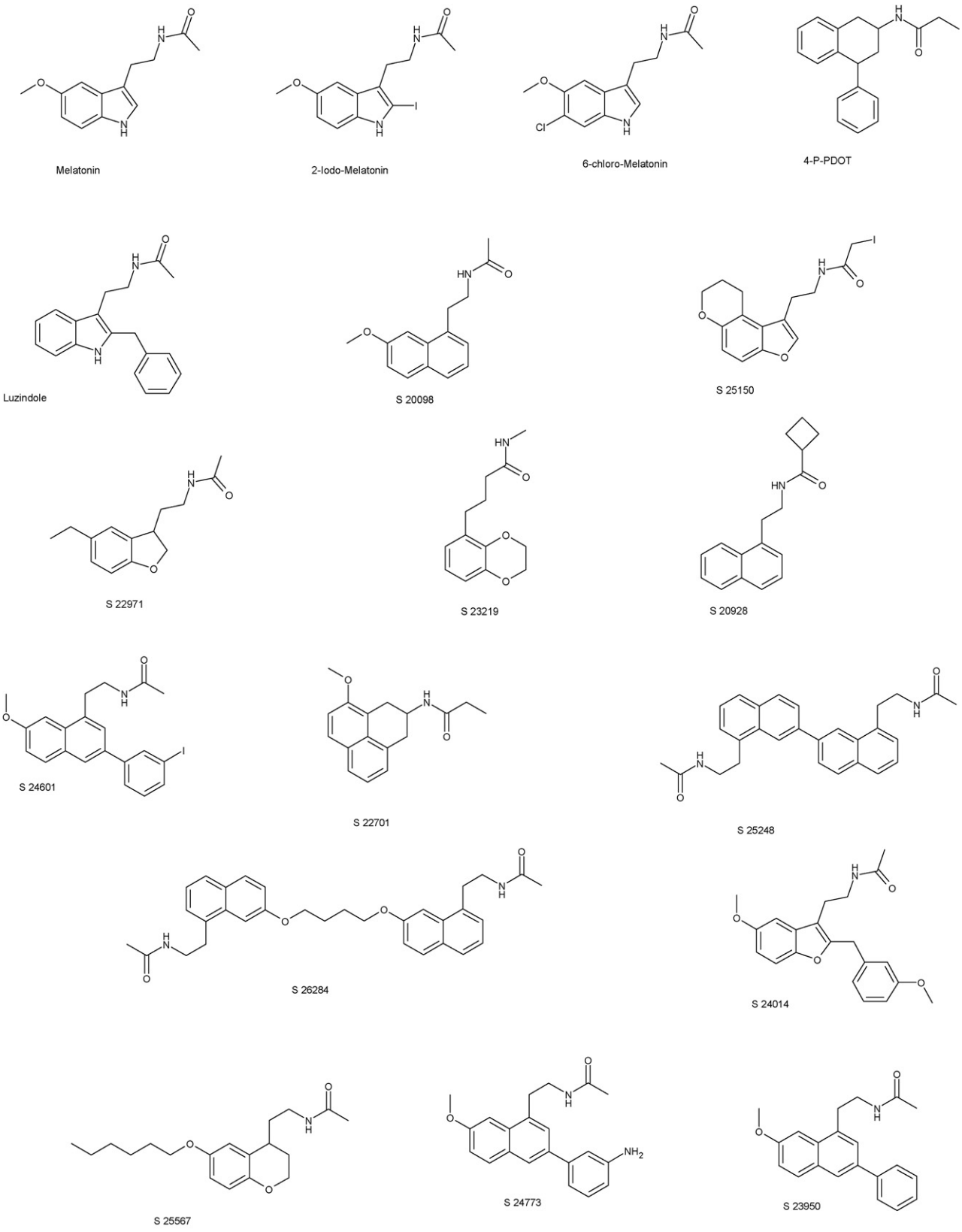

Fig. 1 – Structures of the melatoninergic compounds tested.

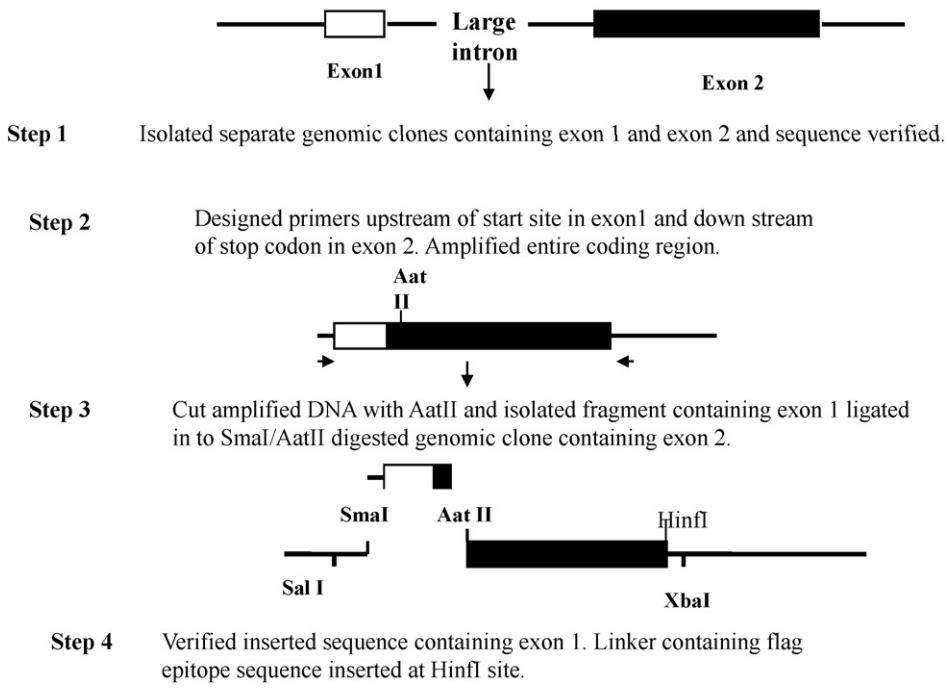

Fig. 2 – Summary outline of the steps in the cloning of rat MT_1 receptor. Exons 1 and 2 were cloned independently from a genomic DNA library and the sequence of each exon was determined. Subsequent PCR amplification of the full sequence from rat brain cDNA was made. The AatII enzyme was identified as a unique site that could be utilized to obtain a fragment of the PCR amplified DNA for ligation with the genomic clone for exon 2.

2.2.2. *Cloning rat MT_2 sequence: exon 1*

A 226 bp DNA fragment of exon 1 of the human MT_2 melatonin receptor (−13 to +212 bp relative to the coding sequence) was amplified from a cloned full length human MT_2 sequence (Accession Number NM_005959.3) with the forward primer: 5′-GGGAGAGTCGCGATGTCA and the reverse primer: 5′-GAGCTTGCGGTTCCTGAG. This fragment was used to screen the rat genomic library at reduced stringency, hybridizing at 60 °C and with a final wash of nylon membranes in 0.2× SSC (1× SSC is 0.15 M NaCl, 15 mM sodium citrate pH 7.0) at 55 °C. Positive clones from this screening were isolated. A 3 kb BamHI fragment was subcloned into pBluescript and sequenced. This was found to contain the entire region of exon 1.

2.2.3. *Cloning rat MT_2 sequence: exon 2*

PCR primers based on a partial rat MT_2 exon 2 sequence (Genbank U28218) were used to amplify a DNA fragment from rat genomic DNA (forward primer: 5′-CTCCCCTCTACATCAGCCTCATCT; reverse primer: 5′-TCAGGAGCCCGTAGACAATAGCAT). This fragment was cloned into pGEM-T (Promega, UK) and verified by sequencing. The PCR fragment was used to screen the rat genomic library. Several positive clones were identified, but only one was used to obtain the full length sequence. Two 3 kb DNA fragments were required to obtain a full length sequence. First a 3 kb BamHI was subcloned in to pBluescript and sequenced (subclone 1). This was found to contain all but 170 bp of the 3′ region of exon 2. The remaining sequence was obtained by subcloning a 3 kb XbaI/SacI fragment (subclone 2). In order to facilitate the addition of the flag sequence at the 3′ end of the receptor, a portion of exon 2 from the XbaI site to the 3′ end of the coding sequence was amplified by PCR with Pfu polymerase (Stratagene, UK). This was achieved by amplifying from sublcone 2 using a primer for the vector T7 sequence and a primer at the 3′ end of the coding sequence in exon 2. The reverse oligonucleotide (5-CC*GGTACCAGA*GAGCACCTTCCTGGACA) spans the last 6 amino acids of the receptor (underlined) and incorportates a KpnI site (italics) for cloning and in frame ligation to a pre-constructed pcDNA3 expression vector containing the FLAG epitope sequence. The PCR fragment was cloned into the XbaI (coding sequence)/Kpn I (vector) site of subclone 1 and sequenced to check no PCR errors had occurred.

2.2.4. *Cloning rat MT_2 sequence: joining exon 1 and 2*

Pfu polymerase was used to amplify exon 1 with the following primers: 5′ primer, extending 18 bp 5′ to the initiation codon and containing a PstI site CCGGGCTGCAGCGTCACCAT; 3′ primer CTGCGTTCCGCAGCTTGCGGTTCCTGA, this primer starts at the first base of the glycine residue at the 3′ border of exon 1. This primer was phosphorylated for the purposes of ligation. Exon 2 was amplified using Pfu polymerase with the following primers: 5′ primer starting at the second base of the glycine at the 5′ border of exon 2 and was phosphorylated for subsequent ligation GTAATTTATTTGTGGTGAATCTGGCCTTGGCTGACCTGG; 3′ primer used was as for the amplification of the XbaI/KpnI PCR fragment above. The resultant PCR amplified exons were then subject to digestion with PstI or KpnI as appropriate and purified on a Qiaquick PCR clean up spin column. These fragments were then combined with PstI/

KpnI cut pBluescript and ligated. A full length clone was obtained and fully sequenced to check no errors were introduced in this procedure. Finally a NheI/KpnI fragment containing the entire sequence of MT_2 was subcloned into the SpeI/KpnI site of the pcDNA3.1-FLAG expression vector.

2.3. Stable CHO cell lines

CHO cells from the European Collection of Animal Cell Cultures, grown in HamF12 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin were seeded at 5×10^6 cells in a T75 cm^2 culture flask. Twenty four hours later, they were transfected with 10 μg of the pcDNA3.1 containing one of the rat melatonin receptor using 30 μl of lipofectamine. The day following transfection, cells were trypsinized, suspended in complete HamF12 medium containing 800 μg/ml of active geneticin and seeded at different dilutions in 96 well plates which were kept during 2–3 weeks in an humidified CO_2 incubator. At the end of this selection period, isolated clones were picked up, amplified and further characterized by binding experiments, in particular, B_{max}'s were measured at this stage. For each cell line, one positive clone was subcloned in limited dilution before being used for binding experiments.

2.4. Membrane preparations

CHO cell lines stably expressing each melatonin receptor were grown at confluence, harvested in phosphate buffer containing 2 mM EDTA and centrifuged at 1000 × *g* for 5 min (4 °C). The resulting pellet was suspended in 5 mM Tris/HCl, pH 7.4, containing 2 mM EDTA, and homogenized using a Kinematica polytron. The homogenate was then centrifuged (20,000 × g, 30 min, 4 °C), and the resulting pellet was suspended in 75 mM Tris/HCl, pH 7.4, containing 2 mM EDTA and 12.5 mM $MgCl_2$. Determination of protein content was performed according to Bradford [29] using the Biorad kit (Bio-Rad SA, Ivry-sur-Seine, France). Aliquots of membrane preparations were stored in binding buffer (Tris/HCl 50 mM, pH 7.4, 5 mM $MgCl_2$) at −80 °C until use.

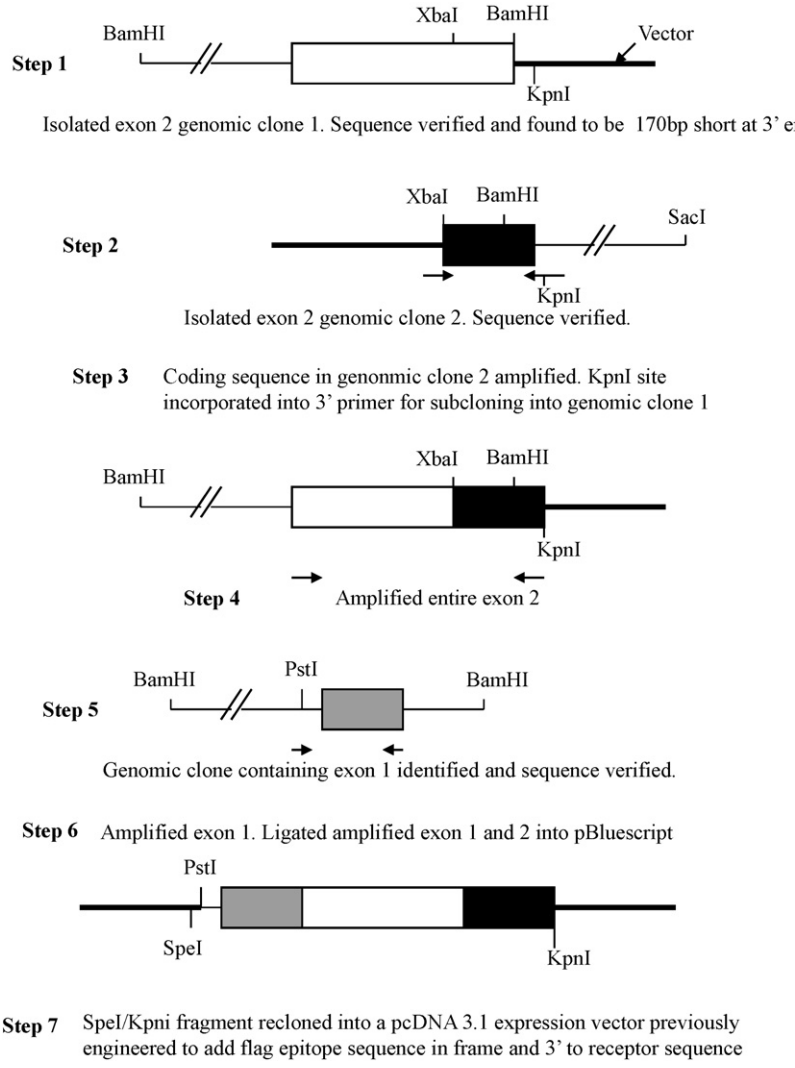

Fig. 3 – Summary outline of the steps in the cloning of rat MT_2 receptor. Exons 1 and 2 were cloned independently from a genomic DNA library and the sequence of each exon was determined. Details of the steps to achieving a full length MT_2 clone can be found in Section 2.

2.5. 2-[^{125}I]-Melatonin binding assay

Membranes were incubated for 2 h at 37 °C in binding buffer (Tris/HCl 50 mM, pH 7.4, 5 mM $MgCl_2$) in a final volume of 250 μl containing 2-[^{125}I]-melatonin 20 pM for competition experiments. The results were expressed as K_i, therefore taking into account the concentration of radioligand used in each experiments. Non-specific binding was defined with 1 μM melatonin. Reaction was stopped by rapid filtration through GF/B unifilters, followed by three successive washes with ice-cold buffer. Data were analysed by using the program PRISM (GraphPad Software Inc., San Diego, CA). The density of binding sites B_{max} and the dissociation constant of the radioligand (K_D) values were calculated using non-linear regression model. For competition experiments, inhibition constants (K_i) were calculated according to the Cheng-Prussof equation: $K_i = IC_{50}/[1 + (L/K_D)]$, where IC_{50} is the Inhibitory Concentration 50% and L is the concentration of 2-[^{125}I]-iodomelatonin [30]. For the correlation analysis of pK_i values, Pearson Product-Moment Correlation Coefficient was employed. All the data showed on human recombinant receptors were obtained either in a previous report [17] or as previously described in this report [17].

2.6. [^{35}S]-GTPγS binding assay

Membranes and drugs were diluted in binding buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 3 mM $MgCl_2$, 3 μM, GDP). For agonist tests, incubation was started by the addition of 0.1 nM [^{35}S]-GTPγS to membranes and ligands, and carried on for 60 min at room temperature in a final volume of 250 μl. To test for antagonist activity, membranes were pre-incubated for 30 min with melatonin (30 nM or 3 nM for rMT_1 and rMT_2 receptors, respectively) and concentration of the tested compound. Reaction was started by the addition of 0.1 nM [^{35}S]-GTPγS and followed by 60 min incubations. Non specific binding was assessed using non radiolabeled GTPγS (10 μM). All reactions were stopped by rapid filtration through GF/B unifilters pre-soaked with distilled water, followed by three successive washes with ice-cold buffer. Data were analysed by using the program PRISM to yield EC_{50} (Effective Concentration 50%) and E_{max} (maximal effect) for agonists. Antagonist potencies were expressed as K_B with $K_B = IC_{50}/1+([ago]/EC_{50}ago)$, where IC_{50} is the inhibitory concentration of antagonist that gives 50% inhibition of [$_{35}$S]-GTPγS binding in the presence of a fixed concentration of agonist ([ago]) and $EC_{50}ago$ is the EC_{50} of the agonist when tested alone [30].

3. Results and discussion

3.1. Rat melatonin receptors

Due to limited distribution and low abundance, rat MT_1 and MT_2 melatonin receptors were cloned using a strategy of screening a rat genomic library for exon 1 and exon 2 followed by ligation of appropriate exons to complete a full length receptor clone (Figs. 2 and 3). Our cloned full length rat MT_1 receptor comprises 353 amino acid in length and found to be identical to a predicted rat MT_1 receptor (Genbank number XP_341442) except for 1 amino acid at position 270, (T270A) located in the 2nd external loop of the receptor. The cloned rat MT_2 receptor is 364 amino acids in length and found to be 100% identical with a predicted sequence for the rat MT_2 receptor (Genbank number XP_345900). Comparing the MT_1 receptor with cloned MT_1 receptors of other species, reveals overall identities of 84% to human, 79.2% to ovine and 84% to mouse MT_1 receptors. The rat MT_2 receptor shows overall identity of 78.2% with human and 88.5% with mouse MT_2 receptors. Transient transfection in Cos 7 cells with preliminary binding studies in the presence of 100 pM ^{125}I-melatonin, demonstrated that both clones encoded for functional melatonin receptors and showed a high level of expression (data not shown).

The rat MT_1 receptor was cloned from genomic DNA and the full length sequence was amplified from whole brain cDNA, confirming the presence of this receptor in rat brain tissue. The rat MT_2 receptor was cloned from genomic DNA only. The full length receptor was not amplified from whole rat brain cDNA. This probably reflects the lower abundance of MT_2 receptors, but may also be exacerbated by a difficulty in one or more steps in the cDNA synthesis and PCR reactions. With the exception of one amino acid difference for MT_1, both receptors were identical to sequences in the Genbank database for predicted rat MT_1 and MT_2 melatonin receptor

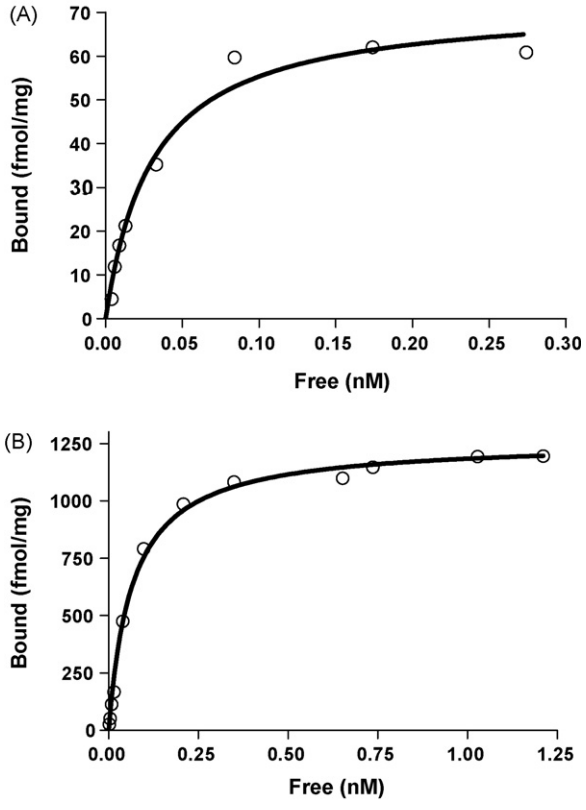

Fig. 4 – Saturation binding experiments of 2-[^{125}I]-iodomelatonin at membranes from CHO cells expressing rMT_1 (A) or rMT_2 (B) receptors. Specific binding is represented. Points shown are from representative experiments performed in triplicates and repeated four times.

Table 1 – Binding affinities (K_D) and levels of receptor expression (B_{max}) in CHO cells transfected with rat or human MT_1 or MT_2 receptor subtypes

	$K_D \pm$ S.E.M. (nM)	$B_{max} \pm$ S.E.M. (fmol/mg)
rMT1	0.042 ± 0.006 (*n* = 5)	85.4 ± 12.1 (*n* = 5)
hMT1	0.021 ± 0.003 (*n* = 4)	435 ± 192 (*n* = 4)
rMT2	0.13 ± 0.032 (*n* = 4)	1485 ± 342 (*n* = 4)
hMT2	0.107 ± 0.011 (*n* = 4)	2656 ± 282 (*n* = 4)

Note: K_D and B_{max} were obtained on the selected clones either from human origin (as described by Audinot et al. [17] or from rat origin, as described in Section 2. Those data were obtained independently, in the same, comparable daily experiments, at 4–5 occasions. Data are mean ± S.E.M., with the number of experiments between parentheses.

sequences. The one amino acid change in MT_1 probably reflects a polymorphism that can be found in melatonin receptors of other species [31,32]. The cloned rat MT_1 and MT_2 receptor have high homology with respective receptors of other species (84% for MT_1 and 78.2% for MT_2 compared to human MT_1 and MT_2, respectively). This includes amino acids that have been shown to be involved in ligand binding, including two serine residues in transmembrane domain 3 (positions 113/117, MT_1 and 123/127, MT_2 [33]) and a valine and histidine residues in transmembrane domain 5 (positions 195/198, MT_1 and 205/208, MT_2 [34]). In addition a glycine residue in transmembrane domain 6 (position 261, MT_1 and 271, MT_2) is conserved. Site directed mutagenesis studies show that this amino acid may be important for facilitating access of melatonin to the binding pocket of the receptor [35]. However, despite these conservations and high amino acid identity between the melatonin receptor subtypes of each species, the present study shows difference in the pharmacological profile between species for both receptor subtypes which confirms a previous study comparing the human and the ovine MT_1 subtype [18].

3.2. *Saturation assays on rat melatonin receptors*

As pointed out before, we checked the absence of melatonin binding capacity in naïve cells. Two clonal CHO cell lines, expressing either rMT_1 or rMT_2 receptors and presenting selective 2-[^{125}I]-iodomelatonin binding were established. On these cell lines, saturation binding experiments were monophasic (Fig. 4) and gave K_D values of 42 ± 6 pM (MT_1) and 130 ± 32 pM (MT_2). These values compare well with that of their human counterparts hMT_1 and hMT_2: 21 ± 3 pM and 107 ± 11 pM, respectively. For the rMT_1 and rMT_2 receptors, the levels of receptor expression (B_{max}) were 85 ± 12 and 1485 ± 342 fmol/mg. This compares with 435 ± 192 and 2656 ± 282 fmol/mg for hMT_1 and hMT_2 receptors, respectively (see Table 1 for direct comparison).

The pharmacological characterization of rat melatonin MT_1 and MT_2 receptors was performed using the same set of molecules, with minor changes, than those used in previous studies in which the pharmacological profile of both human MT_1 receptor subtypes was compared either to the human MT_2 subtype or to the ovine MT_1 receptor [17,18].

Table 2 – Binding affinities at rat MT_1 and MT_2 receptors and comparison with the data obtained at hMT1 and hMT2 receptors

	rMT_1, $K_i \pm$ S.E.M.	rMT_2, $K_i \pm$ S.E.M.	Rat/human*, MT_1/MT_2 ratio	
Reference compounds				
Melatonin	0.37 ± 0.06	0.77 ± 0.16	0.29	0.63
2-Iodomelatonin	0.026 ± 0.005	0.1 ± 0.02	0.26	0.22
Chloromelatonin	1.3 ± 0.43	6.1 ± 1.4	0.21	6.8
4P-PDOT	344 ± 136	36.2 ± 2.2	9.5	126
Luzindole	292 ± 3	337 ± 129	0.87	10
Non selective				
S 22971	14.4 ± 1.9	96.6 ± 23	0.15	1.76
S 20098	2.42 ± 0.72	1.1 ± 0.48	1.1	0.83
S 23219	34.5 ± 11.6	46.3 ± 3.3	0.74	0.33
S 25150	31.7 ± 3.4	1.17 ± 0.32	27.1	0.88
S 20928	327 ± 59	523 ± 61	0.62	5.06
S 22701	11.7 ± 1.7	4 ± 1.5	2.9	8.8
hMT1 selective				
S 24268	1901 ± 411	1090 ± 147	1.74	0.02
S 26284	33.5 ± 6.4	269 ± 72	0.12	0.02
S 25567	36 ± 10.2	160 ± 9.1	0.22	0.16
hMT2 and rMT2 selective				
S 24014	169 ± 49.7	9.18 ± 2	18.4	108
S 24773	188 ± 17.7	5.5 ± 1.2	34.2	109
S 23950	81.9 ± 34	13.6 ± 3.7	68.3	68
S 24601	152 ± 2.6	1.8 ± 0.5	84.4	28

Note: Concentration–response curves were analyzed by non-linear regression. Binding affinities (nM) are expressed as mean $K_i \pm$ S.E.M of at least three independent experiments. The selectivity ratio between hMT_1 and hMT_2 receptors is calculated for each cell line. *Data are from Audinot et al. [17] (except for S 22701).

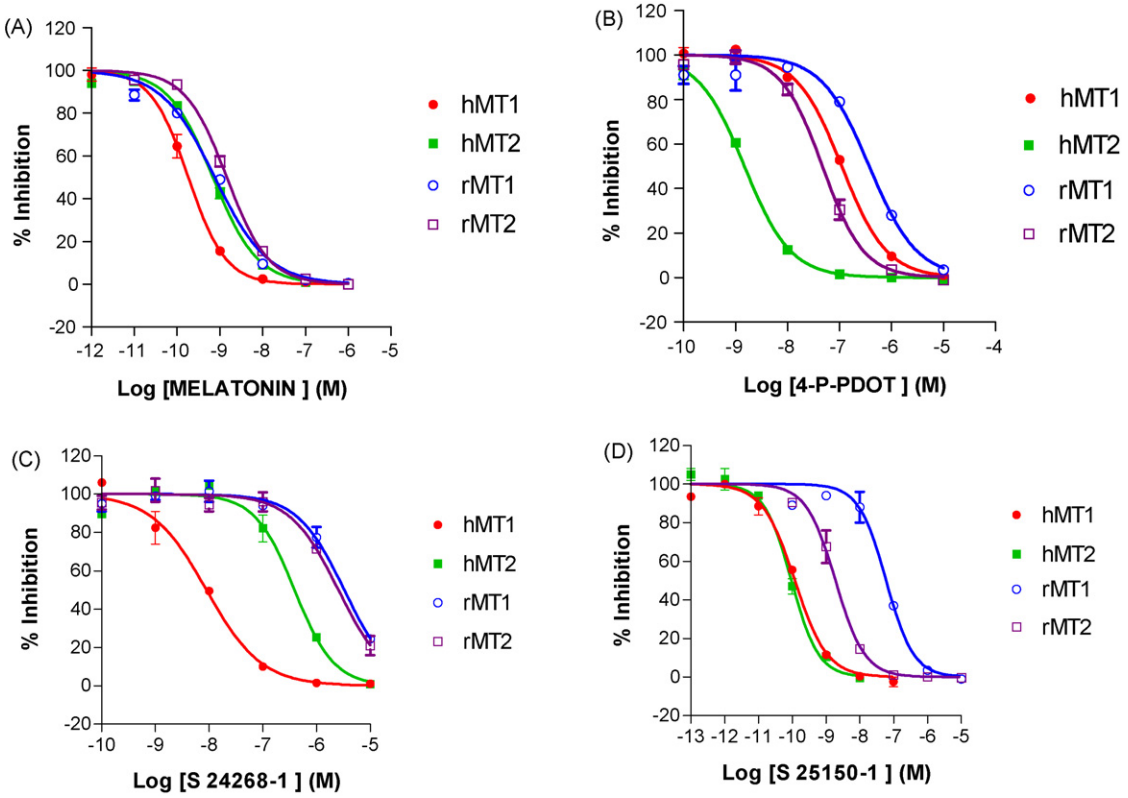

Fig. 5 – Concentration isotherms of 2-[^{125}I]-iodomelatonin specific binding to rat and human MT_1 and MT_2 membranes. (A) Melatonin, (B) 4P-PDOT, (C) S 24268 and (D) S 25150. Points shown are from a representative experiment performed in triplicates and repeated at least three times. Open circles (blue lines), rMT_1; open squares (black lines), rMT_2; closed circles (red lines), hMT_1; and closed squares (green lines), hMT_2.

3.3. *Binding affinities of reference and new ligands*

Despite a slightly higher expression for the rat receptors (as opposed to human ones, Table 1), neither rMT_1 nor rMT_2 showed any constitutive activities. Affinities of a selection of compounds were evaluated for each rat receptor subtypes, these results were systematically compared with those obtained at the human homologues (Table 2). Among the reference agonists, melatonin and 2-iodomelatonin showed a slight preference for rMT_1 compared to rMT_2 (Table 2, Fig. 5). The selective hMT_2 antagonist 4P-PDOT was slightly selective for the rMT_2 and the MT_1/MT_2 ratio decreased from 126 for human to 9.5 for rat receptors (Table 2, Fig. 5). A similar loss of selectivity was observed with luzindole. Among the new

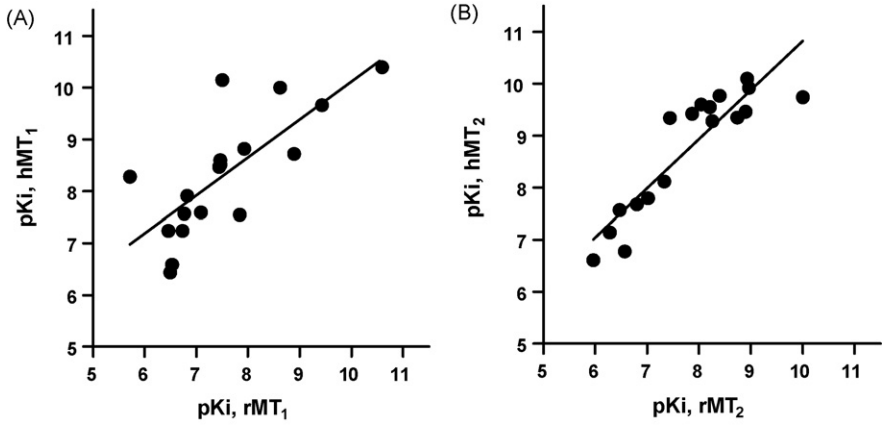

Fig. 6 – Correlation plot of binding affinities (expressed as $pK_i = -\log K_i$) determined at rMT_1 and hMT_1 (A) or at rMT_2 or hMT_2 (B) receptors. Data were calculated from Table 2. The correlation analysis gave, respectively, $r = 0.739$ [95% confidence interval: 0.429–0.894] with a p value of 0.0003 ($n = 18$) for MT_1 receptors, and $r = 0.902$ [95% confidence interval: 0.758–0.962] with p value > 0.0001 ($n = 18$) for MT_2 receptors. Slopes were 0.725 ± 0.160 and 0.945 ± 0.110, respectively, for MT_1 and MT_2 receptors.

compounds, two groups could be distinguished (Table 2, Fig. 1): first, non selective compounds (S 22971, S 20098, S 23219, S 25150, S 22701 and S 20928) which were equally potent at both receptors, with K_i values ranging from 0.1 to 300 nM. For these molecules the K_i values for both rat receptor subtypes were generally lower. This is particularly the case for compound S 25150 for which the K_i for rMT_1 was 450-fold less potent that for hMT_1 whereas the decrease for the MT_2

Table 3 – Compound potency and efficacy as agonist or antagonist, on [$_{35}$S]-GTPγS binding at rMT_1 and rMT_2 receptors and comparison with the data obtained at hMT1 and hMT2 receptors

	MT_1 receptor		MT_2 receptors	
	Agonist EC_{50} (E_{max})	Antagonist K_B (I_{max})	Agonist EC_{50} (E_{max})	Antagonist K_B (I_{max})
References				
Melatonin	(r) 6.78 ± 0.97 (100)	nd	(r) 1.30 ± 0.26 (100)	nd
	(h) 2.24 ± 0.35 (110)	nd	(h) 0.49 ± 0.04 (104)	nd
2-Iodomelatonin	(r) 0.7 ± 0.16 (94)	nd	(r) 0.15 ± 0.03 (111)	nd
	(h) 0.18 ± 0.04 (108)	nd	(h) 0.17 ± 0.05 (121)	nd
6-Chloromelatonin	(r) 17.2 ± 6.9 (101)	nd	(r) 1.5 ± 0.49 (91)	nd
	(h) 5.3 ± 1.4 (93)	nd	(h) 0.55 ± 0.04 (93)	nd
4P-PDOT	(r) Inactive (<10)	(r) 610 ± 52 (83)	(r) Inactive (<10)	(r) 20 ± 5 (90)
	(h) Inactive (<10)	(h) 93 ± 39 (95)	(h) 1.3 ± 0.3 (19)	(h) 1.8 ± 0.8 (75)
Luzindole	(r) Inactive (<10)	(r) 706 ± 146 (113)	(r) Inactive (<10)	(r) 284 ± 102 (99)
	(h) Inactive (<10)	(h) 42 ± 4 (102)	(h) Inactive (<10)	(h) 33 ± 3 (85)
Non selective				
S 22971	(r) 133 ± 7.2 (58)	(r) Inactive (<10)	(r) 55.4 ± 5 (70)	(r) 11.4 ± 4.7 (20)
	(h) 674 ± 323 (24)	(h) 227 ± 24 (96)	(h) 22.8 ± 10.2 (76)	(h) 19 ± 10 (28)
S 20098	(r) 28.7 ± 3.3 (123)	nd	(r) 0.38 ± 0.1 (130)	nd
	(h) 1.6 ± 0.4 (101)	nd	(h) 0.10 ± 0.04 (91)	nd
S 23219	(r) 423 ± 68 (85)	nd	(r) 30 ± 10.6 (93)	nd
	(h) 9.3 ± 7.5 (87)	nd	(h) 2.5 ± 0.6 (90)	nd
S 25150	(r) 37.5 ± 11 (80)	nd	(r) 0.31 ± 0.003 (98)	nd
	(h) 0.23 ± 0.04 (88)	nd	(h) 0.06 ± 0.02 (77)	nd
S 20928	(r) Inactive (<10)	(r) 22 ± 12 (46)	(r) 130 ± 8.8 (23)	(r) 270 ± 96 (78)
	(h) Inactive (<10)	(h) 85 ± 17 (78)	(h) 20 ± 2 (34)	(h) 12 ± 5 (75)
hMT1 selective				
S 24268	(r) Inactive (<10)	nd	(r) 687 ± 54 (40)	nd
	(h) 374 ± 126 (95)	nd	(h) 496 ± 99 (27)	(h) 147 ± 7 (96)
S 26284	(r) Inactive (<10)	(r) 29 ± 8.4 (71)	(r) 149 ± 41 (24)	(r) 31.6 ± 5.8 (30)
	(h) 73 ± 7 (25)	(h) 11 ± 1 (68)	(h) 405 ± 35 (39)	(h) 29 ± 11 (80)
S 25567	(r) 1230 ± 384 (48)	(r) 124 ± 45 (88)	(r) 71.1 ± 5.8 (80)	nd
	(h) Inactive (<10)	(h) 4.3 ± 0.3 (79)	(h) 12 ± 2 (47)	(h) 19 ± 7 (81)
S 22701	(r) 339 ± 96 (70)	(r) Inactive (<10)	(r) 3.7 ± 1.0 (80)	(r) Inactive (<10)
	(h) 9.8 ± 0.5 (38)	(h) 7.2 ± 0.2 (72)	(h) 0.20 ± (101)	(h) Inactive (<10)
hMT2 and rMT2 selective				
S 24014	(r) 682 ± 201 (44)	(r) 227 ± 32 (71)	(r) 2.5 ± 0.65 (38)	(r) 2.8 ± 0.84 (54)
	(h) 97 ± 43 (23)	(h) 45 ± 11 (52)	(h) 0.72 ± 0.13 (27)	(h) 1.5 ± 0.4 (70)
S 24773	(r) Inactive (<10)	(r) 2860 ± 569 (130)	(r) 5.7 ± 0.7 (56)	(r) 8.7 ± 2.7 (45)
	(h) 199 ± 180 (20)	(h) 278 ± 33 (91)	(h) 1.3 ± 0.2 (41)	(h) 0.83 ± 0.3 (37)
S 23950	(r) 316 ± 38 (37)	(r) 1080 ± 440 (88)	(r) 6.3 ± 0.9 (66)	(r) 4 ± 0.53 (24)
	(h) 99 ± 4 (25)	(h) 51 ± 18 (24)	(h) 2.4 ± 0.8 (56)	nd
S 24601	(r) Inactive (<10)	(r) 320 ± 73 (74)	(r) 13 ± 4.6 (52)	(r) 7.9 ± 2.5 (52)
	(h) 391 ± 90 (42)	(h) 770 ± 63 (76)	(h) Inactive (<10)	(h) 6 ± 2.9 (77)

(h) Data are from Audinot et al. [17] (except for S 22701). *Note*: Concentration–response curves were analyzed by non-linear regression. Agonist potency was expressed as EC_{50} ± S.E.M. (nM) while the maximal efficacy, E_{max} ± S.E.M. was expressed as a percentage of that observed with melatonin 1 μM (=100%). Antagonist potency to inhibit the effect of melatonin (30 or 3 nM, respectively, for hMT_1 and hMT_2 receptors) was expressed as K_B ± S.E.M. while the maximal inhibition I_{max} ± S.E.M. was expressed as a percentage of that observed with melatonin 30 or 3 nM (=100%), respectively, for hMT_1 and hMT_2 receptors. Data are mean of at least three independent experiments. Inactive: no dose–response effect until 10^{-5} M and nd: not determined.

subtype was only of 15-fold. These changes led to some selectivity for the rMT_2 receptor (Fig. 5). Second, selective (S 24268, S 26284) and slightly selective (S 25567) hMT_1 compounds with K_i values in the nM range for hMT_1 receptor or selective (S 24014, S 24773, S 23950) or slightly selective (S 24601) hMT_2 compounds exhibiting subnanomolar K_i at hMT_2 receptors, (Table 2, Fig. 5). These compounds showed selectivity ratio hMT_1/hMT_2 of roughly 25–100 on CHO cells. For these selective compounds, the K_i for rat receptors were slightly less potent than for human receptors and generally the selectivity was lower with the exception of S 24601 which became selective for the rMT_2 with the MT_1/MT_2 ratio increased from 28 for human to 85 for rat receptors. The affinity of S 24268 became very low, in the μM range, on rat receptors.

In summary, compounds displayed lower binding affinity at rat versus human melatonin receptors. There was a nice statistical correlation for MT_2 sites ($r = 0.901$, $p < 0.0001$, $n = 18$) with a slope of 0.944 (Fig. 6). Though there was also a significant correlation at MT_1 sites ($r = 0.751$, $p = 0.0003$, $n = 18$), the slope of 0.733 differed from unity (Fig. 6), reflecting a higher variation between rat and human binding affinities at MT_1 receptors.

3.4. *Functional activity of the compounds*

All the compounds were tested alone as agonists, and as antagonists in the presence of a fixed concentration of melatonin (30 nM or 3 nM for rMT_1 and rMT_2 receptors, respectively). The results are summarized in Table 3. For human receptors, affinities (K_i) and potencies (EC_{50} or K_B) were comparable for hMT_2 receptors, while for hMT_1 receptors, the pharmacological potencies were generally lower than the binding affinities. This is also the case for the rat receptors. A shift in the order of potency (5–10-fold) is seen for some compounds including reference compounds melatonin and 2-iodomelatonin (Fig. 7), probably due to a less efficient coupling at this receptor.

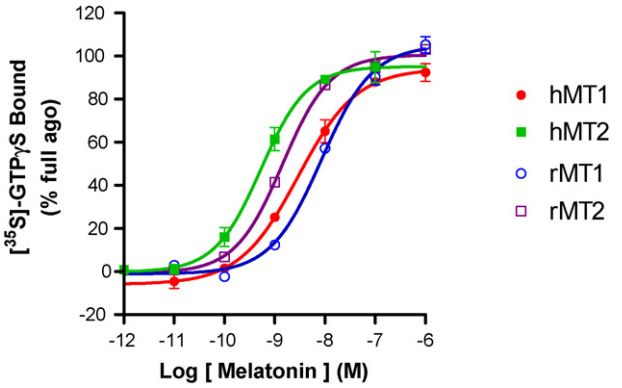

Fig. 7 – Concentration–response curves on [^{35}S]-GTPγS binding of the agonist melatonin at rat and human MT_1 and MT_2 receptors. Data are expressed as a percentage of maximal stimulation of [^{35}S]-GTPγS binding induced by melatonin at 1 μM (=100%). Points shown are from a representative experiment performed in triplicates and repeated at least three times. Open circles (blue lines), rMT_1; open squares (black lines), rMT_2; closed circles (red lines), hMT_1; and closed squares (green lines), hMT_2.

Antagonist activities were also evaluated for partial agonists and the sum of the respective E_{max} and I_{max} were close to 100% (S 24014, S 23950). The reference agonists, including S 20098, 2-iodomelatonin and 6-chloromelatonin, acted as full agonists as compared to melatonin (Table 3). The two reference antagonists 4P-PDOT and luzindole, were devoid of intrinsic activity and acted as antagonists at both rat receptors (Table 3, Fig. 8). The selectivity for MT_2 receptor subtype was similar in both species with a MT_1/MT_2 ratio of 50–60 comparing K_B values. S 20928, another antagonist on human receptors, remained antagonist on rat receptors with only a weak intrinsic activity on rMT_2 as on hMT_2 receptors. S 22971 and S 22701 acted as partial agonists at the rMT_1 and hMT_1 receptor subtypes but with a more potent agonist activity at the rMT_1 as reflected by the E_{max}, whereas their activities on MT_2 subtypes were similar for the two species. For all the other molecules tested for activity the values were similar between the two species.

3.5. *General discussion*

The present study describes for the first time the molecular cloning and the pharmacology of rat MT_1 and MT_2 melatonin receptors stably expressed in CHO cells, and systematically compares binding affinities and pharmacological potencies of 18 compounds with those previously observed at the human MT_1 and MT_2 receptors [17].

Apart for the activity of the molecules themselves, a large number of reports deals with the melatonin pharmacology in rats (see, e.g. [36–41] among the 4000 entries in PubMed®), justifying, if necessary, the present cloning and description of MT_1 and MT_2 receptors.

The binding to the rat receptors subtypes evaluated by the saturation binding experiments with the radioligand 2-[^{125}I]-iodomelatonin or competition experiments with the endogenous ligand, melatonin or the close molecules 2-iodomelatonin or 6-chloromelatonin gave similar results for the two receptor subtypes in the two species. If we compare the results to those obtained for the same reference compound on the ovine MT_1 receptor subtypes, the conclusion is that the endogenous ligand, melatonin, or 2-iodomelatonin which is the radioligand used in most binding studies, have the same binding properties whatever the species considered [17,18, present work].

The series of chemicals studied herein is not diverse neither large enough to draw structure–activity relationships on this species receptors. Nevertheless, based on the molecular pharmacology studies from our laboratory, it is noteworthy to notice that melatonin receptor ligands designed are bioisosteres of melatonin itself. If one considers the molecule from a global point of view, one can segregate it in three parts: the aromatic moiety, the substituent thereof and the acylaminochain which ends for most of the compounds by an ethyl group. Interestingly, as previously noticed, the aromatic part can be substituted by various analogues, such as naphthalene (S 20098, S 20928), benzofurane (S 22971, S 25150), benzodioxane (S 23219) or phenalene (S 22701) without effect on the selectivity of the molecules.

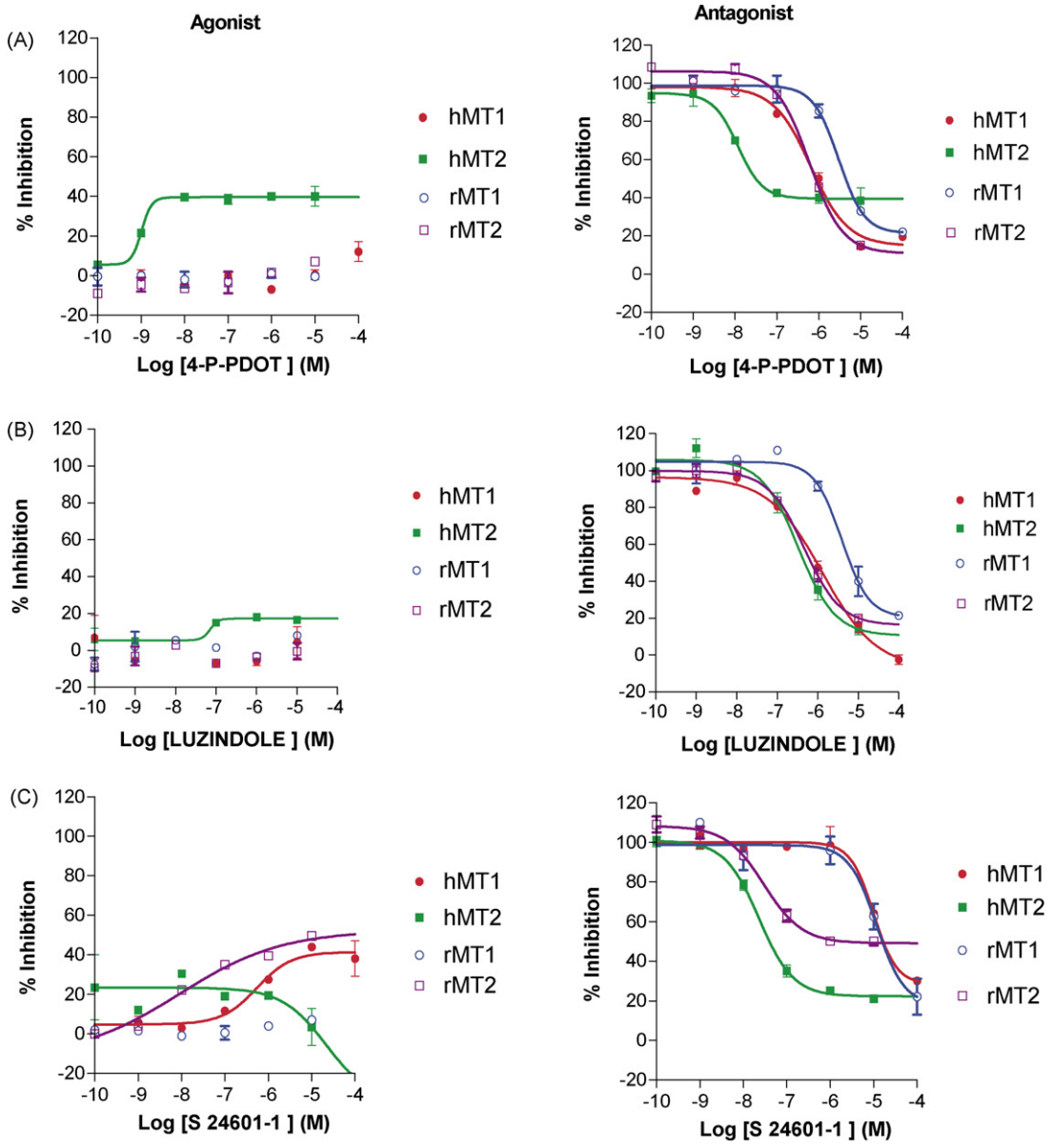

Fig. 8 – Concentration–response curves on [^{35}S]-GTPγS binding to rat and human MT_1 and MT_2 membranes of 4P-PDOT (A) luzindole, (B) and S 24601 (C). For agonist test, data are expressed as a percentage of maximal stimulation of [^{35}S]-GTPγS binding induced by melatonin at 1 μM (=100%). For antagonist test, data are expressed as a percentage of maximal stimulation of [^{35}S]-GTPγS binding induced by melatonin at 30 nM for rMT_1 and 3 nM for rMT_2 (=100%). Representative curves are shown in which each point is the mean of triplicate determinations. Similar results were obtained on at least three independent experiments. Open circles (blue lines), rMT_1; open squares (black lines), rMT_2; closed circles (red lines), hMT_1; and closed squares (green lines), hMT_2.

Furthermore, most of them bear as substituent on the aromatic part of the methoxy group of melatonin, an ethyl chain (S 22971), a long alkoxy side chain (S 25567) or no substituent. Two other compounds are dimeric derivatives (S 24268, S 26284) and are selective for the hMT_1 [17,25]. Lastly three compounds are also bioisosteres of melatonin, bearing an acetylaminoethyl side chain, but in contrast to MT_1 selective ligands, they possess the methoxy group in position 5 and are characterized by the presence of a benzyl or a phenyl substituent on the ortho (benzofurane, S 24014) or meta (naphthalene, S 24773, S 23950) position of the acetylaminoethyl side chain. These compounds are hMT_2 selective with selectivity ratios comprised between 250 and 50 [16,17]. Of interest, the selection of compounds tested in this work brings together reference molecules, non selective and selective ligands, agonists, partial agonists and antagonists. Thus, despite the high amino acid identity between the melatonin receptor subtypes of each species, the present study shows difference in the pharmacological profile between species for both receptor subtypes as it was previously reported in

previous study comparing the human and the ovine MT_1 subtype [18].

In conclusion, we have characterized at the molecular level the rat MT_1 and MT_2 receptors and have settled their pharmacological profile, which interestingly differ from that of their human counterparts. These results are of importance in order to understand the physiological function of the different receptor subtypes MT_1 and MT_2 in rat animal models.

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