Ligand efficacy and potency at recombinant human MT_2 melatonin receptors: evidence for agonist activity of some mt_1-antagonists

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1 NIH3T3 fibroblast cells transfected with the full-length coding region of the MT_2 human melatonin receptor stably expressed the receptor that is coupled to a pertussis toxin-sensitive G protein and exhibits high affinity for melatonin ($K_I = 261$ pM).

2 The order of apparent affinity for selected compounds was: 4-phenyl-2-propionamidotetralin (4P-PDOT) > 2-phenylmelatonin > 2-iodomelatonin > 2-bromomelatonin > 6-chloromelatonin ≥ melatonin > luzindole > N-acetyl-tryptamine ≥ N-[(2-phenyl-1H-indol-3-yl)ethyl]cyclobutanecarboxamide (compound 6) > N-acetylserotonin.

3 4P-PDOT exhibited a very high selectivity (~22,000 times) for the MT_2 receptor with respect to the mt_1 receptor subtype, as tested in comparative experiments with membrane preparations from NIH3T3 cells stably transfected with the human mt_1 receptor.

4 MT_2 melatonin receptors mediated incorporation of [^{35}S]-GTPγS into isolated membranes *via* receptor catalyzed exchange of [^{35}S]-GTPγS for GDP. The relative intrinsic activity and potency of the compounds were subsequently studied by using [^{35}S]-GTPγS incorporation. The order of potency was equal to the order of apparent affinity. Melatonin and full agonists increased [^{35}S]-GTPγS binding by 250% over basal (taken as 100%). Luzindole did not increase basal [^{35}S]-GTPγS binding but competitively inhibited melatonin-stimulated [^{35}S]-GTPγS binding, thus exhibiting antagonist action.

5 The other two mt_1 antagonists used here, 4P-PDOT and N-[(2-phenyl-1H-indol-3-yl)ethyl]cyclo-butanecarboxamide, behaved as partial agonists at the MT_2 subtype, with relative intrinsic activities of 0.37 and 0.39, respectively.

6 These findings show, for the first time, important differences in the intrinsic activity of analogues between the human mt_1 and MT_2 melatonin receptor subtypes.

Keywords: Melatonin; MT_2 receptor; melatonin; relative efficacy; melatonin analogues; melatonin antagonists; [^{35}S]-GTPγS binding

Abbreviations: 4P-PDOT, 4-phenyl-2-propionamidotetralin; Compound 6, N-[(2-phenyl-1H-indol-3-yl)ethyl]cyclobutanecarbox-amide

Introduction

Melatonin is a hormone secreted rhythmically by the mammalian pineal gland. This hormone plays a central role in the regulation of many types of circadian and seasonal behaviour in mammals (Morgan *et al.*, 1994). In man, melatonin elicits circadian, cardiovascular and hypnotic effects (Reppert *et al.*, 1996; Dollins *et al.*, 1994; Lewy *et al.*, 1995; Cagnacci *et al.*, 1998). Melatonin appears to evoke its effects through high affinity, G protein-coupled receptors (Morgan *et al.*, 1994). Currently, two subtypes of human melatonin receptors have been identified: mt_1 (preferentially expressed in the brain) and MT_2 (preferentially expressed in the retina) (Reppert *et al.*, 1994; 1995). Both native and recombinant melatonin receptors are negatively coupled to adenylyl cylase by pertussis toxin-sensitive G proteins (Morgan *et al.*, 1994; Reppert *et al.*, 1994; 1995). Little is known about the relative roles of the two receptor subtypes in the transduction of the melatonin signal. A recent study with mt_1 receptor-deficient mice has suggested that the two melatonin effects on the mouse suprachiasmatic nuclei, inhibition of neuronal firing and entrainment of the circadian rhythm, could be independently mediated by the two receptor subtypes (Liu *et al.*, 1998). The study of the different roles of the two receptor subtypes is further impeded by the lack of selective melatonin receptor agonists. Some melatonin receptor antagonists have been reported to be highly selective for the human MT_2 receptor subtype (Dubocovich *et al.*, 1997), but their efficacy has not yet been tested at the human melatonin receptor subtypes. For this reason we determined the affinity and efficacy of one of these analogues (4P-PDOT) at both human mt_1 and MT_2 receptors in the present study.

Recently we have studied the affinity and efficacy of a number of known melatonin analogues at the mt_1 human melatonin receptor stably expressed in NIH3T3 fibroblast cells, using the agonist-mediated [^{35}S]-GTPγS binding method for evaluation of the efficacy (Nonno *et al.*, 1998). Agonist stimulation of G protein-coupled receptors induces conformational changes in their cognate G proteins, leading to activation of the G protein and effector activation. The essential step for G protein activation is the GDP/GTP exchange (Birnbaumer *et al.*, 1990), that can be quantified by the use of [^{35}S]-GTPγS (Hilf *et al.*, 1989; Lorenzen *et al.*, 1993). The amount of [^{35}S]-GTPγS incorporation into membranes at maximal concentrations of agonist is related to the intrinsic activity of the agonist itself.

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Studies on the efficacy of melatonin analogues at the human MT$_2$ receptor are still lacking. Here we report the pharmacological characterization of the MT$_2$ receptor stably expressed in NIH3T3 mouse fibroblast cells and the activity of known melatonin agonists, partial agonists and antagonists.

Methods

Cloning strategy and transfection

The human melatonin receptor MT$_2$ gene has ben cloned by means of PCR with specific oligonucleotides designed on the GenBank sequence number U25341 (Human MT$_2$ melatonin receptor mRNA, complete coding strand). Two pairs of primers have been used to clone the 5′ half and the 3′ half of the gene from human Hippocampus cDNA (Clontech): 10F (5′ GCG ATG TCA GAG AAC GGC TCC TT) and 535R (5′ ACT CCA GGG ACC CCA CAA AGA AGT) to amplify from nucleotide 9 (including the initiating methionine) to 559; 399F (5′ CAA TAT CAC TGC CAT CGC CAT TA) and 1081R (5′ CTA GAG AGC ATC TGC CTG GTG) to amplify from nucleotide 399 – 1101 (including the Amber codon). The PCR profile was as follows: 95°C for 5 min, (95°C for 1 min; 58°C for 1 min; 72°C for 1 min) for 5 cycles, (95°C for 1 min; 66°C for 1 min; 72°C for 1 min) for 30 cycles; 72°C for 7 min.

The PCR products have both been cloned (pGEM-T Vector System, Promega) and sequenced and they have been found to be identical to the U25341 sequence.

The 10F – 535R sequence overlaps for 160 bp with the 399F – 1081R sequence so that they share a Cfr10 I restriction site that is unique to the entire gene sequence. The two PCR products have been consequently cut by means of Cfr10 I restriction enzyme (Boehringer Mannheim) and ligated at their protruding ends. The final product was cloned in pcDNA3 expression vector (Invitrogen) and transfected in NIH3T3 cells by using liposomal transfection (DOTAP, Boehringer Mannheim) according to the manufacturer's instructions. The NIH3T3 cells were plated at a density yielding approximately 60% confluence at the time of transfection. Cells were cultured in Dulbecco's Modified Eagle's Medium containing high glucose (4.5 g l^{-1}), 10% calf serum, 1 mM sodium pyruvate, in 5% CO$_2$/95% air at 37°C. Selection with G418 (1 mg ml^{-1}) was started 48 h after transfection. Transformed NIH3T3 cells were isolated and the single colonies were selected by using 2-[^{125}I]-iodomelatonin binding (with a radioligand concentration of 100 pM). Colonies expressing apparent B$_{max}$ values higher than 150 fmol mg^{-1} of total cellular protein were plated in 150 cm^2 flasks.

Membrane preparation

NIH3T3 cells stably expressing the cloned human MT$_2$ receptor were grown to confluence. On the day of assay, cells were detached from flasks with 4 mM EDTA in 50 mM Tris-HCl (pH 7.4 at room temperature) and centrifuged at 1000 × g for 10 min at 4°C. The cells were then resuspended in 2 mM EDTA/50 mM Tris-HCl, homogenized in 10 – 15 volumes of ice-cold 2 mM EDTA/50 mM Tris-HCl with Ultra-Turrax and centrifuged at 50,000 × g at 4°C for 25 min. The final pellet was then resuspended in ice-cold 50 mM Tris-HCl assay buffer.

In experiments with pertussis toxin, cells were treated with 100 ng ml^{-1} of pertussis toxin in culture medium for the 24 h preceding the day of assay, and then prepared as described

above. Membrane protein levels were determined according to the method of Bradford (1976).

2-[^{125}I]-iodomelatonin binding

The final membrane concentration was 2 – 4 mg ml^{-1} and the protein concentration was 5 – 10 μg per tube. The binding conditions were described in detail elsewhere (Stankov *et al.*, 1991). The incubation time was 90 min. In preliminary experiments for the selection of colonies and in competition experiments, the total 2-[^{125}I]-iodomelatonin concentration was 100 pM. In saturation studies 2-[^{125}I]-iodomelatonin was added to achieve a concentration range of 10 up to 1000 pM. Nonspecific binding was measured in presence of 0.1 μM cold 2-iodomelatonin.

[^{35}S]-GTPγS binding

Agonist-stimulated [^{35}S]-GTPγS binding was studied by using a modification of previously published methods (Hilf *et al.*, 1989; Lorenzen *et al.*, 1993). The final pellet, obtained as described above, was resuspended in ice-cold 50 mM Tris-HCl assay buffer to give a final membrane concentration of 20 – 30 mg ml^{-1}. Then membranes (30 – 45 μg of protein per tube) were incubated for 30 min at 30°C, with and without various drugs, in assay buffer containing 0.3 – 0.5 nM [^{35}S]-GTPγS, 50 μM GDP, 100 mM NaCl and 3 mM MgCl$_2$.

The final incubation volume was 100 μl. Basal binding was assessed in the absence of drug and nonspecific binding was measured in presence of 10 μM GTPγS. In preliminary experiments, carried out to determine optimal conditions to study basal and melatonin-stimulated [^{35}S]-GTPγS binding to NIH3T3$_{MT2}$ membranes, GDP 0.1 – 100 μM and NaCl 1 – 100 mM were used. The incubation was terminated by adding 1 ml of ice-cold Tris-HCl buffer, pH 7.4, followed by rapid filtration under vacuum through Whatman GF/B glass fibre filters and by three washes with 3 ml of ice-cold Tris-HCl buffer, pH 7.4. Bound radioactivity was determined by liquid scintillation spectrophotometry after overnight extraction in 4 ml Filter-Count scintillation fluid.

Data analysis

Data are reported as means ± s.e.mean of at least three independent experiments that were each performed in duplicate (2-[^{125}I]-iodomelatonin binding experiments) or triplicate ([^{35}S]-GTPγS binding experiments). The IC$_{50}$, EC$_{50}$ and K_D values were determined by using nonlinear curve fitting strategies. Saturation curves were analysed using the one-site model compared with the two-site model. A two-site model was accepted only when the 'goodness-of-fit' was significantly ($P < 0.05$) improved by this model, as tested using a partial *F*-test procedure (De Lean *et al.*, 1978). Hill transformation of the data has been performed to calculate Hill slope values in saturation experiments. K_I values were calculated from the IC$_{50}$ values using the Cheng-Prusoff equation (Cheng & Prusoff, 1973). The data from [^{35}S]-GTPγS binding experiments are given as percentage of basal binding, where the basal binding was fixed as 100%. The relative intrinsic activity values are expressed as a fraction of melatonin maximal net stimulation. The analysis of competitive interaction between melatonin and luzindole was performed by using the equation:

$$pA_2 = \log(DR - 1) - n\log[B] \qquad (1)$$

where DR is the ratio of agonist IC$_{50}$ values with or without antagonist, and [B] is the antagonist concentration. When n

(the slope of the corresponding Schild plot) is not significantly different from 1, the pK_B (instead of pA_2) was calculated by nonlinear fitting by using the following equation, as proposed by Lew & Angus (1995):

$$pEC_{50} = -\log([B] + 10^{-pK_B} - \log c) \tag{2}$$

where the agonist pEC_{50} in the presence of a given antagonist concentration is plotted versus the antagonist concentration [B] and allows the calculation of the pK_B value as a fitted parameter. The parameter $-\log c$ is the difference between the antagonist pK_B and the agonist pEC_{50}.

In order to test the possible presence of 'spare' receptors in our system (defined as the fraction of the total receptor pool not required for maximal G protein activation in NIH3T3 cells) we used the equation proposed by Venter (1997):

$$H = -K_A/K_B\phi H + e^{ES} \tag{3}$$

where H is the height of the concentration effect curve, ϕ is a fixed concentration ratio [antagonist]/[agonist] and e^{ES} is an effect-stimulus parameter, related to efficacy, that may be defined as $e^{ES} = h_m/H_m$, that is maximum height of the concentration stimulus curve/maximum height of the concentration effect curve. An $e^{ES} = 1$ indicates the absence of 'spare' receptors.

Statistical significance of the data was determined by analysis of variance followed by the nonpaired two-tailed Student's *t*-test.

Drugs

2-[125I]-iodomelatonin (specificity activity = 2000 Ci mmol⁻¹) and [35S]-GTPγS (specific activity 1070 Ci mmol⁻¹) were purchased from Amersham (Buckinghamshire, U.K.). Melatonin, N-acetylserotonin, pertussis toxin, GDP and GTPγS were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 2-Iodomelatonin was obtained from RBI (Natick, MA, U.S.A.). 6-chloromelatonin was a gift from Ely Lilly laboratories (Indianapolis, IN, U.S.A.). 2-Bromomelatonin, 2-phenylmelatonin and N-[(2-phenyl-1*H*-indol-3-yl)ethyl]cyclobutanecarboxamide (compound 6) were synthesized as described elsewhere (Duranti *et al*., 1992; Spadoni *et al*., 1993; Garratt *et al*., 1995). Luzindole and 4-phenyl-2-propionamidotetralin (4P-PDOT) were from Tocris Cookson (Bristol, U.K.). Geneticin (G418) was purchased from GIBCO (Grand Island, NY, U.S.A.). General laboratory reagents including TrisHCl, Calf Serum, Dulbecco's Modified Eagle's medium were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Filter-Count scintillation fluid was from Packard (Downers Grove, IL, U.S.A.).

Results

Binding of 2-[125I]-iodomelatonin to the MT₂ receptor and competition studies

A number of clones were obtained and tested for MT₂ receptor expression with 2-[125I]-iodomelatonin (100 pM). The clone expressing the highest 2-[125I]-iodomelatonin binding (single point assay) was chosen for subsequent studies, in order to ensure a good effect-to-noise ratio in the G protein stimulation assay.

Saturation experiments show that, in the absence of sodium ions and GTPγS, 2-[125I]-iodomelatonin binds to a single class of high affinity binding sites (Hill slope = 1.01 ± 1.02), with a pK_D of 10.28 ± 0.05 and a B_{max} of 502 ± 38 fmol mg⁻¹ protein.

In order to investigate receptor-G protein coupling, a series of saturation experiments conducted in the presence of GTPγS and NaCl was performed. In the presence of GTPγS (100 μM) 2-[125I]-iodomelatonin binds to a single class of high affinity binding sites (Hill slope = 0.98 ± 0.02), with a pK_D of 10.1 ± 0.07 (not significantly different from the pK_D value obtained in absence of GTPγS, $t = 2.092$, $df = 4$, $P = 0.0523$) and a B_{max} of 585 ± 21 fmol mg⁻¹ protein, showing little difference to the binding profile in absence of GTPγS. In the presence of 500 mM NaCl, 2-[125I]-iodomelatonin binds to a single class of high affinity binding sites (Hill slope = 0.97 ± 0.03), with a pK_D of 9.92 ± 0.04 (significantly different from the pK_D value obtained in absence of GTPγS, $t = 5.44$, $df = 4$, $P < 0.05$) and a B_{max} of 592 ± 33 fmol mg⁻¹ protein. On the contrary, the saturation curves obtained in the presence of both 500 mM NaCl and 100 μM GTPγS were significantly better described by the two-site model (Hill slope = 0.80 ± 0.04, significantly different from unity, $t = 4.33$, $df = 3$, $P < 0.05$), with 20% of receptors being in the high affinity state, $pK_D = 10.25 ± 0.04$, and 80% in the low affinity state, with a pK_D of 9.38 ± 0.04.

The pharmacological profile of the transfected MT₂ receptor was studied in competition experiments with a number of known melatonin analogues (Figures 1 and 3). Melatonin showed a pK_I of 9.60 ± 0.06 (Table 1). The rank order of apparent affinity was: 4P-PDOT > 2-phenylmelatonin > 2-iodomelatonin > 2-bromomelatonin > 6-chloromelatonin ⩾ melatonin > luzindole > N-acetyl-tryptamine ⩾ N-[(2-phenyl-1*H*-indol-3-yl)ethyl]cylobutanecarboxamide (we will refer to this compound as compound 6) > N-acetylserotonin (pK_I values are shown in Table 1). 4P-PDOT competed with 2-[125I]-iodomelatonin binding (Figure 3) with a slope of −0.50 ± 0.03 (significantly different from unity, $t = 18.51$, $df = 4$, $P < 0.0001$), thus not allowing K_I calculation. The Hill slope values of all other compounds tested were between −0.9 and −1, and they were not significantly different from unity.

G protein activation by melatonin and its analogues

The basal [35S]-GTPγS binding to NIH3T3$_{MT2}$ membranes was 80 ± 5 fmol mg⁻¹ protein. Melatonin caused a dose-dependent increase of the basal binding, to reach a plateau at ≈250 ± 9% with a pEC_{50} value of 9.22 ± 0.04 (Figure 2; pEC_{50} and relative intrinsic activity data are reported in Table 1). 2-Bromomelatonin, 2-iodomelatonin, 2-phenylmelatonin and 6-chloromelatonin also increased basal binding with a similar maximum (262 ± 6, 225 ± 9, 245 ± 3 and 250 ± 2%), pEC_{50} values being 9.55 ± 0.03, 9.97 ± 0.18, 10.24 ± 0.06 and 9.41 ± 0.03, respectively. N-acetyl-serotonin, N-acetyl-tryptamine, compound 6 and 4P-PDOT behaved as partial agonists with relative intrinsic activities ranging from 0.37 to 0.81 (see Table 1 and Figure 1). Luzindole was without any effect on basal [35S]-GTPγS binding with the exception of a very little stimulation over basal (0.07 ± 0.02 relative to melatonin) at the highest concentrations tested.

Luzindole was further able to dose-dependently shift the melatonin concentration-effect curve when added at three different concentrations (10, 100 nM and 1 μM), showing competitive and surmountable antagonism. The slope of the Schild plot was not significantly different from unity therefore suggesting simple competitive antagonism. Analysis of these data with the equation proposed by Lew & Angus (1995) allowed calculation of a pK_B value of 7.94 ± 0.07 (Table 1). Using the equation proposed by Venter (1997), in order to investigate the presence of 'spare' receptors in our system, we
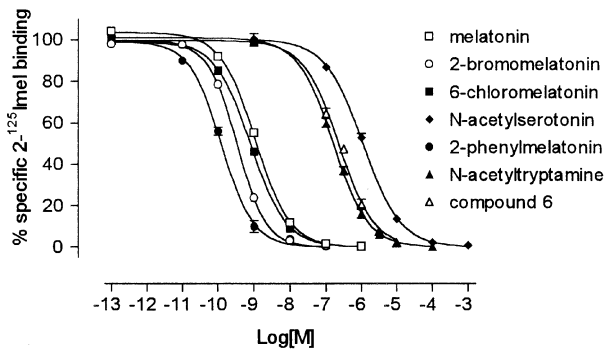
Figure 1 Competition curves of melatonin and its analogues for 2-[^{125}I]-iodomelatonin (2-^{125}Imel) binding to NIH3T3$_{MT2}$ membranes. The data are representative of a single experiment with each point determined in duplicate. The rank order of apparent affinity was 2-phenylmelatonin > 2-bromomelatonin > 6-chloromelatonin ≥ melatonin > N-acetyl-tryptamine ≥ compound 6 > N-acetylserotonin. The experiment was carried out as described in Methods, for 90 min at 37°C. Each point is the mean of duplicate determinations; the error bars indicate the standard deviation.

Table 1 Competition of 2-[^{125}I]-iodomelatonin binding and G protein activation for melatonin and its analogues

Compound	pK_I ± s.e.mean[a]	pEC_{50} ± s.e.mean[a]	IA ± s.e.mean[a]
Melatonin	9.60 ± 0.06	9.22 ± 0.04	1
2-Iodomelatonin	10.25 ± 0.08	9.97 ± 0.18	1.02 ± 0.01
2-Bromomelatonin	9.95 ± 0.06	9.55 ± 0.03	1.05 ± 0.02
2-Phenylmelatonin	10.43 ± 0.07	10.24 ± 0.06	0.98 ± 0.01
6-Chloromelatonin	9.62 ± 0.03	9.41 ± 0.03	1.00 ± 0.02
N-acetyl-serotonin	6.47 ± 0.02	6.51 ± 0.03	0.77 ± 0.03
N-acetyl-tryptamine	7.19 ± 0.04	7.03 ± 0.05	0.81 ± 0.02
Luzindole	7.95 ± 0.07	7.94 ± 0.04[c]	0.07 ± 0.02
4P-PDOT	10.80 ± 0.18[b]	10.91 ± 0.21	0.37 ± 0.01
Compound 6	7.07 ± 0.06	6.94 ± 0.09	0.39 ± 0.01

IA, relative intrinsic activity. [a]Results are expressed as means ± s.e.mean of at least three independent determinations performed in duplicate (competition studies) or in triplicate (G protein activation). [b]pIC$_{50}$ value, slope = −0.50 ± 0.05. [c]pK$_B$ value.

calculated an e^{ES} value of 0.99, consistent with the absence of 'spare' receptors, and a K_A/K_B value of 0.04.

Binding affinity and efficacy of 4P-PDOT and luzindole at the human mt$_1$ receptor

In previous work (Nonno *et al.*, 1998) we characterized the human mt$_1$ melatonin receptor with the same analogues used in this study with the exception of luzindole and 4P-PDOT, so we also studied the interaction of these two compounds with the mt$_1$ receptor for comparison with MT$_2$ (Figure 3). 4P-PDOT and luzindole inhibited 2-[^{125}I]-iodomelatonin binding to membrane preparation obtained from NIH3T3 cells stably expressing 600 fmol mg^{-1} protein of the mt$_1$ human receptor, with pK_I values of 6.98 ± 0.05 and 6.72 ± 0.04, respectively; comparison of the competition curves obtained with mt$_1$ and MT$_2$ membrane preparation reveals a high selectivity for 4P-PDOT (IC$_{50mt1}$/IC$_{50MT2}$ = 22,000) and, to a lesser extent, also for luzindole (K$_{Imt1}$/K$_{IMT2}$ = 16.6).

Both analogues behaved as antagonists in the G protein activation assay, being unable to significantly affect basal [^{35}S]-GTPγS binding to NIH3T3$_{mt1}$ membranes at any concentration tested. In these experiments melatonin (100 nM) increased [^{35}S]-GTPγS binding to 350 ± 10% over basal.
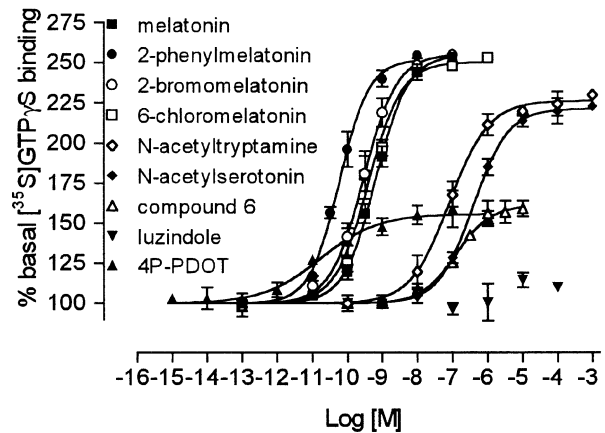

Figure 2 Comparison of the stimulation of [^{35}S]-GTPγS binding to NIH3T3$_{MT2}$ membranes by melatonin and its analogues. The data are representative of a single experiment with each point determined in triplicate. The order of agonist potency (with relative intrinsic activities obtained in this experiment reported in brackets) was: 4P-PDOT (0.37) > 2-phenylmelatonin (0.98) > 2-bromomelatonin (1.03) > 6-chloromelatonin (0.97) > melatonin (1) > N-acetyl-tryptamine (0.82) > compound 6 (0.41) > N-acetylserotonin (0.78). Luzindole was without any effect on basal [^{35}S]-GTPγS binding. The experiment was carried out as described in Methods, for 30 min at 37°C. Each point is the mean of triplicate determinations; the error bars indicate the standard error. Values represent percentage of basal binding, defined as 100%.
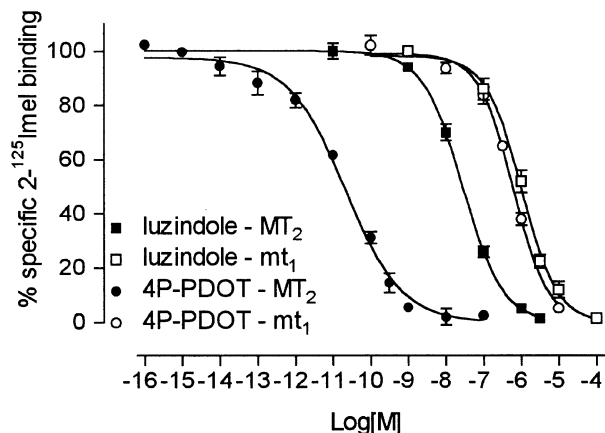

Figure 3 Comparison of competition curves for 4P-PDOT and luzindole to 2-[^{125}I]-iodomelatonin (2-^{125}Imel) binding to NIH3T3$_{MT2}$ (closed symbols) and NIH3T3$_{mt1}$ (open symbols) membranes. The data are representative of a single experiment in which each time point is determined in duplicate. Both 4P-PDOT and luzindole show higher affinity for the MT$_2$ receptor subtype. Note the slope of the competition curve of 4P-PDOT (−0.58) in NIH3T3$_{MT2}$ membranes with respect to the slope of luzindole (−0.91) in NIH3T3$_{MT2}$ membranes and to the slope of 4P-PDOT (−0.96) and luzindole (−0.94) in NIH3T3$_{mt1}$ membranes. The experiment was carried out as described in Methods, for 90 min at 37°C. Each point is the mean of duplicate determinations; the error bars indicate the standard deviation.

Pertussis toxin sensitivity

A 24 h pretreatment of NIH3T3$_{MT2}$ cells with 1 μg ml^{-1} pertussis toxin (PTX) completely abolished the melatonin induced increase in [^{35}S]-GTPγS binding. Basal [^{35}S]-GTPγS binding was significantly lower in membranes prepared from PTX-pretreated cells (45 ± 6%) than in membranes prepared

from control cells (100%). Melatonin stimulated binding was $245 \pm 12\%$ in control cells while it was not significantly different from basal values in PTX-pretreated cells (the basal [^{35}S]-GTPγS binding to PTX-pretreated membranes was 37 ± 3 fmol mg^{-1} protein and the melatonin-stimulated binding was 40 ± 2 fmol mg^{-1} protein).

Discussion

MT₂ human melatonin receptors stably expressed in NIH3T3 cells bound melatonin and its analogues with high affinity and mediated incorporation of [^{35}S]-GTPγS into isolated membranes *via* receptor-catalyzed exchange of [^{35}S]-GTPγS for GDP. G protein activation was completely abolished by pertussis toxin treatment, showing Gi/o coupling with MT₂ receptors.

2-[^{125}I]-iodomelatonin bound to a single class of high affinity binding sites and this binding was resistant to modulation by GTPγS. Both GTPγS and NaCl, when not coincubated, reduced the affinity of 2-[^{125}I]-iodomelatonin for the MT₂ melatonin receptor, probably by inducing a partial dissociation of R/G complexes, but the Hill slopes (0.98 and 0.97, respectively) calculated from these saturation curves indicate a homogeneous receptor population. On the contrary, receptor/G protein complexes were efficiently dissociated in presence of both GTPγS and sodium chloride (Hill slope of 0.8 and significant improvement of the 'goodness of fit' with the two-site model). These data indicate that most of the melatonin receptors expressed in NIH3T3$_{MT2}$ cells were coupled with G proteins in the presence of a full agonist (radiolabeled 2-iodomelatonin), suggesting that in our system the G protein concentration is not limiting; this conclusion is supported by the finding that no spare receptors were present in our system, as measured with the method proposed by Venter (1997). GTPγS-insensitivity indicates that the receptors form very stable receptor/G protein complexes. A GTPγS-insensitivity has been only reported for the melatonin receptors expressed in bovine (Nonno *et al.*, 1995) and in the ovine hippocampus (Barrett *et al.*, 1994), but not in other native tissues that express melatonin binding sites (Morgan *et al.*, 1994), nor in the human cerebellum (Fauteck *et al.*, 1994) that expresses the mt₁ subtype (Mazzucchelli *et al.*, 1996). We recently reported insensitivity to modulation of receptor coupling to G proteins by GTPγS also for the mt₁ subtype stably expressed in NIH3T3 cells (Nonno *et al.*, 1998), while both recombinant mt₁ and MT₂ subtypes were sensitive to guanine nucleotides when transiently expressed in COS-7 cells (Dubocovich *et al.*, 1997). In that sense, the GTPγS-sensitivity of melatonin receptors seems to be tissue- or system-dependent. Similar findings have been reported for other PTX-sensitive G protein-coupled receptors, such as A₁ adenosine (Nanoff *et al.*, 1995) and the human 5HT$_{1a}$ receptor (Varrault *et al.*, 1992). Furthermore, Nanoff and coworkers (1997) reported the partial purification of a membrane protein which stabilizes a tight receptor/Gi protein coupling mode of the A₁ adenosine receptor; this protein, 'coupling cofactor', is responsible for the resistance of R/G complexes to modulation by guanine nucleotides. Wreggett & De Lean (1984) have also suggested that stability of R/G complexes could be a common feature for many Gi/o-coupled receptors.

The pharmacological profile obtained with competition studies reveals that melatonin and 6-chloromelatonin share the same apparent affinity for the MT₂ receptor, that is different from the findings with the mt₁ subtype, to which melatonin binds with a 6–7 times higher affinity than 6-chloromelatonin

(Nonno *et al.*, 1998), thus confirming previous reports (Reppert *et al.*, 1994; 1995; Dubocovich *et al.*, 1997). 4P-PDOT was the compound with the highest affinity to the MT₂, while it showed only a modest affinity for the mt₁ subtype, thus revealing a very high mt₁/MT₂ selectivity (∼22,000 fold). Studies of affinity of melatonin analogues for recombinant human mt₁ and MT₂ receptor subtypes are limited. Dubocovich *et al.* (1997) studied the affinity of a number of melatonin agonists, partial agonists and antagonists for both the mt₁ and MT₂ human melatonin receptor expressed in COS-7 cells. They reported that 4P-PDOT and some structurally-related analogues bound with ∼300 times higher affinity to the MT₂ subtype, 4P-PDOT pK_I values being 8.8 at MT₂ and 6.3 at the mt₁ subtype, while in the present study 4P-PDOT showed a pIC$_{50}$ of 10.8 for the MT₂ receptor and a pK_I value of 6.98 for the mt₁ subtype. We cannot exclude that the MT₂ affinity of 4P-PDOT reported in the present work could be overestimated due to the very low slope value of its competition curves, accounting in part for the differences observed between the two studies and for the very high selectivity that we measured. In the case of luzindole, in contrast, our results for both mt₁ and MT₂ subtypes (pK_I values of 6.72 and 7.95, respectively) were very similar to those reported by Dubocovich *et al.* (1997) (pK_I values of 6.8 and 8).

To our knowledge the present work is the first study that reports the efficacy of melatonin and its analogues for the MT₂ human melatonin receptor. The results of the G protein activation assay show that the human MT₂ melatonin receptor expressed in NIH3T3 cells is efficiently coupled to a PTX-sensitive G protein. Melatonin induced [^{35}S]-GTPγS binding in a dose-dependent manner with a pEC$_{50}$ value of 9.22. This value, similar to the other pEC$_{50}$ values reported here for agonists and partial agonists (see Table 1), is slightly lower than the pK_I value. This finding probably reflects the different binding conditions of the two assays: in fact the [^{35}S]-GTPγS binding experiments were performed in the presence of 50 μM GDP and 100 mM NaCl, that appears to induce the shift of a portion of the melatonin receptor to the low-affinity state, while the membrane preparations used for 2-[^{125}I]-iodomelatonin binding experiments appear to contain virtually only high-affinity binding sites (see data from 2-[^{125}I]-iodomelatonin saturation binding isotherms). This interpretation is supported by the fact that, according to the ternary complex model (Costa *et al.*, 1992), the differences induced by the presence of GDP and NaCl are higher for full agonists than for partial agonists and antagonist (the pK_I value and the pK_B value of luzindole are identical).

Luzindole was the only antagonist at the MT₂ subtype, while other putative melatonin antagonists such as compound 6 and 4P-PDOT behaved as partial agonists (with relative intrinsic activities of 0.39 and 0.37, the last showing very high potency (pEC$_{50}$ = 10.91). In a previous study we reported that compound 6 showed antagonist activity at the mt₁ receptor stably transfectd in NIH3T3 cells (K_B = 67 nM) (Nonno *et al.*, 1998). In the present work we show that luzindole and 4P-PDOT are also mt₁ antagonists as well. Luzindole and compound 6 are generally considered low affinity melatonin antagonists (Takaki *et al.*, 1997; Garratt *et al.*, 1995). In a recent work Dubocovich *et al.* (1997) studied the efficacy of 4P-PDOT and some structually related compound in the rabbit retina and found them to be high-affinity antagonists. However, the above considerations are based on efficacy studies performed in tissues or cells that express native melatonin receptors and may contain a heterogeneous receptor population. Therefore, they do not lend themselves to the unequivocal characterization of an analogue action at a

specific receptor subtype. For example, the antagonist activity of compound 6 has been tested on pigment aggregation in *Xenopus laevis* melanophores (Garratt *et al.*, 1995) that contain the Mel$_{1c}$ receptor, a melatonin receptor subtype that is not present in mammals (Reppert *et al.*, 1996), while luzindole antagonizes the functional responses to melatonin in the calcium-dependent dopamine release assay from the rabbit retina (Dubocovich, 1988) and in the pigment aggregation in *Xenopus laevis* melanophores (Sugden, 1992). The receptor subtype composition of the rabbit retina is not definitively known. Comparison of the antagonist affinity constants obtained from their ability to inhibit dopamine release mediated by melatonin with their affinity constants (2-[^{125}I]-iodomelatonin binding) determined in COS-7 cells expressing the human MT$_2$ receptors, suggested that the rabbit retina may contain MT$_2$ melatonin receptors (Dubocovich *et al.*, 1997). The difference between the results obtained with 4P-PDOT in the rabbit retina and the present data (obtained in NIH3T3 cells stably expressing human MT$_2$ melatonin receptors) could be due to diversity between the human and the rabbit MT$_2$ melatonin receptors; another possible explanation is that 4P-PDOT expresses residual intrinsic activity in our system because of the high receptor number, and that such activity could not be detected in native tissue.

On the basis of the data obtained with the human mt$_1$ and MT$_2$ receptors expressed in the same cell type (NIH3T3) and characterized with the same method for the evaluation of relative intrinsic activities (stimulation of [^{35}S]-GTPγS binding to membranes) we are able to draw some conclusions regarding the properties of compounds considered melatonin receptor antagonists, as tested in our system: (i) luzindole appears to antagonize both the human melatonin receptor subtypes, with low affinity to the mt$_1$ and moderate affinity for the MT$_2$; (ii) compound 6 behaves as a low-affinity mt$_1$-antagonist and as a low-affinity MT$_2$-partial agonist and (iii) 4P-PDOT is a low-affinity mt$_1$-antagonist and a high-affinity MT$_2$-partial agonist. These conclusions indicate that there are different requirements for antagonist action between the two human melatonin receptor subtypes and that those differences must be taken into account when studying the analogue's action in tissues for which the subtype composition is unknown.

In summary, agonist-stimulated [^{35}S]-GTPγS binding in NIH3T3 cells is a sensitive method to study the human MT$_2$ receptor activation of G proteins. MT$_2$ receptor activation is measured at a point preceding second messenger generation and allows one to distinguish compounds of differing intrinsic activity and potency.

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