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Molecular pharmacology of the ovine melatonin receptor: comparison with recombinant human MT_1 and MT_2 receptors

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Abstract

The variations of the pharmacological properties of melatonin receptors between different mammalian species in transfected cell lines have been poorly investigated. In the present study, melatonin analogues have been used to characterize the pharmacology of the recombinant ovine melatonin receptor (oMT_1) expressed in CHO cell lines and the native oMT_1 from the *pars tuberalis* (PT). Studies with selective ligands on native and transfected oMT_1 showed similar properties for binding affinities [r^2(PT/CHO) = 0.85]. The affinities and the functional activities of these ligands were compared with the human receptors (hMT_1 or hMT_2) expressed in CHO cells as well. The oMT_1 and hMT_1 receptors had similar pharmacological profiles ($r^2 = 0.82$). Nevertheless, some of the selective compounds at the human receptor presented a reduced affinity at the ovine receptor. Furthermore, some compounds showed marked different functional activities at oMT_1 vs. hMT_1 receptors. Our findings demonstrated differences in the pharmacological properties of melatonin receptors in ovine and human species.

Keywords: Melatonin; Recombinant human melatonin receptors; Recombinant ovine melatonin receptor; Molecular pharmacology; *Pars tuberalis*; *CHO cells*

1. Introduction

The daily light/dark cycle entrains the mammalian master biological clock located in the suprachiasmatic nucleus and as a consequence the rhythmic release of the pineal melatonin (MLT)[1] hormone (*N*-acetyl-5-methoxytryptamine). This hormone is involved in the regulation of numerous physiological functions [1,2]. The high-affinity agonist radioligand 2-^{125}I-iodomelatonin (2-[^{125}I]-MLT) has been widely used to map the localization of MLT binding sites in the brain and peripheral tissues [3]. MLT binding sites were classified on the basis of kinetic and pharmacological properties into ML_1 and ML_2 classes [4,5]. The ML_1 site has been identified as a high-affinity membrane associated receptor sensitive to guanine nucleotides (G protein-coupled receptors: GPCRs) and coupled to the inhibition of adenylyl cyclase *via* a pertussis toxin sensitive G-protein [3]. Three melatonin receptor subtypes

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Abbreviations: CHO, Chinese Hamster Ovary; GPCRs, G protein-coupled receptors; hMT_1, human recombinant MT_1 melatonin receptor subtype; hMT_2, human recombinant MT_2 melatonin receptor subtype; 2-I-MLT, 2-iodomelatonin; 2-[^{125}I]-MLT, radioligand 2-^{125}I-iodomelatonin; MLT, melatonin; oMT_1, ovine recombinant MT_1 melatonin receptor subtype; [^{35}S]-GTPγS, guanosine-5′[γ-^{35}S]-triphosphate; PT, *pars tuberalis*; SCN, suprachiasmatic nucleus.

[1] The abbreviation of melatonin is conventionally written "MLT".

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have now been cloned in this class. The first of these was cloned from *Xenopus laevis* melanophores [6] and was named Mel_{1c}. Subsequently, two further subtypes, MT_1 and MT_2 (previously called Mel_{1a} and Mel_{1b})[2] have been isolated from different species including human [7–10]. MT_1 and MT_2 are expressed in mammals whereas Mel_{1c} was detected only in birds and Xenopus [9,11]. The MT_1 and MT_2 melatonin receptor subtypes show relatively high identity at the amino acid level (about 55% overall and 70% within transmembrane domains) [7,8] and present similar pharmacological properties (e.g. picomolar range affinity, [12]). Selective ligands for MT_1 and MT_2 melatonin receptor subtypes have recently been identified and constitute valuable pharmacological tools to study the respective functional role of these receptor subtypes [13,14]. Finally, a low affinity site MT_3 (previously called ML_2) has been recently described in hamster as the human homologue of the cytoplasmic protein quinone reductase 2 [15].

The MT_1 and MT_2 subtypes of MLT receptors have been cloned and present high homology among different species [7–11,16]. However, the comparison of the pharmacological properties of melatonin receptor subtypes from different mammalian species has been poorly studied but is important for several reasons. Firstly, it may provide valuable information about the functional importance of the parts of the sequence differing among species. Secondly, some species, like sheep, seem to express only one MLT receptor subtype [17], cloned from the *pars tuberalis* (PT) and called MT_1 from its higher identity (i.e. 79% of homology in amino acid sequence with hMT_1 compared with 56% for the hMT_2 receptor). However, it is important to check whether oMT_1 is pharmacologically closer to hMT_1 than to hMT_2. Thirdly, pharmacology properties of specific MLT ligands need to be tested against the receptor(s) of the species of interest before considering their use in physiological studies in the corresponding species.

A cellular system stably expressing a single receptor subtype allows studies of both the ligand affinity and its functional (agonist or antagonist) activity. The aim of the present study was, on one hand, to compare the pharmacological profiles of the ovine MT_1 in native tissue (PT) or expressed in CHO cells and, to the other, to compare the pharmacological properties of the ovine MT_1 and of the human MT_1 and MT_2 receptors expressed in CHO cells. Binding affinities were determined with 2-[^{125}I]-MLT and functional response on transfected CHO cell lines was evaluated using the [^{35}S]-GTPγS binding assay.

[2] The official nomenclature and classification of melatonin receptors approved by the Nomenclature Committee of the International Union of Pharmacology (Dubocovich *et al*. [38]) was used in the present work. The recombinant melatonin receptors subtypes previously known as Mel_{1a} and Mel_{1b} [7,8] are referred here as "MT_1" and "MT_2" because respective functions have been defined and these two melatonin receptor subtypes have been pharmacologically characterized in native tissues [14,51].

2. Materials and methods

2.1. Reagents and drugs

2-[^{125}I]-MLT (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 Amersham Pharmacia Biotech. Melatonin (MLT or *N*-acetyl-5-methoxytrytamine), *N*-acetyl-serotonin (*N*-acetyl-5-hydroxytryptamine), 2-I-MLT (2-iodomelatonin) were obtained from Sigma whereas 4P-PDOT (4-phenyl 2-propionamidotetraline) and luzindole (2-benzyl-*N*-acetyltryptamine) were purchased from Tocris. Thirteen compounds loosely resembling melatonin were evaluated. Their synthesis were reported in the following reports, mostly patents. Compound 5 (*N*-[2-(7-methoxynapth-1-yl)ethyl] propionamide) and compound 6 (*N*-[2-(7-methoxy-1-naphtyl) ethyl] 2-butenamide) were synthesized by Depreux *et al*. [18], compound 7 (*N*-[2-(1-naphtyl)ethyl] cyclobutanecarboxamide) by Leclerc *et al*. [19], compound 8 (*N*-[2-(7-hexyloxy-1-naphtyl) ethyl]acetamide) by Andrieux *et al*. [20], compound 9 (*N*-ethyl (8-chloro-7-methoxy-1-naphtyl)butanamide) by Lesieur *et al*. [21], compound 10 (*N*-methyl-[4-(2,3-dihydro-1,4-benzodioxin-5-yl)]butanamide) by Charton *et al*. [22], compound 11 (*N*-[2-(2-benzyl-5-methoxybenzo(*b*)furan-3-yl)ethyl]acetamide) and compound 12 (*N*-[2-(2-(3-methoxybenzyl)5-methoxybenzo(*b*)furan-3-yl)ethyl]acetamide) by Wallez *et al*. [23], compound 13 (*N*-[2-(7-{8-[2-(acetylamino)ethyl]-2-naphtyl}-1-naphtyl)]ethyl]acetamide) by Lesieur *et al*. [24], compound 14 (*N*-[2-(8,9-dihydro-7*H*-furo[3,2-*f*]chromen-1-yl)ethyl]-2-iodoacetamide) by Lesieur *et al*. [25], compound 15 (*N*-methyl(5-ethylbenzo(*b*)thien-3-yl) butanamide) by Lesieur *et al*. [21], compound 16 ((*R*,*S*)*N*-[2-(6-hexyloxy-3,4 dihydro-2*H*-1-benzopyran-4-yl)ethyl]acetamide) by Guillaumet *et al*. [26] and compound 17 (*N*-(2-{7-[4-({8-[2-acetylamino)-ethyl]-2-naphtyl}oxy)butoxy]-1-naphtyl}ethylacetamide) by Lesieur *et al*. [24]. All compounds were dissolved in DMSO at a stock concentration of 10 mM and stored at −20°. All other chemicals were obtained from Sigma.

2.2. PT tissues

Lambs obtained from SODEM slaughterhouse were killed between 06:00 and 12:00 hr between December and July (late night and morning). PT was cut off ventrally from the brain, frozen immediately in liquid nitrogen vapours and stored at −80°. The interval between slaughter and freezing was less than 10 min.

2.3. Establishment of stable transfected cell lines

The CHO cell line from the European Collection of Animal Cell Cultures was used to express melatonin receptors. The CHO cell lines stably expressing the human

receptors (hMT_1 or hMT_2) have been previously described [13]. In addition, a stably transfected CHO cell line expressing the oMT_1 was investigated. Total RNA from PT was isolated by the guanidinium thiocyanate method [27]. One microgram of total RNA was reverse transcribed (SuperscriptII reverse transcriptase) with Oligo dT primers. One-tenth of the reaction provided the template for the polymerase (Platinium Taq) to amplify the partial coding sequence with forward and reverse primers 5′-CTCATCTTCACCATCGTGG-3′ and 5′-AGCTTTAAACGGAGTCCACC-3′, respectively. The 5′ end of the coding region was obtained by amplification of genomic DNA with forward and reverse primers 5′-ATGGCGGGGCGGCTGTGGGGCT-3′ and 5′-TTCCCTGCGTTCCTCAGCTTC-3′, respectively. PCR was performed according to a high GC content method [28] using a heat stable thermal polymerase (Deep Vent). The band was ligated into pGEM (Promega) in frame with the corresponding downstream coding region of the cDNA. Sequencing experiments have confirmed the cloning of the MT_1 receptor [29]. Then, the oMT_1 sequence was inserted in an expression vector and transfected in CHO cells as described for the human receptors in Audinot *et al*. [13].

2.4. Membrane preparations

The CHO cells stably expressing the hMT_1 or hMT_2 or oMT_1 receptor, respectively, were grown to confluence, harvested in phosphate buffer containing 2 mM EDTA and centrifuged at 1000 *g* for 5 min (4°). 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°), and the resulting pellet was suspended in 75 mM Tris–HCl, pH 7.4, containing 2 mM EDTA and 12.5 mM $MgCl_2$. Membranes from PT were simultaneously harvested in 5 mM Tris–HCl, pH 7.4, containing 2 mM EDTA, centrifuged at 1000 *g* for 10 min (4°) and the supernatant was centrifuged at 10,000 *g* for 20 min (4°). The resulting pellet was suspended in 50 mM Tris–HCl, pH 7.4, containing 4 mM $CaCl_2$ and 6 mM ascorbic acid, and homogenized using a Kinematica polytron. Determination of protein content was performed according to the Bradford method [30] using a Biorad kit (Bio-Rad SA). Aliquots of membrane preparations were stored at −80° until use.

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

Membranes of CHO cells (50 μg/mL) were incubated with 2-[^{125}I]-MLT (Perkin-Elmer, 2000 Ci/mmol) for 120 min at 37° in 50 mM Tris–HCl, pH 7.4, containing 5 mM $MgCl_2$. Membranes from PT tissue (100 μg/mL) were incubated with 2-[^{125}I]-MLT for 18 hr at ambient temperature in 50 mM Tris–HCl, pH 7.4, 4 mM $CaCl_2$ and 6 mM ascorbic acid [31]. In saturation assays, 2-[^{125}I]-MLT was used with concentrations ranging from 1 pM to 1 nM. In competitive assays, 2-[^{125}I]-MLT concentration was maintained at 0.03 nM and compounds were used in the range of 0.1 pM–10 μM. Nonspecific binding was determined with 10 μM MLT. 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.). For saturation experiments, the maximal concentration of binding site (B_{max}) and the dissociation constant of the radioligand (K_D) values were calculated according to the method of Scatchard [32]. For displacement 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]-MLT [33]. For the correlation analyses, the K_i values of the different chemicals for the three melatonin receptors were expressed as pK_i corresponding to the logarithmic expression of K_i [$pK_i = -\log(K_i)$]. To calculate the correlations between binding affinities, Pearson product–moment correlation coefficients were employed.

2.6. [^{35}S]-GTPγS binding assay in transfected cell lines

Membranes and drugs were diluted in binding buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 3 mM $MgCl_2$, 3 μM, GDP). Saponin (20 μg/mL) was used to improve the signal/noise ratio for hMT_1 and hMT_2 while it was not necessary for oMT_1. For agonist tests, incubation was started by the addition 0.1 nM [^{35}S]-GTPγS to membranes and drugs, and lasted for 30 min at room temperature in a final volume of 250 μL. For antagonist tests, membranes were preincubated 45 min with 100 nM MLT for oMT_1, 30 nM for hMT_1 and 3 nM for hMT_2 and the antagonist. Reaction was started by the addition of 0.1 nM [^{35}S]-GTPγS and incubation lasted 30 min. Nonspecific binding was assessed using nonradiolabelled GTPγS (10 μM). All reactions were stopped by rapid filtration through GF/B unifilters presoaked with distilled water, followed by three successive washes with ice-cold buffer. Data were analysed using nonlinear regression from the program PRISM (GraphPad Software Inc.). Agonist efficacy (E_{max}) is expressed relative to that of MLT (=100%) which was tested at a maximally effective concentration of 1 μM in each experiment. Potency of agonists are expressed as the mean EC_{50} obtained from at least three independent experiments. Antagonist potencies are 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. I_{max} of the antagonist is the maximal inhibition of MLT effect (100 nM MLT for oMT_1, 30 nM for hMT_1 and 3 nM for hMT_2).

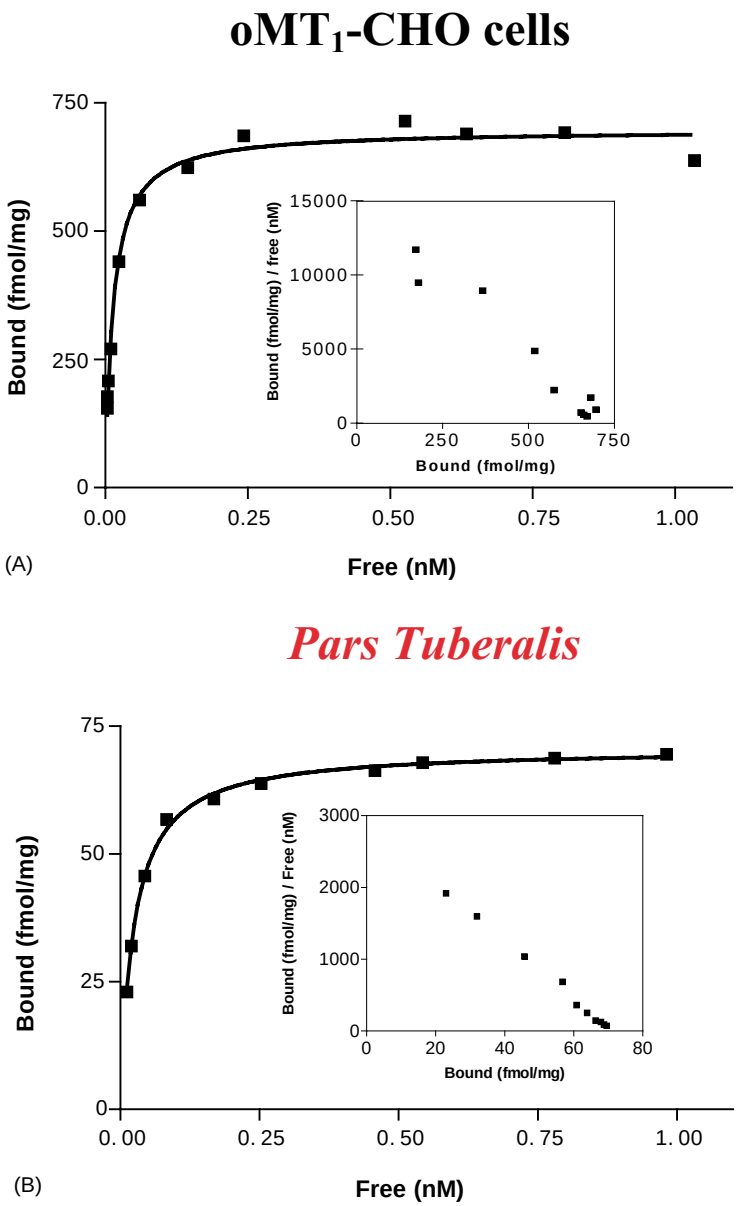

Fig. 1. Saturation binding experiments of 2-[^{125}I]-iodomelatonin at membranes from CHO cells expressing oMT$_1$ (A) and at ovine *pars tuberalis* (B). Specific binding is represented. (Inset) Scatchard plot of the specific binding. Points shown are from representative experiments performed in duplicates and repeated three times.

3. Results

3.1. Saturation assay on ovine melatonin receptors

Saturation studies (N = 3) using 2-[^{125}I]-MLT revealed a single binding site (*F* test, GraphPad Prism) in both the CHO cell lines and in ovine PT membranes (Fig. 1). The concentration of melatonin receptors (B_{max}) was 670 ± 25 fmol/mg protein for recombinant oMT$_1$ expressed in CHO cells and 71 ± 1 fmol/mg in PT, while the hMT$_1$-expressing CHO cells showed a B_{max} of 435± 192 fmol/mg protein (not shown). Moreover, the K_D of 2-[^{125}I]-MLT in PT (21 ± 6 pM) was comparable to that of oMT$_1$ when expressed in CHO cells (14 ± 1 pM) and to that of hMT$_1$ (not shown, 21 ± 3 pM). At hMT$_2$ receptors, a higher K_D value of 107 ± 11 pM has been reported (not shown).

3.2. Ligand competition on 2-[^{125}I]-iodomelatonin binding

MLT, 2-I-MLT, 4P-PDOT, luzindole and 13 ligands of MLT were tested for their binding properties at ovine and human melatonin receptors. For all these compounds, competition curves were monophasic on membranes from cell lines expressing hMT$_1$, hMT$_2$ or oMT$_1$ as well as in PT, meaning that all curves were best fitted by a one-site analysis than by a two-site analysis (*F* test, GraphPad Prism).

Table 1
Comparison of Equilibrium binding constants (K_i) determined by competitive inhibition of 2-[^{125}I]-iodomelatonin between *pars tuberalis* (PT) and ovine MT_1 expressed in CHO cells

	K_i (nM)		
	PT	CHO oMT_1	Ratio PT/CHO
MLT	0.04 ± 0.03	0.18 ± 0.15	0.24
2-Iodo-MLT	0.024 ± 0.003	0.035 ± 0.007	0.68
4P-PDOT	83.5 ± 59.87	141.0 ± 54.0	0.59
Luzindole	71.2 ± 12.65	157.0 ± 32.0	0.45
Compound 5	0.15 ± 0.10	0.07 ± 0.01	2.07
Compound 6	14.7 ± 2.6	10.5 ± 3.2	1.40
Compound 7	nd	142 ± 25	–
Compound 8	2.0 ± 1.8	0.46 ± 0.33	4.39
Compound 9	4.6 ± 0.41	4.6 ± 1.1	1.01
Compound 10	nd	2.0 ± 0.8	–
Compound 11	1.6 ± 0.50	1.3 ± 0.3	1.20
Compound 12	0.5 ± 0.18	7.3 ± 1.4	0.07
Compound 13	110.2 ± 28.91	30.1 ± 13.6	3.66
Compound 14	0.1 ± 0.01	0.088 ± 0.024	0.94
Compound 15	4.5 ± 1.46	6.0 ± 2.4	0.75
Compound 16	15.9 ± 8.41	28.9 ± 25.5	0.55
Compound 17	12.9 ± 4.77	70.7 ± 47.5	0.18

Inhibition constant K_i values were calculated from IC_{50} obtained from competition curves by the method of Cheng and Prussof [33]. Ratio of PT/CHO represents folds difference in affinity of each compound for 2-[^{125}I]-iodomelatonin binding to recombinant ovine MT_1 and *pars tuberalis* melatonin binding sites. Results are expressed by mean ± SEM. nd: not determined.

The pharmacological profile was compared between the different melatonin receptors by an analysis of the K_i ratios for each ligand (Tables 1 and 2) and correlation analyses including all ligands (Figs. 2 and 3). With the exception of compound 12 (K_i ratio of 0.07), binding affinities were comparable on membranes from PT and from CHO cells expressing oMT_1 (Table 1). There was indeed a highly significant correlation [$r^2 = 0.85$, $P < 0.0001$, N = 15, Fig. 2]. Eleven of the seventeen compounds have nonselective affinities on human melatonin MT_1 and MT_2 receptors (Table 2): MLT, 2-I-MLT, compounds 5, 6, 7, 8, 9, 10, 14, 15 and 16 ($0.16 <$ ratio $hMT_1/hMT_2 < 5.4$). In contrast, compounds 13 and 17 are selective at hMT_1 (ratio $hMT_1/hMT_2 = 0.02$ for both compounds) whereas 4P-PDOT, luzindole, compounds 11 and 12 are selective or slightly selective at hMT_2 receptors ($10 <$ ratio $hMT_1/hMT_2 < 126$) (Table 2). No significant correlation was observed between affinities determined at these two receptors ($r^2 = 0.28$, $P > 0.05$, N = 17, Fig. 3A).

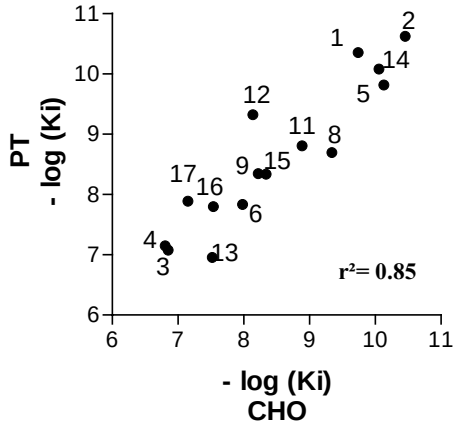

Fig. 2. Correlation analysis between the ligand affinities (expressed as pK_i) at membranes from CHO cells expressing oMT_1 and ovine *pars tuberalis* (PT). The correlation coefficient (r^2) was 0.85. Compounds were referred to as follows: 1: melatonin, 2: 2-iodomelatonin, 3: 4P-PDOT and 4: luzindole. For other number correspondence, see Section 2.

Table 2
Comparison of equilibrium binding constants (K_i) determined by competitive inhibition of 2-[^{125}I]-iodomelatonin between ovine MT_1 and human MT_1 and MT_2 melatonin receptor expressed in CHO cells

	Ovine MT_1 K_i (nM)	Human MT_1 K_i (nM)	Human MT_2 K_i (nM)	Ratio hMT_1/hMT_2	Ratio oMT_1/hMT_1	Ratio oMT_1/hMT_2
MLT	0.18 ± 0.15	0.22 ± 0.01	0.35 ± 0.04	0.63	0.82	0.51
2-Iodo-MLT	0.035 ± 0.007	0.04 ± 0.01	0.18 ± 0.01	0.22	0.98	0.20
4P-PDOT	141.0 ± 54.2	58 ± 8	0.46 ± 0.14	126	2.4	306
Luzindole	157.4 ± 31.5	258 ± 8	27.0 ± 1.0	10	0.61	5.8
Compound 5	0.07 ± 0.01	0.10 ± 0.04	0.09 ± 0.01	1.11	0.74	8.2
Compound 6	30.7 ± 0.89	13.6 ± 0.5	2.78 ± 0.03	5.4	5.4	4.2
Compound 7	142.7 ± 24.5	364 ± 10	72 ± 11	5.1	0.39	1.98
Compound 8	0.5 ± 0.33	0.52 ± 0.01	3.1 ± 0.3	0.17	0.88	0.15
Compound 9	4.6 ± 1.1	3.2 ± 0.7	2.2 ± 0.03	1.45	1.43	2.08
Compound 10	2.0 ± 0.79	2.5 ± 0.1	7.6 ± 0.9	0.33	0.8	0.26
Compound 11	1.3 ± 0.26	3.8 ± 0.05	0.26 ± 0.04	15	0.34	5
Compound 12	7.3 ± 1.43	27.1 ± 4.7	0.250 ± 0.04	108	0.27	9
Compound 13	30.1 ± 13.6	5.2 ± 1.7	246 ± 46	0.02	5.7	0.1
Compound 14	0.09 ± 0.02	0.07 ± 0.01	0.08 ± 0.01	0.88	1.2	1.1
Compound 15	6.0 ± 2.4	2.2 ± 0.2	3.6 ± 0.08	0.61	2.7	1.7
Compound 16	28.9 ± 25.51	3.4 ± 0.1	21.0 ± 4.0	0.16	8.5	1.4
Compound 17	70.7 ± 47.5	3.1 ± 1.3	167 ± 18	0.02	23	0.4

Inhibition constant K_i values were calculated from IC_{50} obtained from competition curves by the method of Cheng and Prussof [33]. Ratio of hMT_1/hMT_2 or ratio oMT_1/hMT_1 or hMT_2 (K_i/K_i) represents folds difference in affinity of each compound for 2-[^{125}I]-iodomelatonin binding to the ovine MT_1 and human MT_1 or MT_2 melatonin binding sites. Results are expressed by mean ± SEM.

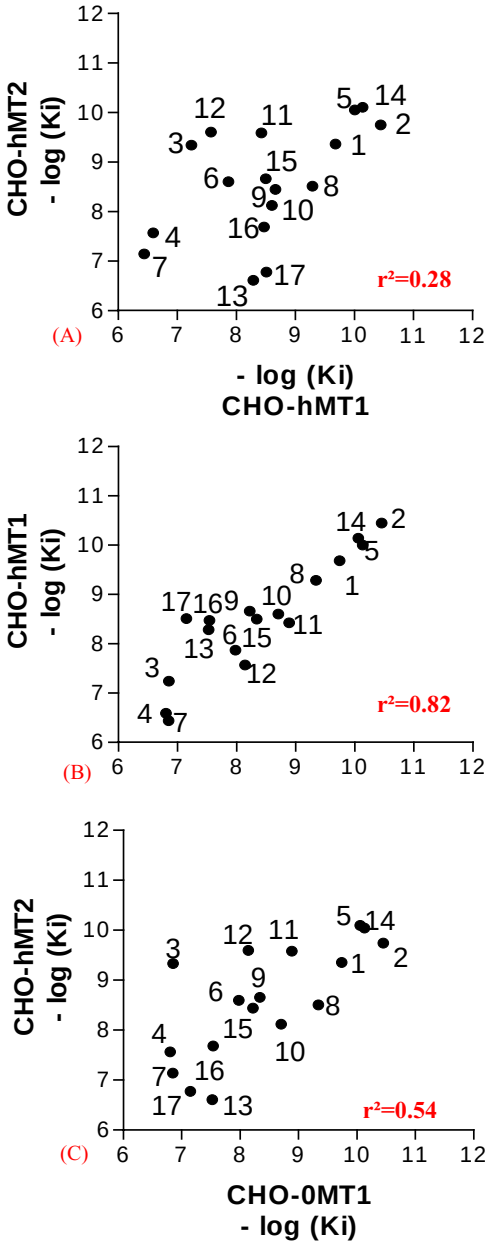

Fig. 3. Correlation analysis between ligand affinities (expressed as pK_i) measured at melatonin receptors expressed in CHO cells: hMT_1 and hMT_2 (A), oMT_1 and hMT_1 (B), oMT_1 and hMT_2 (C). Compounds were referred to as follows: 1: melatonin, 2: 2-iodomelatonin, 3: 4P-PDOT and 4: luzindole. For other number correspondence, see Section 2.

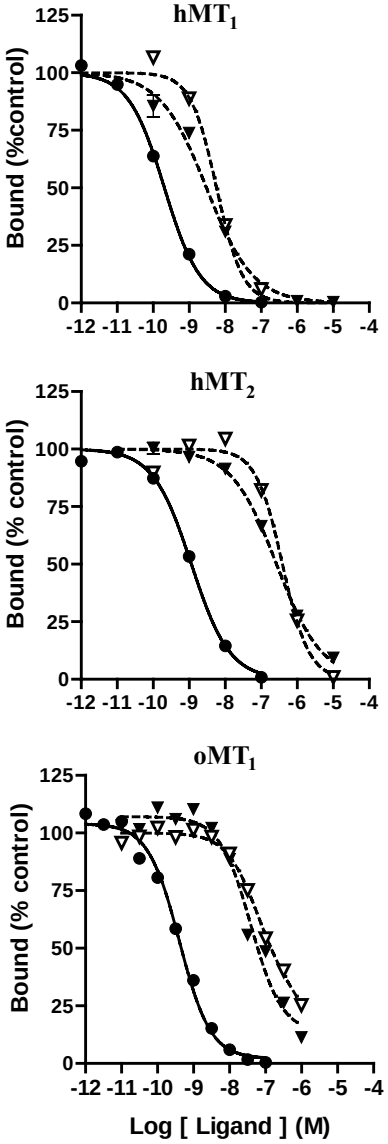

Fig. 4. Concentration–response curves on 2-[^{125}I]-iodomelatonin binding at hMT_1, hMT_2 and oMT_1 receptors expressed in CHO cells of melatonin (●) and the selective hMT_1 compound 13 (▼) and compound 17 (▽). Representative curves are shown in which each point is the mean of triplicate determinations. Similar results were obtained on at least three independent occasions.

The comparison between hMT_1, hMT_2 and oMT_1 receptor subtypes expressed in CHO cells showed a significant correlation between oMT_1 and hMT_1 ($r^2 = 0.82$, $P < 0.0001$, N = 17; Table 2; Fig. 3B) but a weaker correlation between oMT_1 and hMT_2 ($r^2 = 0.54$, $P < 0.0008$, N = 17; Table 2; Fig. 3C). In a detailed analysis, the selective hMT_1 ligands (compounds 13 and 17) presented different affinities on oMT_1 and hMT_1 (ratio oMT_1/hMT_1: 5.7 and 23, respectively) (Table 2; see Fig. 4). Similarly, the ratio oMT_1/hMT_2 for the selective hMT_2 compounds was lower than the hMT_1/hMT_2 ratio (compound 11: 5 vs. 15; compound 12: 9 vs. 108, respectively; see Table 2). Moreover, the nonselective compound 16 (ratio $hMT_1/hMT_2 = 0.16$) presented a different affinity between oMT_1 and hMT_1 (ratio $oMT_1/hMT_1 = 8.5$) (Table 2).

3.3. Functional assays: [^{35}S]-GTPγS binding on human and ovine melatonin receptors

The agonist or antagonist potency of all the ligands tested in the present study were measured by using the binding of the nonhydrolysable radionucleotide [^{35}S]-GTPγS on membranes of hMT_1-, hMT_2- and oMT_1-expressing CHO

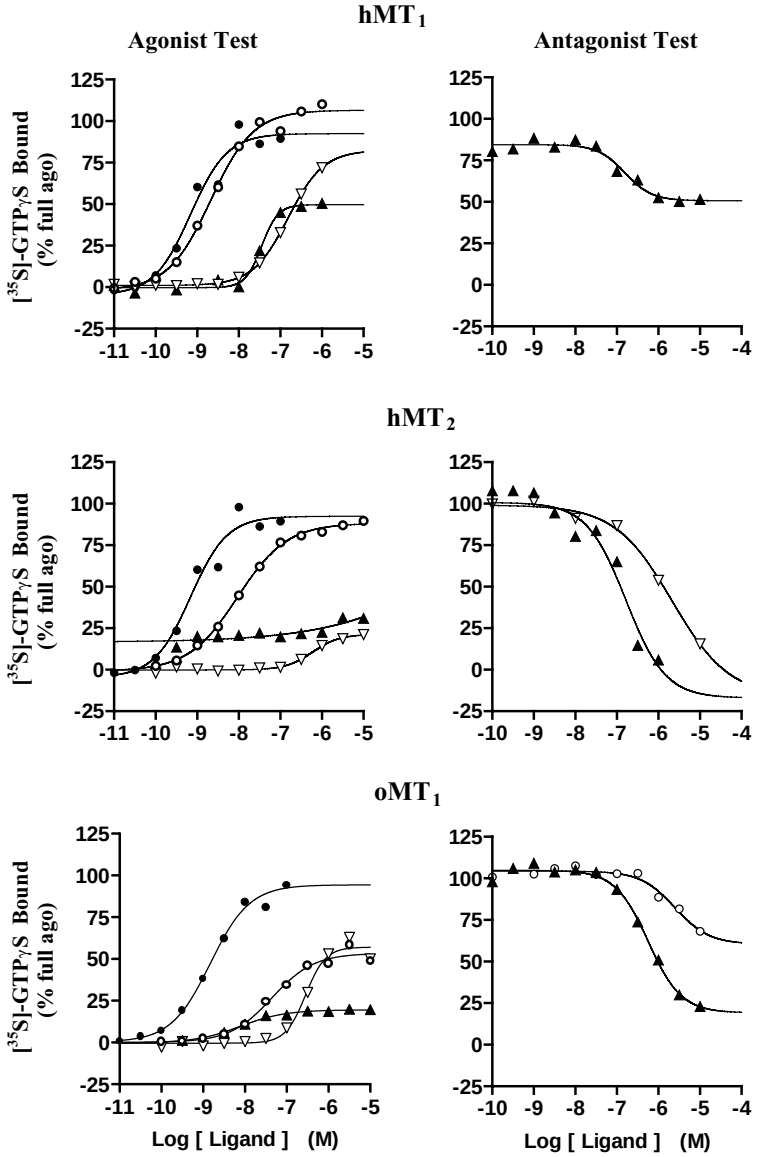

Fig. 5. Concentration–response curves on [^{35}S]-GTPγS binding at hMT_1, hMT_2 and oMT_1 receptors expressed in CHO cells of melatonin (●), compound 11 (▲), compound 10 (○) and compound 13 (▽) when tested alone (left panel) or in the presence of a fixed dose of melatonin (30 nM for hMT_1, 3 nM for hMT_2 and 100 nM for oMT_1, right panel). Data are expressed as a percentage of maximal stimulation of [^{35}S]-GTPγS binding induced by melatonin (=100%) at 1 μM in the agonist assay or by 30 nM for hMT_1, 3 nM for hMT_2 or 100 nM for oMT_1 in the antagonist assay. Representative curves are shown in which each point is the mean of triplicate determinations. Similar results were obtained on at least three independent occasions.

cells (Fig. 5). An increase in the [^{35}S]-GTPγS binding to membranes was observed with compounds 5, 6, 14 and 2-I-MLT whereas 4P-PDOT and luzindole decreased MLT activity on hMT_1, hMT_2 and oMT_1 receptors. As compared to MLT, compounds 12 and 13 exerted agonist activity of the [^{35}S]-GTPγS binding on hMT_1 and oMT_1 whereas compounds 7, 9, 15, 16 and 17 inhibited the MLT agonism at those two receptors, i.e. are antagonists. A few compounds presented different efficacies on [^{35}S]-GTPγS binding as a function of the receptor subtypes. Compounds 8 and 10 exerted full agonists effects on hMT_1 (E_{max} = 99 and 87%, respectively) and hMT_2 (E_{max} = 102 and 90%, respectively) receptors and partial activity on oMT_1 (E_{max} = 48% for both compounds; see Fig. 5). Those partial activities were confirmed in the antagonist protocol in which compounds 8 and 10 decreased MLT activity by 61 and 47%, respectively. Compound 13 exerted a partial agonist effect on oMT_1 (E_{max} = 68%) and hMT_2 (E_{max} = 27%) receptors while a full agonist activity was observed at hMT_1 (E_{max} = 95%). Compound 11 exerted a partial agonist effect on hMT_1 (E_{max} = 48%) while antagonist activity was observed fully on hMT_2 (I_{max} = 100%) and on oMT_1 (I_{max} = 97%) (Table 3).

4. Discussion

The use of melatonin receptor ligands allowed us to demonstrate that a cellular system stably expressing a single receptor subtype, i.e. oMT_1, is a valuable tool for

Table 3
Comparison of functional response to ligands at hMT_1, hMT_2 and oMT_1 expressed in CHO cells

	Ovine MT_1 K_i (nM)				Human MT_1 K_i (nM)				Human MT_2 K_i (nM)			
	Agonist		Antagonist		Agonist		Antagonist		Agonist		Antagonist	
	EC_{50} (nM)	E_{max} (%)	K_B (nM)	I_{max} (nM)	EC_{50} (nM)	E_{max} (%)	K_B (nM)	I_{max} (nM)	EC_{50} (nM)	E_{max} (%)	K_B (nM)	I_{max} (nM)
MLT	2.1 ± 0.2	100	nd	nd	2.2 ± 0.4	110 ± 2	nd	nd	0.49 ± 0.04	104 ± 6	nd	nd
2-Iodo-MLT	0.36 ± 0.02	110 ± 7	nd	nd	0.18 ± 0.04	108 ± 3	nd	nd	0.17 ± 0.05	121 ± 13	nd	nd
4P-PDOT	Inactive	<10	17 ± 5	89 ± 6	Inactive	<10	93 ± 39	95 ± 7	1.3 ± 0.3	19 ± 2	1.8 ± 0.8	075 ± 13
Luzindole	Inactive	<10	16 ± 3	58 ± 9	Inactive	<10	42 ± 4	102 ± 7	Inactive	<10	33 ± 3	85 ± 4
Compound 5	1.6 ± 0.2	118 ± 16	nd	nd	1.1 ± 0.2	95 ± 7	nd	nd	0.08 ± 0.01	102 ± 5	nd	nd
Compound 6	395 ± 109	90 ± 14	nd	nd	327 ± 170	117 ± 10	nd	nd	1.42 ± 0.2	97 ± 5	nd	nd
Compound 7	Inactive	<10	12 ± 1	48 ± 1	Inactive	<10	53 ± 18	85 ± 6	20 ± 2	34 ± 5	12 ± 5	75 ± 3
Compound 8	3.5 ± 0.5	48 ± 6	1.7 ± 0.4	61 ± 4	1.8 ± 0.3	99 ± 8	nd	nd	0.82 ± 0.13	102 ± 1	nd	nd
Compound 9	Inactive	<10	3.1 ± 1.1	109 ± 7	Inactive	<10	5.4 ± 2.9	83 ± 10	1.1 ± 0.2	31 ± 8	2.9 ± 1.5	53 ± 2
Compound 10	57 ± 19	48 ± 2	9.9 ± 3.5	47 ± 4	9.3 ± 7.5	87 ± 6	nd	nd	2.5 ± 0.6	90 ± 9	nd	nd
Compound 11	Inactive	<10	4.1 ± 1.5	97 ± 9	17 ± 9	48 ± 4	0.35 ± 0.13	38 ± 8	Inactive	<10	0.25 ± 0.01	100 ± 7
Compound 12	141 ± 32	31 ± 1	8.3 ± 2.8	38 ± 8	97 ± 43	23 ± 5	45 ± 11	52 ± 9	0.72 ± 0.13	27 ± 1	1.5 ± 0.4	70 ± 9
Compound 13	675 ± 161	68 ± 5	nd	nd	374 ± 126	95 ± 8	nd	nd	496 ± 99	27 ± 8	147 ± 7	96 ± 4
Compound 14	1.2 ± 0.2	87 ± 3	nd	nd	0.23 ± 0.04	88 ± 14	nd	nd	0.06 ± 0.02	77 ± 2	nd	nd
Compound 15	Inactive	<10	5.7 ± 1.8	99 ± 14	nd	nd	5.8 ± 1.6	88 ± 5	2.8 ± 0.5	37 ± 4	1.5 ± 0.5	52 ± 2
Compound 16	Inactive	<10	5.8 ± 2.3	98 ± 2	Inactive	<10	4.3 ± 0.3	79 ± 12	12 ± 2	47 ± 17	19 ± 7	81 ± 26
Compound 17	Inactive	<10	13 ± 5	62 ± 4	73 ± 7	25 ± 1	11 ± 1	68 ± 10	405 ± 35	39 ± 3	29 ± 11	80 ± 4

The agonist or antagonist activities of the compounds at the recombinant melatonin receptors were evaluated by using the [^{35}S]-GTPγS binding assay. EC_{50} and E_{max} were determined through agonist activity of the compounds increasing the basal [^{35}S]-GTPγS binding level (see Fig. 4). When compounds failed to this level in the agonist test (see Fig. 4), K_B and I_{max} were calculated in the antagonist test. nd: not determined.

studying the pharmacology of native ovine melatonin receptor. Indeed, similar binding profiles for a series of ligands were obtained with PT membranes and with oMT_1-expressing CHO cells. In comparison to human melatonin receptors expressed in CHO cells, oMT_1 presented a different binding profile to hMT_2 but similar to hMT_1. However, differences in the affinities and in the functional activities of some compounds were observed between the human and ovine MT_1 receptors.

A single melatonin receptor has been cloned from sheep PT [17] and has been classified as MT_1 on the basis of its closer homology of amino acid sequence with hMT_1 than with hMT_2 (79 and 56%, respectively) [7,8]. However, the lack of selective compounds for either hMT_1 or hMT_2 has not allowed the characterization of the pharmacological profile of this receptor present in high density in the PT [7,34–36]. Saturation of 2-[^{125}I]-MLT binding revealed that recombinant oMT_1 expressed in CHO cell lines and PT sites were saturable with dissociation constants in the picomolar range (14 and 21 pM, respectively). Those values are similar to those previously published [31,36,37]. This high affinity compares well with the affinity recorded for the recombinant hMT_1 receptor expressed in CHO cells [13]. Recently, new selective analogues have been identified that could discriminate between the human MT_1 and MT_2 receptors [13]. The availability of these new specific MT_1 or MT_2 compounds thus constitutes a critical step in the pharmacological characterization of MLT binding sites and in the understanding of their role in the multiple MLT actions.

The data clearly showed that the binding in PT membranes and in recombinant oMT_1 receptor are identical. Furthermore, for melatonin, iodo-melatonin and S 20750 (compound 6), the data presented in the present work are similar to those previously reported in the literature on the native ovine receptor [34,37]. The comparison of the binding profiles of oMT_1, hMT_1 and hMT_2 showed that oMT_1 is pharmacologically much closer to hMT_1 than to hMT_2. The fact that the oMT_1 receptor presents a similar binding profile than hMT_1 results probably from the higher degree of identity within the amino acid sequence of oMT_1 with hMT_1 than with hMT_2. Nevertheless, despite an overall good correlation between oMT_1 and hMT_1 affinities, a detailed analysis showed that the two selective hMT_1 ligands, compounds 13 and 17, were less potent at oMT_1. These differences are probably due to the chemical structures of these two ligands. Indeed these two compounds have dimeric structures and the presence of a bulky group in position 7 instead of the methoxy group may be less favourable for the ligand binding pocket of the oMT_1 than for that of hMT_1 receptor. The same argument may explain the selectivity of these two ligands for hMT_1 towards hMT_2.

The agonist/antagonist activities of molecules have been evaluated in CHO cells using [^{35}S]-GTPγS binding assay. Our results show that the pharmacology of oMT_1 melatonin receptor is different for some compounds than either human melatonin receptors. For example, compounds 8 and 10 exerted full agonist activity at hMT_1 and hMT_2 while they are partial agonists at oMT_1 (*circa* 50% of the melatonin effect). In the presence of either product, the efficacy of MLT was decreased by *circa* 50%, in agreement with the definition of a partial agonist. Moreover, compound 11 exerts partial agonist effects at hMT_1 ($E_{max} = 48\%$, $I_{max} = 38\%$) while its antagonist activity was observed at both hMT_2 and oMT_1 with a maximal inhibition of 100 and 97%, respectively.

This observation is consistent with differences in activity profile previously observed between species. For example, ML23 (*N*-(2,4-dinitrophenyl)-5-methoxytryptamine) blocks the effect of MLT on dopamine release from rat hypothalami and inhibits MLT enhancement of sexual maturation in rats [39,40]. However, ML23 does not prevent the testicular regression in golden hamster nor the release of dopamine in chick retina, both induced by MLT [41,42]. Furthermore, ML23 presents neither agonist or antagonist effects on Xenopus melanophore pigment aggregation [43]. Nevertheless, this last observation could be due to species differences or could reflect differences in metabolism of ML23. In the present study, our compounds permit to demonstrate for the first time, obvious pharmacological differences between oMT_1 and hMT_1 receptors. Pharmacological differences between species have been reported for other receptors [44–47].

The comparison between hMT_1 and oMT_1 amino acid sequences showed a high homology in the intracellular (81%) and transmembrane domains (86%) in opposition to extracellular domains (59%). Recent studies using mutagenesis approaches have demonstrated the importance of some of the conserved residues that are involved in G protein modulation or in affinity of ligands [48–50]. Mutations at the Ala^{252} and Gly^{258} residues located in the transmembrane domain VI abolished the high affinity of 2-[^{125}I]-MLT to the mutated hMT_1 expressed in COS-7 cells [50]. Moreover, Val^{208} and His^{211} of the ovine receptor that correspond to the Val^{192} and His^{195} in hMT_1 (according to Reppert *et al*. [7]) are involved in affinities and intracellular responses of ligands [48,49]. In contrast, higher variability of the sequences between hMT_1 and oMT_1 was observed in the N- and C-terminal regions (73 and 24% respectively), in the third intracellular loop (33%) and the fourth transmembrane domain (24%). The differences in the amino acid sequence between oMT_1 and hMT_1 could account for the different responses to [^{35}S]-GTPγS binding induced by the ligands and could point to the importance of specific residues of the receptor, yet to be determined precisely.

In conclusion, the results of this pharmacological study demonstrated that the only ovine melatonin receptor cloned to date and classified as MT_1, presents a binding profile closer to hMT_1 than to hMT_2. However, despite the high degree of homology between the oMT_1 and

hMT_1 receptors, differences in their functionality have been observed.

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