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Characterisation of human melatonin mt_1 and MT_2 receptors by CRE-luciferase reporter assay

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Abstract

A cyclic AMP response element (CRE)-luciferase reporter gene assay was used to characterise the functional responses of human melatonin mt_1 and human melatonin MT_2 receptors, stably expressed in the human embryonic kidney cell line HEK293, to a series of six naphthalenic analogues of melatonin. By comparison to the observed melatonin-mediated inhibition of stimulated luciferase levels the naphthalenic series was identified as comprising agonists, partial agonists and one antagonist of melatonin mt_1 and melatonin MT_2 receptor function. Three of the agonist/partial agonist members of this series were also identified as displaying a functional selectivity for the melatonin MT_2 receptor. Competitive displacement of 2-[^{125}I]iodomelatonin binding to the ovine pars tuberalis melatonin ML_1 receptor demonstrated a close correlation to the observed functional luciferase responses of the human melatonin mt_1 receptor. We conclude that the CRE-luciferase reporter gene assay provides an effective functional screening method for the pharmacological characterisation of human melatonin receptor subtypes.

Keywords: Melatonin receptor; cAMP response element; Luciferase; Reporter gene assay; HEK293 cell

1. Introduction

During the hours of darkness melatonin is synthesised and released by the pineal gland (Reiter, 1991). The resultant increase in the concentration of circulating melatonin produces a systemic 'time-signal', identifying both the time of onset and the duration of the night. This hormonal signal however appears to modulate cellular effects in only a limited number of tissues (Morgan et al., 1994). It is now known that melatonin functions through the activation of G protein-coupled melatonin receptors (Ebisawa et al., 1994), and that the tissue specific expression of these receptors defines where melatonin-mediated processes occur (Reppert et al., 1996). Two tissues that express melatonin receptors are the hypothalamic suprachiasmatic nucleus (Weaver and Reppert, 1996) and the pars tuberalis of the pituitary (Morgan et al., 1989a; Reppert et al., 1994). The suprachiasmatic nucleus is the site of the biological circadian clock (Reppert et al., 1988) and the pars tuberalis has been implicated in the regulation of seasonal changes in plasma prolactin levels in seasonally breeding mammals (Morgan and Williams, 1996). Both tissues are sensitive to melatonin. The suprachiasmatic nucleus is an important potential therapeutic site as it has been demonstrated that melatonin mediates effects on both acute neuronal inhibition and phase shifting of mouse suprachiasmatic nucleus, potentially through the activation of two distinct melatonin receptor subtypes (Liu et al., 1997). In man the potential benefits from the modulation of melatonin receptors expressed in the suprachiasmatic nucleus are demonstrated by evidence that administration of melatonin can alleviate the symptoms of jet-lag and alter the sleep patterns of shift workers and the blind (Arendt et al., 1997).

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The cloning of melatonin receptors has identified at least three molecular subtypes, Mel_{1a}, Mel_{1b} and Mel_{1c} (Ebisawa et al., 1994; Reppert et al., 1994, 1995). Pharmacologically the Mel_{1a} and Mel_{1b} receptors have now been defined as melatonin mt_1 and melatonin MT_2 receptors.[1] The expression of these subtypes is known to be species specific. Mammals express either melatonin mt_1 receptor alone (e.g., sheep), or melatonin mt_1 and MT_2 receptors in combination (e.g., humans), whereas lower vertebrates appear to express melatonin mt_1, MT_2 and Mel_{1c} receptors (Reppert et al., 1996). All of these subtypes, previously described as displaying melatonin ML_1 receptor pharmacology (Dubocovich, 1988), have similar binding affinities for melatonin (K_i ca. 5×10^{-9} M) and display similar rank orders for the binding of common melatonin receptor ligands (Reppert et al., 1995; Dubocovich et al., 1997). Functionally, all of the cloned receptor subtypes mediate inhibition of cellular cyclic AMP levels by melatonin, presumably through activation of G_i proteins (Reppert et al., 1996). The reasons why three essentially identical subtypes have evolved are not currently understood; however, evidence about the tissue distribution of these receptors suggests that they may indeed possess intrinsic differences that are required to perform specific functions (Reppert et al., 1995). Recent studies have identified some drugs that are selective for melatonin MT_2 receptor binding, suggesting some differences in the structure, or flexibility, of the melatonin MT_2 receptor compared to the melatonin mt_1 receptor (Reppert et al., 1995; Conway et al., 1997; Dubocovich et al., 1997). Clearly more investigations are required to fully characterise any differences in melatonin receptor subtype structure, function and pharmacology.

Here we report the characterisation of the effects of a set of six naphthalenic bioisosteres of melatonin upon human melatonin mt_1 and human melatonin MT_2 receptors stably expressed in the human embryonic kidney cell line HEK293 (Conway et al., 1997). Included within this set of naphthalenic drugs were the previously reported melatonin receptor agonist S20098 (*N*-[2-(7-methoxy-1-naphthyl)-ethyl]acetamide) (Ying et al., 1996), the putative antagonist S20928 (*N*-[2-(1-naphthyl)ethyl]cyclobutylcarboxamide) (Ying et al., 1996) and S20642 (*N*-[2-(7-methoxy-1-naphthyl)ethyl]cyclobutylcarboxamide) a drug identified as displaying selective effects upon the displacement of 2-[^{125}I]iodomelatonin binding in various chicken tissues (Pang et al., 1997). Functional responses were measured using a cyclic AMP response element (CRE)-luciferase reporter gene assay, which provides an indirect measurement of cellular cyclic AMP levels by quantification of the cyclic AMP dependant enhancement of luciferase protein expression (Stratowa et al., 1995). This methodology is significantly faster and less labour intensive than assaying cyclic AMP directly, and has been demonstrated as accurately reporting the functional pharmacology of a number of G protein-coupled receptors (Himmler et al., 1993; Stratowa et al., 1995; George et al., 1997). We present data demonstrating that the CRE-luciferase reporter assay provides an effective method for the pharmacological characterisation of human melatonin mt_1 and MT_2 receptor subtypes. Our studies demonstrate that 3 naphthalenic agonist and partial agonist drugs display an apparent functional selectivity for the melatonin MT_2 receptor.

2. Materials and methods

2.1. Materials

Tissue culture media, supplements and sera were purchased from Life Technologies. 2-[^{125}I]iodomelatonin (2200 Ci/mmol) was obtained from NEN DuPont. Melatonin, forskolin and [Nle^4-D-Phe^7]α-melanocyte stimulating hormone ([Nle^4-D-Phe^7]α-MSH) were purchased from Sigma. Naphthalenic compounds, S20098 S20928, S20642, S22365 (*N*-[2-(7-propoxy-1-naphthyl)ethyl]acetamide), S22480 (4-(7-methoxy-1-naphthyl)-*N*-methylbutanamide) and S22029 (3-(7-methoxy-1-naphthyl)-*N*-methylpropanamide) (Fig. 1), were obtained from the Institut de Recherches Internationales Servier and synthesised as previously reported (Adam et al., 1992; Yous et al., 1992; Leclerc et al., 1998). Other general reagents were from Sigma or Life Technologies.

2.2. Cell lines

The generation and tissue culture requirements of HEK293 cells engineered to stably express human melatonin receptors has been previously reported (Conway et al., 1997). Since this previous report Mel_{1a} melatonin receptors have been reclassified as melatonin mt_1 receptors and Mel_{1b} melatonin receptors are reclassified as melatonin MT_2 receptors.[1] The HEK293 cell lines expressing human melatonin mt_1 and human melatonin MT_2 receptors are therefore renamed HEK-hmt_1 and HEK-hMT_2, respectively.

2.3. CRE-luciferase reporter plasmid construction

The plasmid p$(CRE)_2$TKCAT (Steger et al., 1991) was restricted with *Xba*I and *Xho*I and the ~220 bp fragment, containing two cyclic AMP response elements (CRE_2) upstream of the Herpes virus thymidine kinase promoter (TK), was purified and ligated into *Xba*I and *Xho*I restricted SK Bluescript (Stratagene). The CRE_2-TK

[1] Melatonin receptor classification recommended by NC_IUPHAR and published in receptor and ion channel nomenclature supplement 8, Trends. Pharmacol. Sci., 1998, pp. 52.

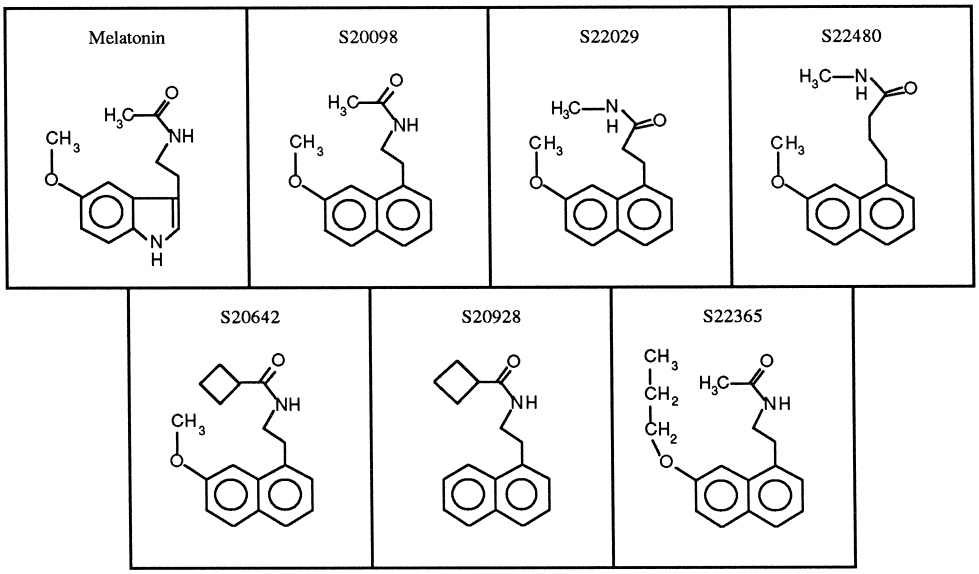

Fig. 1. Chemical structures of compounds used in this study.

encoding DNA was recovered from this construct as a *Sac*I and *Xho*I fragment (~240 bp) and ligated into *Sac*I and *Xho*I restricted pGL3-basic (Promega). This construction generated the CRE-luciferase reporter plasmid pGL3(CRE_2-TK), which was confirmed by DNA sequencing.

2.4. CRE-luciferase reporter assay

HEK-hmt_1 and HEK-hMT_2 cells were cultured to ~80% confluence at which time they were co-transfected with plasmids pGL3(CRE_2-TK) and pcDOR8 (Barrett et al., 1994) using the lipid transfection reagent FuGENE™ 6 (Boehringer Mannheim) as recommended by the manufacturer. Cells were cultured for 24 h post-transfection before being washed with phosphate buffered saline (pH 7.4), recovered in phenol red free Dulbecco's modified Eagle medium (supplemented with 10% (v/v) foetal calf serum) and seeded as 50 μl aliquots (5×10^4 cells) into white 96 well tissue culture plates. Following a further 24 h incubation, drug treatments were added in a 5.6 μl volume to triplet wells, and plates were left to incubate overnight (16 h). The level of expressed luciferase was measured by the addition of 50 μl of 'Constant Light Signal Reagent' (Boehringer Mannheim) to each well, and plates were briefly placed in the dark (30 min, room temperature) before being quantified on a Packard LumiCount™ Reader (1 s per well). Where experiments were designed to measure drug-mediated dose-response effects a control melatonin dose-response was always included. Data from the triplet wells was averaged and the control data normalised from a value of 100 (no melatonin) to a value of 0 (maximum response, 10^{-5} M melatonin). Data obtained for other drug treatments were normalised relative to the control values determined in that experiment. All experiments were performed on three or more separate occasions, and mean values ± S.E.M. determined. IC_{50} values and apparent efficacies were calculated by fitting a four-parameter logistic curve (Grafit software, Sigma) through the mean normalised data. For antagonists pA_2 values were determined by performing Schild analysis (Arunlakshana and Schild, 1959).

2.5. Cyclic AMP assay of ovine pars tuberalis cells

Ovine pars tuberalis cells were prepared as previously reported (Morgan et al., 1989b). Assays were performed in duplicate by the addition of 2×10^5 cells to 1.5 ml microcentrifuge tubes holding a 50 μl volume of forskolin (to

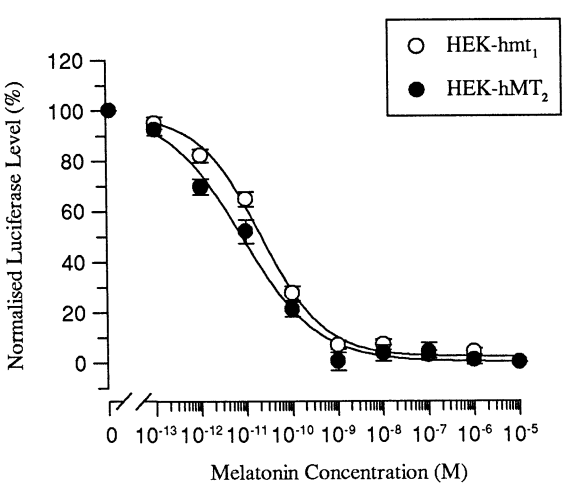

Fig. 2. Inhibition of [Nle^4-D-Phe^7]α-MSH stimulated luciferase levels by melatonin. Transfected HEK-hmt_1 and HEK-hMT_2 cells were exposed to 10^{-6} M [Nle^4-D-Phe^7]α-MSH and melatonin for 16 h. Data represent means ± S.E.M. of normalised values from 21 experiments performed in triplicate.

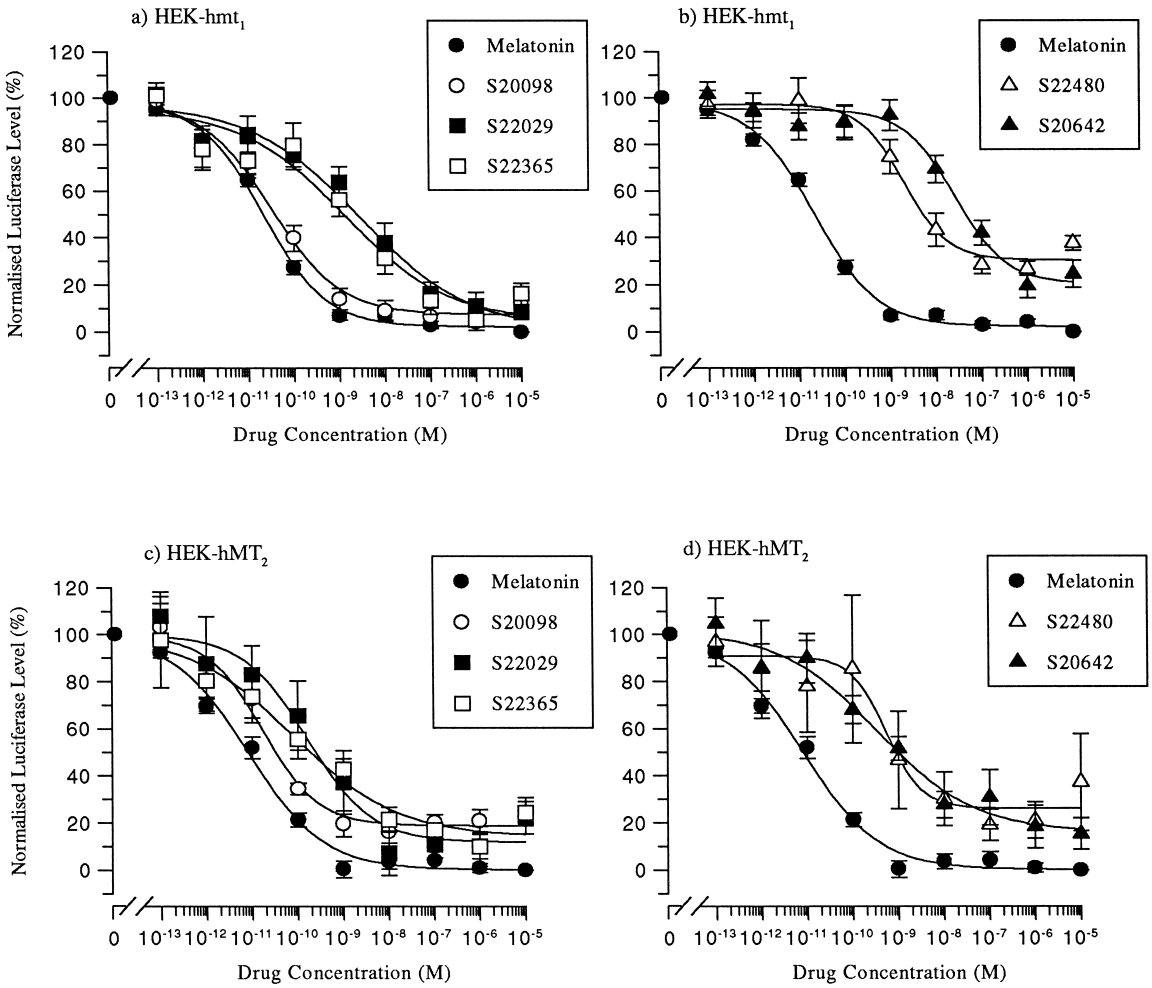

Fig. 3. Inhibition of [Nle^4-D-Phe^7]α-MSH stimulated luciferase levels by naphthalenic analogues of melatonin. Transfected HEK-hmt$_1$ and HEK-hMT$_2$ cells were exposed to 10^{-6} M [Nle^4-D-Phe^7]α-MSH and naphthalenic analogues of melatonin for 16 h. Data represent means ± S.E.M. of normalised values from at least three experiments performed in triplicate. IC_{50} values and apparent efficacies were determined by fitting a four-parameter logistic curve (Grafit software, Sigma) and are listed in Table 1. For clarity the data for HEK-hmt$_1$ (a,b) and HEK-hMT$_2$ (c,d) are split over two panels each. For reference the melatonin data from Fig. 2 is also plotted.

give 10^{-5} M final concentration) and other appropriate drugs. Reactions were incubated for 15 min at 37°C before being stopped by immersion in boiling water for 2 min. Measurement of cyclic AMP by radioimmunoassay was performed as previously reported (Morgan et al., 1990; Howell et al., 1994). Data were manipulated as stated

Table 1
Functional response parameters for melatonin receptor agonist and partial agonist effects upon [Nle^4-D-Phe^7]-α-MSH stimulated luciferase levels in transfected HEK-hmt$_1$ and HEK-hMT$_2$ cells. IC_{50} affinity constants were calculated by fitting a four-parameter logistic curve (Grafit software, Sigma) to the mean data presented in Fig. 3

Drug	Human melatonin mt$_1$			Human melatonin MT$_2$			
	IC_{50} (nM)	IC_{50}/IC_{50} Mel[a]	Efficacy (%)[b]	IC_{50} (nM)	IC_{50}/IC_{50} Mel[a]	Efficacy (%)[b]	mt_1/MT_2[c]
Melatonin	0.0212	1.0	100 ± 1.6	0.00807	1.0	100 ± 2.4	2.6
S20098	0.0317	1.5	94.8 ± 4.6	0.017	2.1	81.3 ± 5.4	1.9
S22029	2.76	130.2	102.1 ± 10.9	0.16	19.8	88.4 ± 7.8	17.3
S22365	1.25	59.0	97.4 ± 13.4	0.0772	9.6	86.0 ± 7.4	16.2
S20642	25.5	1202.8	81.7 ± 7.1	0.347	43.0	84.2 ± 8.0	73.5
S22480	1.94	91.5	71.7 ± 4.4	0.0535	66.3	74.0 ± 7.5	3.6

[a] Ratio of affinity constants (IC_{50}/IC_{50} Mel) represents fold difference in affinity of each analogue with respect to that of melatonin.
[b] The apparent efficacy calculated by the four-parameter logistic curve ± S.E.M.
[c] Ratio of fold difference in affinity (mt_1/MT_2) of each analogue for human melatonin mt$_1$ and melatonin MT$_2$ receptors.

above for the luciferase reporter assay. All experiments were repeated on three or more occasions and mean values calculated. IC_{50} values and apparent efficacies were calculated by the methods stated above.

2.6. 2-[^{125}I]iodomelatonin equilibrium binding

2-[^{125}I]iodomelatonin equilibrium binding experiments on HEK-hmt$_1$, HEK-hMT$_2$ and ovine pars tuberalis cell membranes were performed in a final reaction volume of 200 μl of 'ligand binding buffer' (10 mM Tris-HCl pH 7.5, 1 mM EGTA), for 2 h at 37°C. Separation of membrane bound 2-[^{125}I]iodomelatonin was performed by vacuum filtration through 25 mm diameter Whatmann GFC filters (pre-soaked in ligand binding buffer, 1% (v/v) polyethylamine). Following a 10 ml wash with ice-cold ligand binding buffer, the filters were briefly blotted on Whatmann 3MM paper, uniformly folded into small pellets and counted on a Packard Cobra γ-counter. All treatments were performed in duplicate and average values calculated. IC_{50} values were calculated by fitting a four-parameter logistic curve (Grafit software, Sigma) and were subsequently corrected to K_i values (Cheng and Prusoff, 1973). All experiments were repeated on two or more occasions and mean K_i values ± S.E.M. calculated.

3. Results

3.1. CRE-luciferase based characterisation of melatonin effects on human melatonin mt$_1$ and melatonin MT$_2$ receptors

HEK-hmt$_1$ and HEK-hMT$_2$ cells were co-transfected with plasmids pGL3(CRE$_2$-TK) and pcDOR8 (encoding

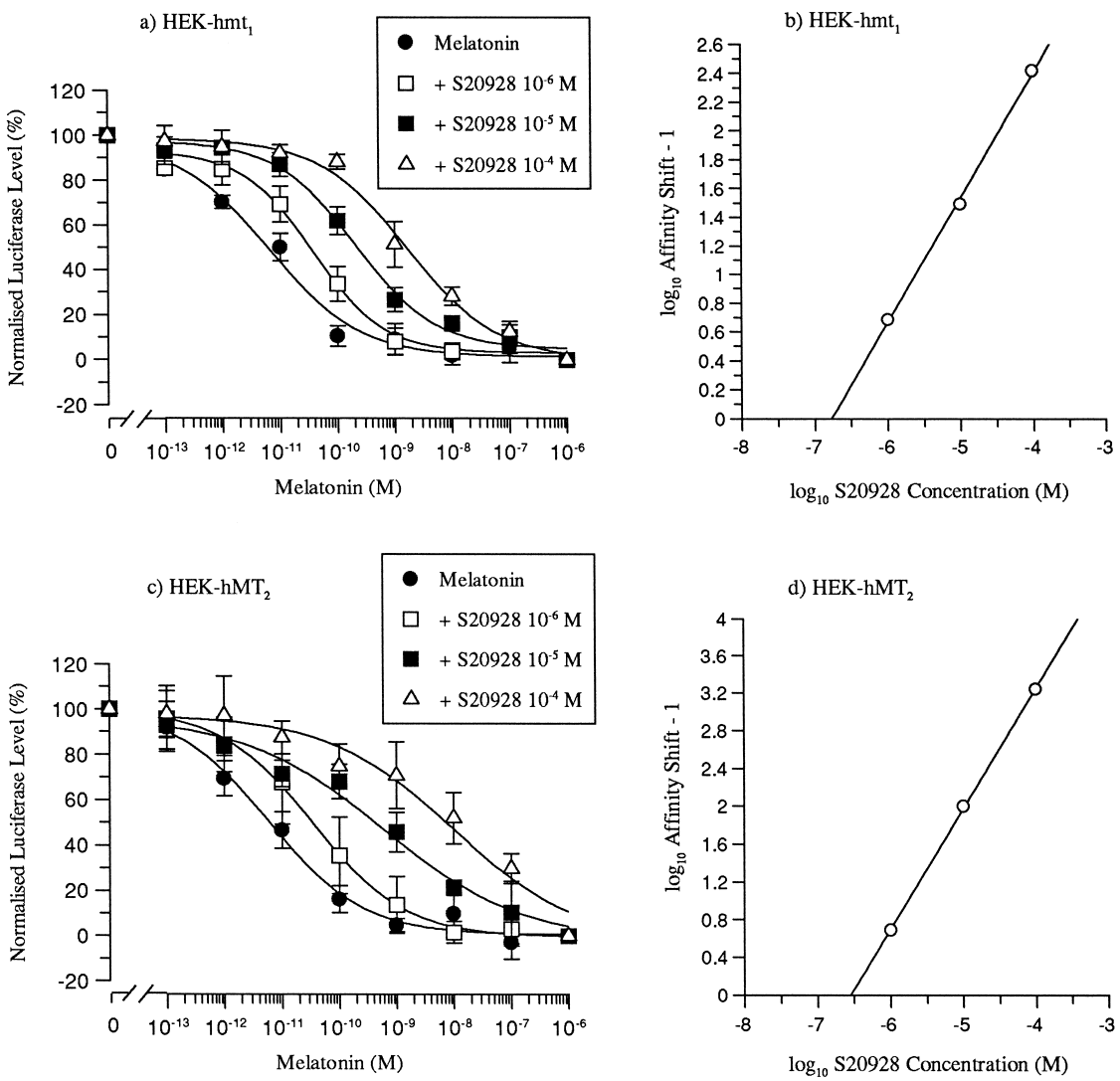

Fig. 4. Antagonism of the melatonin-mediated inhibition of [Nle^4-D-Phe^7]α-MSH stimulated luciferase levels by S20928. (a,c) Transfected HEK-hmt$_1$ and HEK-hMT$_2$ cells were exposed to 10^{-6} M [Nle^4-D-Phe^7]α-MSH and melatonin, in the presence or absence of S20928, for 16 h. Data represent means ± S.E.M. of normalised values from at least three experiments carried out in triplicate. (b,d) Schild plots of data shown in panels (a) and (c) respectively.

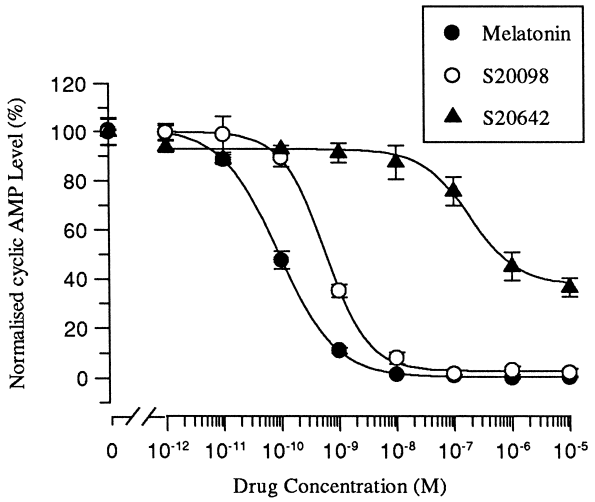

Fig. 5. Inhibition of forskolin stimulated cyclic AMP levels by melatonin, S20098 and S20642. Ovine pars tuberalis cells were exposed to 10^{-5} M forskolin and melatonin, S20098 or S20642 for 15 min. Data represent means ± S.E.M. of normalised values from at least three experiments performed in duplicate.

for the expression of ovine melanocortin MC5 receptor) using the lipid transfection reagent FuGENE™ 6. Transfected HEK-hmt$_1$ cells demonstrated a three to fourfold stimulation of luciferase levels following 16 h incubation with 10^{-6} M [Nle^4-D-Phe^7]α-MSH, whereas transfected HEK-hMT$_2$ cells typically displayed a two to threefold stimulation (data not shown). Co-incubation with melatonin produced a dose-dependent inhibition of the [Nle^4-D-Phe^7]α-MSH stimulated luciferase levels in both transfected cell lines (IC_{50} values; HEK-hmt$_1$ 21.2 pM, HEK-hMT$_2$ 8.1 pM) (Fig. 2). The maximum inhibition observed in HEK-hmt$_1$ cells reduced luciferase levels to ~50% of the [Nle^4-D-Phe^7]α-MSH stimulated value, whereas HEK-hMT$_2$ cells displayed a smaller melatonin-mediated inhibition, typically reducing luciferase levels to ~75% of their stimulated value (data not shown).

3.2. CRE-luciferase based characterisation of 6 naphthalenic analogues of melatonin on human melatonin mt$_1$ and melatonin MT$_2$ receptors

When compared to the melatonin-mediated effects upon luciferase levels in HEK-hmt$_1$ and HEK-hMT$_2$ cells, five naphthalenic drugs (S20098, S22029, S22365, S20642 and S22480) were shown to act as agonists or partial agonists (Fig. 3). The affinities and apparent efficacies of these drugs are listed in Table 1. The sixth drug, S20928, was shown to act as an antagonist of melatonin-mediated effects on both HEK-hmt$_1$ and HEK-hMT$_2$ cells (HEK-hmt$_1$ pA_2 6.77, slope 0.87; HEK-hMT$_2$ pA_2 6.55, slope 1.27) (Fig. 4). Three of the characterised agonists and partial agonists, S22029, S22365 and S20642, were shown to display functional selectivity for the human MT$_2$ receptor (Table 1).

3.3. Characterisation of drug effects on cyclic AMP levels in ovine pars tuberalis cells

Melatonin, S20098, and S20642 were tested for inhibition of 10^{-5} M forskolin stimulated cyclic AMP levels in ovine pars tuberalis cells. Compared to the observed melatonin dose response (IC_{50} 87.4 pM) S20098 was identified as an agonist (IC_{50} 565 pM) and S20642 was identified as a partial agonist (IC_{50} 191 nM, apparent efficacy 62.8 ± 3.3%) (Fig. 5).

3.4. 2-[^{125}I]iodomelatonin equilibrium binding

All of the drugs used in this study were tested for competitive displacement of 2-[^{125}I]iodomelatonin (100 pM) in HEK-hmt$_1$, HEK-hMT$_2$ and ovine pars tuberalis cells (Table 2). The determined equilibrium affinity constants (K_i) were transformed to pK_i ($\log_{10} K_i$) values, and comparisons plotted for all three receptor systems (Fig. 6). All of the comparisons displayed linear correlations with r^2 values ≥ 0.9.

Table 2
Equilibrium binding constants determined by competitive inhibition of 2-[^{125}I]-iodomelatonin binding in HEK-hmt$_1$ and HEK-hMT$_2$ and ovine pars tuberalis cells. K_i values were calculated from IC_{50} values using the method of Cheng and Prusoff (1973). K_i values listed are mean values ± S.E.M. from two or three experiments performed in duplicate

Drug	Human melatonin mt$_1$		Human melatonin MT$_2$		Ovine pars tuberalis ML$_1$	
	K_i (nM)	K_i/K_i Mel[a]	K_i (nM)	K_i/K_i Mel[a]	K_i (nM)	K_i/K_i Mel[a]
Melatonin	0.164 ± 0.035	1.0	0.266 ± 0.036	1.0	0.457 ± 0.062	1.0
S20098	0.202 ± 0.051	1.2	0.087 ± 0.024	0.4	0.38 ± 0.01	0.8
S22029	11.6 ± 2.9	70.7	3.90 ± 1.27	17.3	30.6 ± 12.9	67.0
S22365	5.17 ± 0.66	31.5	1.87 ± 0.35	8.3	43.2 ± 7.1	94.5
S20642	22.1 ± 2.0	134.8	4.84 ± 0.90	21.4	77.8 ± 31.4	170.2
S22480	3.69 ± 0.61	22.5	5.10 ± 1.12	22.6	17.4 ± 1.5	38.1
S20928	206 ± 42	1256.1	100 ± 28	442.5	459 ± 290	1004.4

[a]Ratio of affinity constants (K_i/K_i Mel) represents fold difference in affinity of each analogue with respect to that of melatonin.

3.5. Correlation of ligand binding to the ovine pars tuberalis melatonin receptor and the functional luciferase responses of human melatonin mt_1 and melatonin MT_2 receptors

Linear correlations were calculated for pK_i ($\log_{10} K_i$) values of ovine pars tuberalis ligand binding plotted against

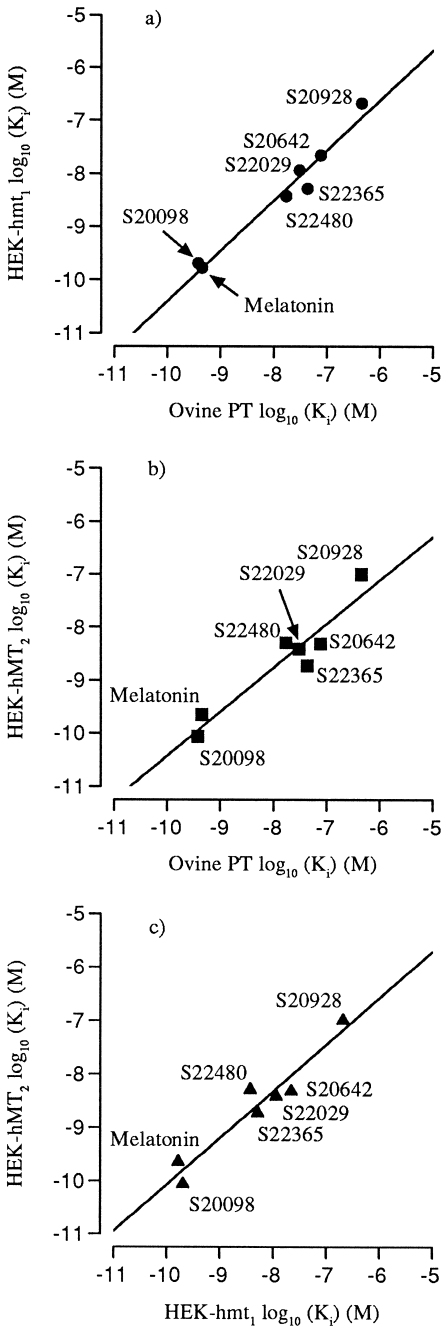

Fig 6. Correlations between the equilibrium affinity constants (K_i) for agonist and partial agonist binding to ovine pars tuberalis, HEK-hmt$_1$ and HEK-hMT$_2$ cells. Data shown represent mean values from two or three experiments and are listed in Table 2. Linear correlations; (a) HEK-hmt$_1$ vs. ovine pars tuberalis, r^2 0.96, slope 0.95 ± 0.08, (b) HEK-hMT$_2$ vs. ovine pars tuberalis, r^2 0.90, slope 0.82 ± 0.13, (c) HEK-hmt$_1$ vs. HEK-hMT$_2$, r^2 0.93, slope 0.87 ± 0.11.

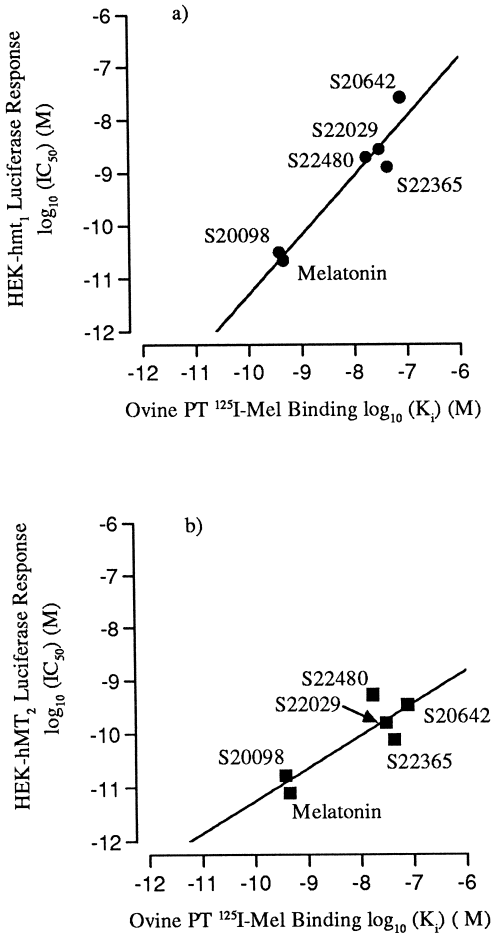

Fig 7. Correlations between the equilibrium affinity constants (K_i) for agonist and partial agonist binding to ovine pars tuberalis cells and their potency (IC_{50}) determined by luciferase responses in HEK-hmt$_1$ and HEK-hMT$_2$ cells. Data shown represent mean values as listed in Tables 1 and 2. Linear correlations; (a) ovine pars tuberalis vs. HEK-hmt$_1$, r^2 0.92, slope 1.12 ± 0.17, (b) ovine pars tuberalis vs. HEK-hMT$_2$, r^2 0.74, slope 0.61 ± 0.18.

pIC_{50} ($\log_{10} IC_{50}$) for agonist and partial agonist functional luciferase responses in HEK-hmt$_1$ and HEK-hMT$_2$ cells (Fig. 7). The data for the ovine pars tuberalis vs. HEK-hmt$_1$ displayed a r^2 linear correlation value of 0.92 with a slope of 1.12 ± 0.17. The ovine pars tuberalis vs. HEK-hMT$_2$ displayed a r^2 value of 0.74 with a slope of 0.61 ± 0.18.

4. Discussion

The pharmacological characterisation of receptors has been greatly advanced with the development of gene cloning and expression technologies. The advantages of constructing relatively simple cell systems expressing high levels of a specific receptor are clear, providing the means for the rapid characterisation of receptor-mediated ligand binding and functional responses. Such studies may complement or even substitute for studies performed upon receptors expressed in native tissue models. Recent ad-

vances have enabled further refinement of engineered cell systems, eliminating the need for direct quantification of some second messenger molecules which are used as the measure of the receptor-mediated functional response (Stratowa et al., 1995). One such system utilises the CRE-enhanced expression of firefly luciferase as an indirect method to observe receptor-mediated changes to cellular cyclic AMP levels (Himmler et al., 1993). Published data have demonstrated the validity of using such a system, with ligand induced changes acting through the 5-HT receptor presenting identical dose-responses when measuring either expressed luciferase or cyclic AMP levels (George et al., 1997). Quantification of luciferase levels is also significantly faster and less labour intensive than assaying cyclic AMP directly and therefore CRE-luciferase technology provides an effective system for the detailed pharmacological characterisation of receptors that function via modulation of cyclic AMP levels. Here we report the use of engineered human embryonic kidney (HEK293) cell lines, stably expressing human melatonin mt_1 or melatonin MT_2 receptors, in which the functional responses induced by six naphthalenic ligands (Fig.1) were measured by cyclic AMP-dependent expression of firefly luciferase protein. This represents the first application of this method in the pharmacological characterisation of melatonin receptor subtypes.

We first constructed the CRE-luciferase reporter plasmid pGL3(CRE_2-TK) in which DNA encoding for firefly luciferase was placed under the control of two octanucleotide CRE sequences (TGACGTCA) and the Herpes simplex virus thymidine kinase promoter (data not shown). As melatonin receptors are known to inhibit stimulated levels of cyclic AMP we transiently transfected HEK-hmt_1 and HEK-hMT_2 cells, stably expressing the human melatonin mt_1 and MT_2 receptors respectively, with pGL3(CRE_2-TK) and plasmid pcDOR8 encoding for the expression of the cyclic AMP stimulatory ovine melanocortin MC5 receptor (Barrett et al., 1994). Melatonin was shown to display a dose-dependent inhibition of 10^{-6} M [Nle^4-D-Phe^7]α-MSH (melanocortin receptor agonist) stimulated luciferase levels in both transfected cell lines (HEK-hmt_1, IC_{50} 21.2 pM, and HEK-hMT_2, IC_{50} 8.1 pM) (Fig. 2). An incubation time of 16 h was utilised in these experiments as preliminary studies identified that changes in luciferase expression only became evident at approximately 8 h and increased up to 16 h (data not shown). The requirement for this relatively long incubation time was probably due to the reporter construct being introduced in a transient transfection, and the fact that it contains only two CRE enhancer sequences. We limited the reporter to two CREs in these studies to try and maintain as representative a reporter system as possible. For this reason we also elected to stimulate cyclic AMP levels through the agonist activation of a second receptor, the melanocortin MC5 receptor, instead of using forskolin. The possible process of receptor desensitisation during the 16 h incubation period does not appear to have had a significant effect on the determined IC_{50} affinity values, which are in close agreement to those previously reported for other melatonin receptor model systems (Reppert et al., 1994; Dubocovich et al., 1997). The transfected HEK-hmt_1 cells demonstrated a three to fourfold stimulation of luciferase levels upon incubation with 10^{-6} M [Nle^4-D-Phe^7]α-MSH, whereas the transfected HEK-hMT_2 cells typically displayed a 2 to 3 fold stimulation (data not shown). The maximum inhibition observed in HEK-hmt_1 cells reduced luciferase levels to ~50% of the [Nle^4-D-Phe^7]α-MSH stimulated value, whereas HEK-hMT_2 cells displayed a smaller melatonin-mediated inhibition, typically reducing luciferase levels to ~75% of their stimulated value (data not shown). These levels of response probably reflect the balance between the concurrent cyclic AMP stimulatory and inhibitory processes.

Subsequent to the characterisation of melatonin-mediated responses the effects mediated by six naphthalenic analogues of melatonin were tested on HEK-hmt_1 and HEK-hMT_2 cells. Five of these drugs (S20098, S20642, S22029, S22365 and S22480) acted as agonists or partial agonists (Fig. 3, Table 1), whereas the sixth drug, S20928, was shown to act as antagonist on both receptor subtypes (Fig. 4). The HEK-hmt_1 cells were found to discriminate three agonists, S20098, S22365 and S22029, and two partial agonists S20642 (apparent efficacy 81.7%) and S22480 (apparent efficacy 71.7%). HEK-hMT_2 cells identified partial agonist behaviour for all five drugs (apparent efficacies 74.0 to 88.4%). In a recent report on the functional characterisation of human melatonin receptors expressed in Chinese hamster ovary cells, 6-hydroxymelatonin produced a maximal inhibition of cyclic AMP levels on the mt_1 receptor but only an 82% response on the MT_2 receptor (Browning et al., 1998). This is similar to the effects of S20098, S22365 and S22029 identified in our study. Together these findings may suggest that some compounds that evoke a maximal response of the melatonin mt_1 receptor do not produce maximal activation of the melatonin MT_2 receptor. As the reductions in apparent efficacies reported in these studies, are however, relatively small caution should be given to this possible interpretation until more rigorous investigations are performed. Three of the naphthalenic compounds were identified in our study as displaying a functional selectivity for the melatonin MT_2 receptor (S20642, 73.5 fold; S22365, 16.2 fold; and S22029, 17.3 fold) (Table 1). It is interesting that S20642 was found to display a relatively large functional selectivity as this naphthalenic compound has been previously identified as displaying binding selectivity in various chicken tissues (Pang et al., 1997). This may illustrate that S20642 is a useful partial agonist compound for the pharmacological discrimination and characterisation of melatonin receptor subtypes.

To further characterise the engineered CRE-luciferase/HEK293 model system experiments were de-

signed to allow comparison with the pharmacology of ovine pars tuberalis tissue, which is known to express melatonin ML_1 receptors (Morgan et al., 1989a). Functional characterisation of the ovine pars tuberalis melatonin ML_1 receptors was performed by measuring the effects of melatonin, S20098, S20642 and S20928 for the inhibition of forskolin stimulated cyclic AMP levels. Results identified S20098 as an agonist and S20642 as a partial agonist (apparent efficacy 62.8%) (Fig. 5), while S20928 was identified as an antagonist (data not shown). These findings are identical to the functional responses determined in the HEK-hmt_1 cells by the CRE-luciferase reporter assay. The identification of S20098 as an agonist and S20928 as an antagonist are also in agreement with the previously reported functions of these two naphthalenic compounds on hamster brain and suprachiasmatic nucleus (Ying et al., 1996). These data demonstrate therefore that the engineered CRE-luciferase/HEK293 model system accurately reports the functional pharmacology of melatonin receptor ligands. Competitive displacement of 2-[^{125}I]iodomelatonin binding by melatonin and the naphthalenic drugs was performed on ovine pars tuberalis, HEK-hmt_1 and HEK-hMT_2 cells (Table 2). Comparison of the calculated K_i affinity constants identified that the ovine pars tuberalis melatonin ML_1 receptor displayed a good linear correlation with both the human melatonin mt_1 and melatonin MT_2 receptors (ovine pars tuberalis vs. HEK-hmt_1, r^2 0.96, slope 0.95 ± 0.08; ovine pars tuberalis vs. HEK-hMT_2, r^2 0.90, slope 0.82 ± 0.13) (Fig. 6). A linear correlation was also determined for the K_i affinity constants of ligand binding to the ovine pars tuberalis cells and the agonist and partial agonist IC_{50} functional luciferase responses of HEK-hmt_1 and HEK-hMT_2 cells. The correlation between the pars tuberalis melatonin ML_1 receptor and the human mt_1 receptor was very close (r^2 0.92, slope 1.12 ± 0.17), however, the correlation with the human melatonin MT_2 receptor was identified as being less close (r^2 0.74, slope 0.61 ± 0.18) (Fig. 7). The sum of these data suggests that the ovine pars tuberalis receptor displays a common pharmacological profile with the human melatonin mt_1 receptor. This conclusion is supported by molecular evidence to suggest that the ovine pars tuberalis receptor is a melatonin mt_1 receptor sub-type. Genomic Southern blots have demonstrated that sheep possess only one melatonin receptor gene (data not shown), and cloning has demonstrated that this is the melatonin mt_1 receptor (Reppert et al., 1994; Barrett et al., 1997).

We have demonstrated that the CRE-luciferase reporter system can accurately determine the functional pharmacology of drug action upon human melatonin mt_1 and melatonin MT_2 receptors. This methodology therefore has the potential to provide for the high-throughput screening of drug action on human melatonin receptors. We propose to further refine this system by engineering the HEK-hmt_1 and HEK-hMT_2 cells to stably co-express the CRE-luciferase reporter and the melanocortin MC5 receptor, and also to investigate if the insertion of additional CRE elements in the reporter construct may provide enhancement of response levels. Studies using these model cell systems may help to provide a detailed understanding of the differences between the human melatonin mt_1 and MT_2 receptor sub-types.

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