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Analogues of diverse structure are unable to differentiate native melatonin receptors in the chicken retina, sheep pars tuberalis and *Xenopus* melanophores

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1 The pineal hormone melatonin exerts its biological effects through specific, high affinity G-protein coupled receptors. Recently, three melatonin receptor subtypes (Mel_{1a}, Mel_{1b} and Mel_{1c}) have been cloned. Neither the cloned subtypes, nor the native receptors have yet been compared in a detailed pharmacological analysis.

2 The present study examined the structure-activity relationships of a series of 21 melatonin analogues, by comparing their potency on the pigment aggregation response in *Xenopus laevis* melanophores with their affinity in radioligand binding competition studies in chicken retina and sheep pars tuberalis (PT), two tissues in which melatonin is known to mediate a biological response.

3 All but four of the analogues were full melatonin receptor agonists producing a concentration-related redistribution of pigment granules in cultured *Xenopus* melanophores. The remaining analogues produced little pigment aggregation at 10 μM.

4 Saturation studies with 2-[^{125}I]-iodomelatonin identified a single binding site in the chicken retina and sheep PT membranes, with a K_D of 36.6 ± 2.8 and 37.3 ± 4.3 pM, and a maximal number of binding sites (B_{max}) of 16.6 ± 0.5, and 40.1 ± 1.7 fmol mg^{-1} protein, respectively.

5 Comparison of the potency/affinity of the analogues for the binding sites gave a highly significant correlation in each case, retina/melanophore, $r = 0.97$ ($P < 0.001$, $n = 17$), PT/melanophore, $r = 0.97$ ($P < 0.001$, $n = 17$) and PT/retina, $r = 0.98$ ($P < 0.001$, $n = 21$).

6 Despite their large range in affinity and structural diversity these melatonin agonists were unable to distinguish between melatonin receptors in the chicken retina, sheep pars tuberalis and *Xenopus* melanophores.

Keywords: Melatonin receptor subtypes; melanophores; pars tuberalis; chicken retina; 2-[^{125}I]-iodomelatonin binding

Introduction

Melatonin (N-acetyl 5-methoxytryptamine) was first identified by Lerner and co-workers almost 40 years ago, as a secretory product of the bovine pineal gland. The hormone was isolated and characterized as a causative agent in the process of amphibian skin lightening (Lerner *et al.*, 1959). Of the chromatophores present in the amphibian dermis, only the melanophores, which contain the black pigment melanin, respond to melatonin. Melatonin acts through a specific, high affinity receptor to trigger the centripetal movement of thousands of pigment granules within the dermal melanophores towards the perinuclear region. This redistribution of pigment granules by melatonin provides the cellular basis of physiological colour change. The response to melatonin is highly specific, occurs rapidly on the application of very low concentrations of melatonin ($< 10^{-8}$M), is completely reversible and quantifiable (for review see Rollag, 1988). Melanophores obtained from *Xenopus laevis* neural crest explants have already been exploited to investigate structure-activity relationships at the melatonin receptor (Sugden, 1991; 1994).

Melatonin also regulates various neural, endocrine and behavioural functions that are cued by variations in photoperiod, including the timing of seasonal changes in the reproductive axis of photoperiodic species such as sheep, hamster and deer, via an action on the hypothalamus and the pars tuberalis (PT) of the pituitary gland (for review see Morgan *et al.*, 1994). In addition to the control of reproductive activity, melatonin also regulates seasonal adaptations in pelage composition and energy balance (Bartness *et al.*, 1993). The hormone is also involved in the entrainment of circadian rhythms. The foetal biological clock of rodents is entrained by maternal melatonin (Reppert & Weaver, 1991), and in adults, small doses of melatonin are able to entrain circadian rhythms in motor activity, drinking and body temperature (Redman *et al.*, 1983). A third well-documentated role of melatonin is as a local hormone in the retina, where a small quantity of melatonin is synthesized, and acts locally to regulate photoreceptor outer disc shedding and retinomotor movements (Besharse *et al.*, 1988).

Recently, a high affinity melatonin receptor has been cloned from an immortalized *Xenopus laevis* melanophore cell line by use of an expression cloning strategy (Ebisawa *et al.*, 1994). The predicted amino acid sequence shows seven hydrophobic segments which probably form the transmembrane regions. When expressed in mammalian cells, the complementary DNA (cDNA) encodes a specific melatonin receptor which, like the native receptor, is negatively coupled to adenylyl cyclase through a pertussis toxin-sensitive G-protein (Morgan *et al.*, 1994). By using degenerate primers designed from the sequence of the melanophore receptor, three distinct subtypes of the melatonin receptor have now been identified – Mel_{1a}, Mel_{1b} and Mel_{1c}. Both birds and mammals express the Mel_{1a} subtype; the Mel_{1c} subtype has not yet been cloned in mammals; however, the Mel_{1b} subtype has been identified. It was initially thought that birds did not posses the Mel_{1b} subtype but recently a fragment of receptor cDNA has been isolated from the chicken which closely resembles the human Mel_{1b} receptor (Reppert *et al.*, 1994; 1995a,b; Liu *et al.*, 1995). Comparison of the sequences of the cloned melatonin receptors with other G-protein coupled receptors identifies some common features, but indicates that melatonin receptors form a distinct group

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within the G-protein coupled receptor superfamily. All three cloned melatonin receptor subtypes inhibit adenosine 3′:5′-cyclic monophosphate (cyclic AMP) synthesis when expressed in mammalian cells, and, despite their structural divergence, appear to have very similar binding characteristics. An initial pharmacological evaluation of the cloned subtypes with a small number of melatonin analogues could not discriminate between the three receptor subtypes (Reppert *et al.*, 1995a,b).

In the present study, a number of melatonin analogues, of diverse chemical structure were tested on the pigment aggregation response in isolated *Xenopus laevis* melanophores and in radioligand binding studies in two tissues in which melatonin is known to mediate a biochemical and physiological response – the sheep PT and the chicken retina. In cultured sheep PT cells melatonin inhibits forskolin stimulated cyclic AMP synthesis (Morgan *et al.*, 1990). Circulating melatonin is thought to act directly on the PT to regulate the effects of photoperiod on prolactin secretion (Lincoln & Clarke, 1994). In the chicken retina, the hormone inhibits Ca^{2+}-dependent release of dopamine from amacrine cells (Dubocovich, 1985). In addition to the modulation of dopamine release within the retina, melatonin is implicated in photoreceptor disc movement (Besharse *et al.*, 1988), and has been shown to inhibit forskolin stimulated cyclic AMP synthesis (Iuvone & Gan, 1994; Osborne & Chidlow, 1994). A comparison of the potency/affinity of the analogues in each tissue/cell may therefore provide a valuable insight into the structural requirements for distinct physiological actions of melatonin. The development of novel, high affinity melatonin receptor agonists and/or antagonists will provide useful tools for defining the physiological role(s) of melatonin, investigating its cellular actions and identifying and characterizing melatonin receptor subtypes.

Methods

Tissue culture

Xenopus laevis embryos were obtained from adult frogs induced to lay by injection of human chorionic gonadotrophin (400 i.u./male, 600 i.u./female). Embryos were reared in tap water until they reached stage 20 (late neurula, as assessed from the normal table of *Xenopus* development, Nieuwkoop & Faber, 1956). The neural plate was then dissected out and the constituent cells dispersed as described previously (Messenger & Warner, 1977). After dispersion, cells were grown in Culture Medium (C1): Leibovitz modified L-15 medium without glutamine, (ICN Laboratories Ltd., Bucks, UK), diluted (1:1) with deionized water containing 10% heat inactivated foetal calf serum (Imperial Labs., UK), 2 mM L-glutamine, 100 i.u. ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin (Sigma), 2.5 $\mu g\ ml^{-1}$ amphotericin B (Sigma) and 100 nM α-melanocyte stimulating hormone (αMSH, Sigma) and maintained at room temperature. After 48 h clearly visible melanophores had migrated to the periphery of a central area of dispersed neural crest cells. Also present in culture were other neurally-derived cells such as neurones, muscle cells and fibroblasts. Fibroblasts were usually the predominant cell type present; melanophores making up a smaller proportion of the total cell population. After 48 h, unattached cells were removed, and the remaining cells washed with fresh C1. Melanophores were fed every 4 days with C1, and were used in experiments between 7 and 14 days after initial cell dissociation. At this time melanophores were responsive to melatonin, and the growth of contaminating cells (mainly fibroblasts) was not so great that it inhibited the growth of melanophores. On the day preceding an experiment, culture media was replaced with C1 containing 30 nM αMSH.

Quantification of pigment aggregation

Typically, 10 cells per dish were chosen for each experiment. Those chosen were usually at the periphery of the culture, where fewer neighbouring cells allowed a clearer definition of the edge of individual cells and more accurate measurement of the pigmented area in individual melanophores. Cells were observed under bright field illumination, through a Leitz compound microscope (×100 magnification), and digitized images captured and analysed by the DIGIT programme (Hayes & Fitzke, 1987). The area occupied by pigment in each cell was measured before the addition of any compound and designated as 100%. Increasing concentrations (10^{-12}–10^{-5} M) of compounds (from 100× concentrated stock solution in distilled water) were added at 20 min intervals, and the area occupied by pigment in each cell measured 15 min following each successive addition. The area of each cell occupied by pigment following each drug addition was expressed as a percentage of the original area. This value generally reached a lower limit of 20–30% when the pigment appeared fully aggregated around the cell centre. The concentration of a compound required to cause half maximal aggregation was defined in each case as the EC_{50}. If less than 70% aggregation was seen following the addition of 10^{-5} M of a compound, and the subsequent addition of 10^{-6} M melatonin caused no further aggregation, the cell was considered to be unresponsive and was excluded from the analysis of results. Typically, less than 10% of cells tested were unresponsive. If the addition of 10^{-6} M melatonin caused complete aggregation of the pigment, the EC_{50} of the original compound was taken to be greater than 10^{-5} M. The pigmented area in cells treated with vehicle did not change significantly over the 150 min time period needed to complete a concentration-response curve: control (before vehicle) – 100%, 150 min after vehicle – 100.6± 6.0 (mean±s.e.mean, $n=16$).

Membrane preparation

Chickens (*Gallus Domesticus*, White Leghorn) were obtained from Orchard Farms (Buckinghamshire) at one day of age, and were housed under diurnal lighting conditions (12 h light:12 h dark, lights on at 06 h 00 min) until killed during the light phase (11 h 00 min–15 h 00 min) at 14 to 21 days of age. Whole eyes were removed and retina dissected free of pigment epithelium. The retina were then frozen immediately in liquid nitrogen, and stored at −70°C until required. Sheep pars tuberalis (PT) were kindly provided by Dr P.J. Morgan (Rowett Research Institute, Aberdeen). Tissues were homogenized in 20 vols Tris-HCl buffer (50 mM, pH 7.4, assay buffer) containing phenylmethylsulphonyl fluoride (PMSF, 1 mM), leupeptin (50 $\mu g\ ml^{-1}$) and ethylene glycol bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA, 1 mM) (tissue preparation buffer), then centrifuged (4°C, 100,000 *g*, 20 min). The resultant pellets were then pooled, rehomogenized in tissue preparation buffer and recentrifuged; this process was then repeated. The final membrane pellet was suspended in tissue preparation buffer. Protein concentration was determined by a dye-binding method (Bradford, 1976), with bovine serum albumin as standard. Aliquots of retina membranes were frozen in liquid nitrogen until required; PT membranes were used on the day of preparation.

2-[^{125}I]-iodomelatonin binding to membrane preparations

For saturation analysis, duplicate membrane aliquots (protein concentrations per 30 μl; retina, 40 μg; PT, 8 μg), were incubated with 2-[^{125}I]-iodomelatonin (4–800 pM). Non-specific binding was determined in the presence of cold melatonin (1 μM). In competition assays to determine IC_{50} values for melatonin and related analogues, membranes were incubated with 60–80 pM 2-[^{125}I]-iodomelatonin, in the presence of at least five concentrations of competing compound (10^{-5}–10^{-12} M). For each analogue tested, IC_{50} values were determined with duplicate aliquots of a single membrane preparation due to the small amount of tissue available. Samples

were incubated either at 25°C for 90 min (retina) or 37°C for 120 min (PT); under these conditions equilibrium was reached (Sugden & Chong, 1991; data not shown). Reactions were terminated by the addition of 2 ml ice-cold assay buffer to each tube, and immediate filtration through glass fibre filters (GF/C; Whatman Ltd., Maidstone, Kent), soaked in 0.1% polyethyleneimine (PEI; Sigma). Each tube was then rinsed with a further 2 ml of assay buffer, and each filter was washed (2 × 5 ml), then assayed for radioactivity with a Packard Gamma counter (Cobra 2).

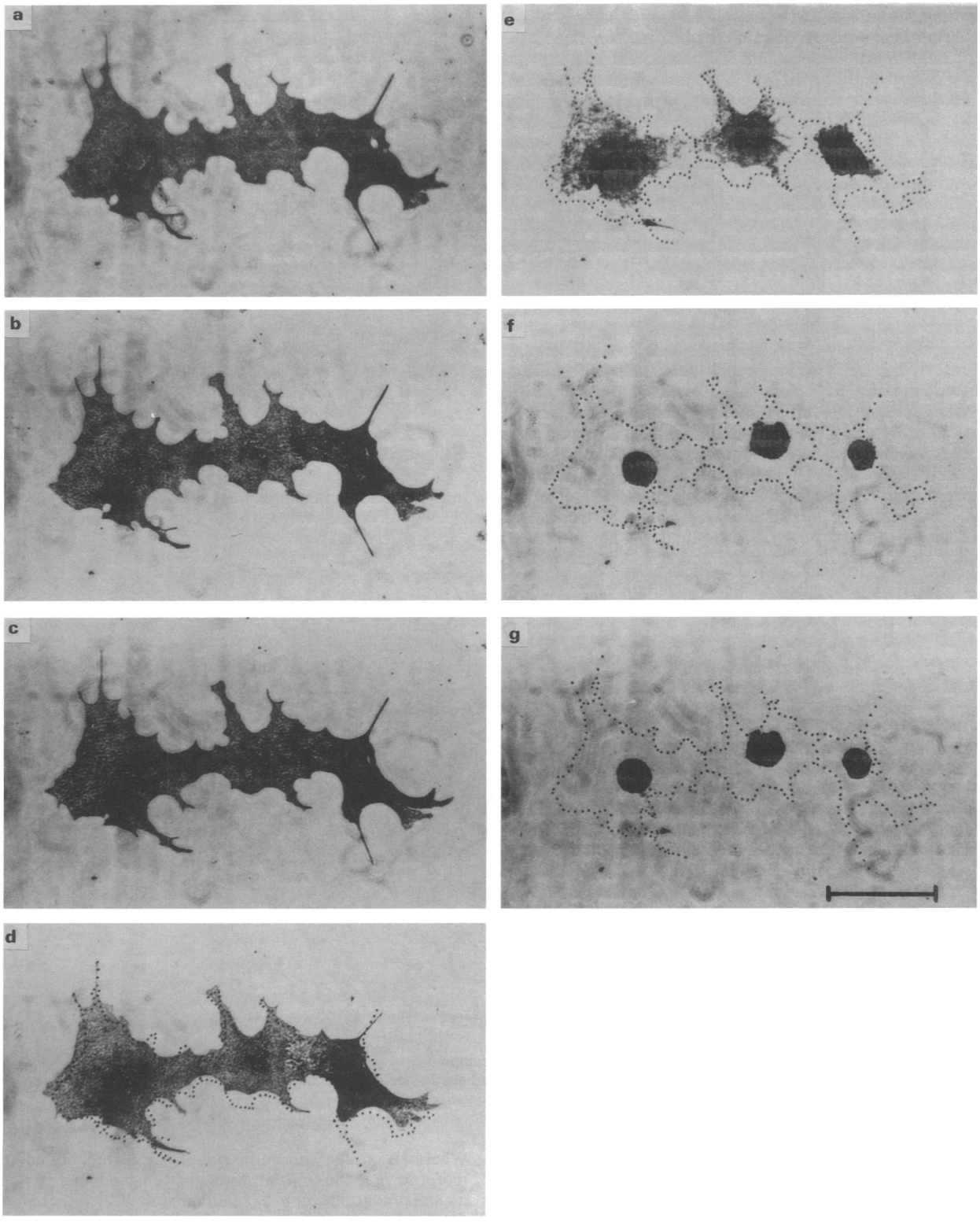
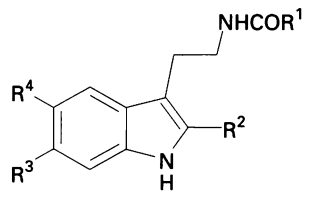


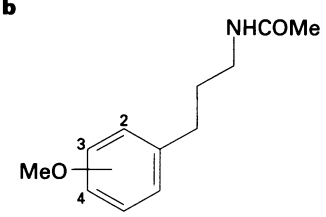
Figure 1 Photographs showing the response of three *Xenopus* melanophores to increasing concentrations of melatonin. (**a**) The cells before the addition of melatonin, final concentrations of melatonin are then as follows: (**b**) 10^{-12} M, (**c**) 10^{-11} M, (**d**) 10^{-10} M, (**e**) 10^{-9} M, (**f**) 10^{-8} M and (**g**) 10^{-7} M. Identical responses were seen in melanophores not in contact with one another. Images were captured 15 min after each drug addition. The dotted line in (d)–(g) shows the original position of pigment within the melanophores. The scale bar represents 100 μm.

a



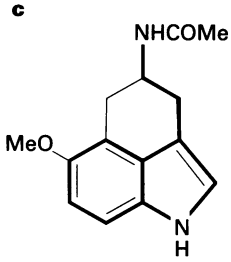
Compound number	R^1	R^2	R^3	R^4
1	H	H	H	OMe
2	Me	H	H	OMe
3	Et	H	H	OMe
4	Pr	H	H	OMe
5	CycloPr	H	H	OMe
6	Me	H	F	OMe
7	Me	H	OH	OMe
8	Me	H	Cl	OMe
9	Me	H	H	OCH_2Ph
10	Me	H	H	Me
11	Me	H	H	OH
12	Me	H	H	H
13	Me	C_6C_5	H	H
14	Me	Br	H	H

b

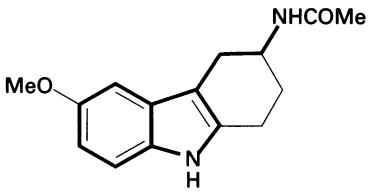


Compound number	Position		
	2	3	4
15	MeO	H	H
16	H	MeO	H
17	H	H	MeO

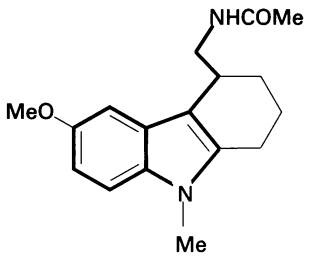
c



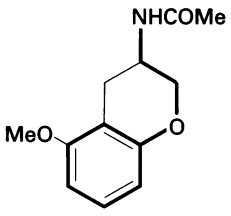
Compound 18



Compound 19



Compound 20



Compound 21

Figure 2 Structures of compounds tested: (**a**) tryptamines (compounds 1–14), (**b**) methoxy substituted phenylalkylamides (compounds 15–17) and (**c**) melatonin analogues with a rigid ethanamide side chain (compounds 18–21). For compound identity see Table 1. The bold lines in (c) show the similarity of the analogue to melatonin.

Data analysis

Saturation studies were analysed by non-linear regression with the ENZFITTER programme (Leatherbarrow, 1987), which fits the following equation:

$$B = B_{max}{}^{*}F/(K_D + F)$$

Where: B = the concentration of the ligand bound to the receptor, F = the concentration of free ligand, and B_{max} = the maximal concentration of binding sites. IC_{50} values were determined in competition assays by the ALLFIT programme (De Lean *et al.*, 1978), which fits the equation:

$$Y = \frac{A - D}{1 + (X/C)^B} + D$$

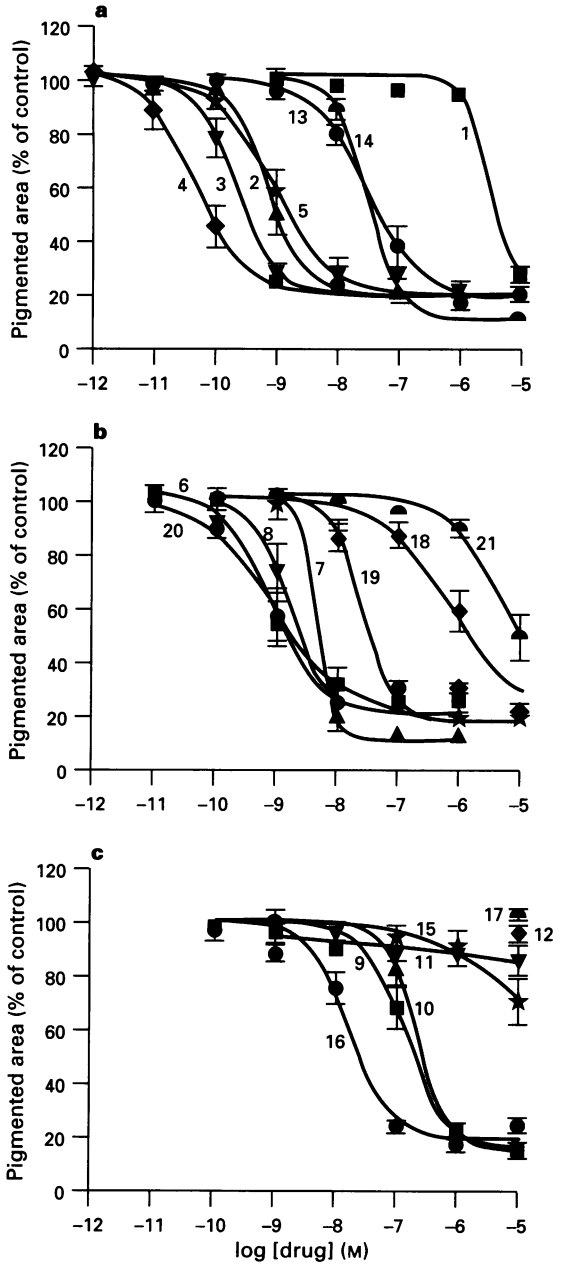


Figure 3 (**a**, **b** and **c**) Concentration-related pigment aggregation in *Xenopus laevis* melanophores. For compound identity, see Table 1, compounds are grouped such that each concentration-response curve may be seen clearly. The area of each cell occupied by pigment was measured 15 min after addition of the compound, and determined as a percentage of the initial pigment area. Each point represents the mean of 8–10 cells; vertical lines show s.e.mean. Where the error is <2% no error bars are shown.

Where: X = the concentration of inhibitor, Y = the percentage inhibition of specific 2-[^{125}I]-iodomelatonin binding, A = maximal specific binding, B = the slope factor, C = IC_{50} and D = the minimal specific binding. The K_i values were determined for each compound from the calculated IC_{50} value by use of the Cheng-Prusoff equation (Cheng & Prusoff, 1973).

Drugs

2-[^{125}I]-iodomelatonin (2200 Ci mmol^{-1}) was purchased from NEN Dupont (Stevenage, Herts). The structures of the melatonin analogues used are shown in Figure 2. Melatonin (**2**), N-acetylserotonin (**11**) and 6-hydroxymelatonin (**7**) were obtained from Sigma Chemical Co., (Poole, Dorset). 6-Chloromelatonin (**8**) was obtained from Tocris-Cookson (Bristol, U.K.), 6-fluoromelatonin (**6**) and N-acetyl 4-amino-6-methoxy-1,3,4,5-tetrahydrobenz[cd]indole (**18**) were provided by Dr M.E.Flaugh (Lilly Research Laboratories, Indianapolis, U.S.A.). N-Acetyltryptamine (**12**), N-acetyl 2-phenyltryptamine (**13**), N-acetyl 2-bromotryptamine (**14**), N-propanoyl 5-methoxytryptamine (**3**), N-acetyl 3-amino-6-methoxy-1,2,3,4-tetrahydrocarbazole (**19**) and N-acetyl 4-aminomethyl-6-methoxy-9-methyl-1,2,3,4-tetrahydrocarbazole (**20**) were synthesized as described previously (Garratt *et al.*, 1994a,b; 1995). N-Acetyl 2-methoxy-1-(3-aminopropyl)benzene (**15**), N-acetyl 3-methoxy-1-(3-aminopropyl)benzene (**16**) and N-acetyl 4-methoxy-1-(3-aminopropyl)benzene (**17**) were synthesized from commercially available 3-(X-methoxyphenyl)propionic acids (Garratt *et al.*, 1996). Finally, N-formyl 5-methoxytryptamine (**1**), N-butanoyl 5-methoxytryptamine (**4**), N-cyclopropanecarbonyl 5-methoxytryptamine (**5**) N-acetyl 5-methoxychroman (**21**), N-acetyl 5-benzyloxytryptamine (**9**) N-acetyl 5-methyltryptamine (**10**) were synthesized from the free amines, 5-methoxy and 5-methyltryptamine (Sigma) or 3-amino-5-methoxychroman (generously supplied by Dr Seth-Olov Thorberg, Astra Alab AB, Sodertalje, Sweden), as described previously (Ho *et al.*, 1968; Sugden, 1994). All drugs were dissolved in methanol at a stock concentration of 10^{-2} M, and stored at −20°C until required. On the day of the experiment serial dilutions of the compounds were made in distilled water for melanophore studies and methanol for radioligand binding assays. At the maximum concentration used, methanol (0.1% v/v) did not produce any pigment aggregation, nor did it inhibit 2-[^{125}I]-iodomelatonin binding in the radioligand binding studies.

Results

Pigment aggregation studies

Melatonin (**2**) produced a concentration-related aggregation of pigment granules within melanophores, with an EC_{50} value of 0.67 nM (Figure 1). This value agrees with previous results (Sugden, 1994), in which the same experimental conditions were employed, i.e. αMSH was present in the culture medium to ensure that pigment was fully dispersed before the addition of any compounds.

The compounds tested may be considered as belonging to one of three groups (Figure 2): (a) tryptamines, (b) simple methoxy substituted phenylalkylamides (in which the N- and C-1 position carbon atoms of the indole ring have been removed) and (c) melatonin analogues in which the ethanamide side chain has a more rigid conformation than that of melatonin.

With the exception of N-acetylserotonin (**11**) and N-acetyltryptamine (**12**), all of the tryptamines tested were full melatonin receptor agonists and caused complete aggregation of pigment in *Xenopus* melanophores (Figure 3). N-Acetylserotonin (**11**), the metabolic precursor of melatonin, and N-acetyltryptamine (**12**) had little activity as agonists, even at a concentration of 10^{-5} M (pigmented area >80% of control). The lack of agonist effect may reflect the low affinity of these analogues in binding assays, although other compounds with similarly low affinity (for example compounds **1**, **18**, **21**), were clearly agonists. Conceivably these two compounds could act as antagonists. N-Acetyltryptamine (**12**) has been shown to be a weak melatonin receptor antagonist, blocking melatonin-induced inhibition of [^{3}H]-dopamine release from the chicken retina (Dubocovich, 1984). In melanophores it has been found to be a partial agonist (Sugden, 1992). Increasing the length of the N-acyl side-chain caused a progressive increase in potency (EC_{50} values; N-formyl (**1**) = 2200 nM, N-acetyl (**2**) = 0.67 nM, N-propanoyl (**3**) = 0.15 nM, N-butanoyl (**4**) = 0.05 nM). These results agree well with those previously obtained in *Xenopus* melanophores (Sugden, 1991). However, changing a linear (N-butanoyl, **4**) to a cyclic (N-cyclopropanecarbonyl, **5**) side chain caused an 18 fold reduction in potency (EC_{50} = 0.05 nM compared to 0.9 nM).

Table 1 Identity, potency and affinity of analogues used in pigment aggregation and radioligand binding studies

Number	Compound name	-log Melanophore EC_{50}	-log Retina K_i	-log PT K_i
1	N-Formyl 5-methoxytryptamine	5.66±0.09	5.61±0.12	5.33±0.11
2	Melatonin	9.17±0.05	9.12±0.07	9.22±0.1
3	N-Propanoyl 5-methoxytryptamine	9.83±0.1	9.21±0.08	9.04±0.21
4	N-Butanoyl 5-methoxytryptamine	10.31±0.04	9.75±0.15	9.59±0.1
5	N-Cyclopropanecarbonyl 5-methoxytryptamine	9.04±0.03	9.12±0.02	8.67±0.01
6	6-Fluoromelatonin	9.15±0.1	9.38±0.1	9.00±0.16
7	6-Hydroxymelatonin	8.39±0.08	7.92±0.07	8.01±0.18
8	6-Chloromelatonin	8.81±0.09	8.88±0.08	8.98±0.12
9	N-Acetyl 5-benzyloxytryptamine	6.85±0.08	7.29±0.14	6.66±0.13
10	N-Acetyl 5-methyltryptamine	6.67±0.1	6.45±0.02	7.05±0.21
11	N-Acetylserotonin	ND	5.91±0.11	6.23±0.11
12	N-Acetyltryptamine	ND	5.89±0.1	6.51±0.19
13	N-Acetyl 2-phenyltryptamine	7.54±0.03	7.76±0.11	7.87±0.13
14	N-Acetyl 2-bromotryptamine	7.49±0.04	7.42±0.08	7.23±0.21
15	N-Acetyl 2-methoxy-1-(3-aminopropyl)benzene	ND	5.75±0.14	5.76±0.2
16	N-Acetyl 3-methoxy-1-(3-aminopropyl)benzene	7.77±0.11	7.24±0.08	7.56±0.11
17	N-Acetyl 4-methoxy-1-(3-aminopropyl)benzene	ND	5.15±0.11	5.22±0.03
18	N-Acetyl 4-amino-6-methoxy-1,3,4,5-tetrahydrobenz[cd]indole	6.02±0.2	6.03±0.11	6.00±0.02
19	N-Acetyl 3-amino-6-methoxy-1,2,3,4-tetrahydrocarbazole	7.56±0.04	6.92±0.13	7.99±0.11
20	N-Acetyl 4-aminomethyl-6-methoxy-9-methyl-1,2,3,4-tetrahydrocarbazole	9.21±0.17	9.06±0.13	9.00±0.13
21	N-Acetyl 5-methoxychroman	5.76±0.07	5.07±0.07	5.21±0.12

For each analogue the negative logarithm of potency (EC_{50}) in the *Xenopus* melanophore pigment aggregation assay, and the affinity constant (K_i) in radioligand binding assays in chicken retina and sheep pars tuberalis (PT) is given ± the standard error of the computer derived estimate. EC_{50} values were calculated from the data shown in Figure 3. ND= not determined, these compounds did not reduce the pigmented area to <70% of the control at 10 μM. EC_{50} and K_i values were calculated as described in the Methods.

Although removal of the 5-methoxy group from the indole nucleus (N-acetyltryptamine, (**12**) produced a compound with no discernable agonist activity, the addition of a phenyl or bromo group at the 2-position of the indole nucleus restored much of this loss, producing full melatonin receptor agonists (EC_{50} values, N-acetyl 2-phenyltryptamine (**13**) = 28.8 nM; N-acetyl 2-bromotryptamine (**14**) = 32.4 nM). Other C5 position substitutions produced much weaker agonists than melatonin, the benzyloxy derivative (**9**) being only marginally more potent than the methyl analogue (**10**) (EC_{50} values = 141 nM and 213 nM, respectively). Substitution of a hydroxy, chloro or fluoro group at C6 had variable results: 6-hydroxymelatonin (**7**) was the least active (EC_{50} = 4.07 nM), 6-chloromelatonin (**8**) slightly less potent (EC_{50} = 1.55 nM) and 6-fluoromelatonin (**6**) was equipotent to melatonin (**2**) (EC_{50} values = 0.71 nM and 0.67 nM, respectively).

Four of the analogues tested retained the 5-methoxy and N-acetyl groups of melatonin, but the indole nucleus was replaced by an alternate heterocyclic system (compounds **18**–**21**). All of these compounds were agonists in the pigment aggregation assay. However, there was a considerable difference in their potency. Compounds **18**–**20** are rigid tricyclic analogues of melatonin in which the ethanamide side chain is incorporated into a third ring and differ in the positioning of the N-acetyl moiety. Compound **20** was equipotent to melatonin (EC_{50} = 0.62 nM), **19** had a much lower biological activity (EC_{50} = 27.5 nM) and **18** was even less potent (EC_{50} = 956 nM). Compound **21** which has the ethanamide side chain incorporated into a chroman nucleus and the N-acetyl group in a similar orientation to that of compound **18**, was the least potent (EC_{50} = 1740 nM).

The series of methoxy N-acetyl phenylpropanamides (compounds **15**–**17**) had very low potency in the pigment aggregation assay. Of the three analogues tested, only compound **16**, in which the position of the methoxy and N-acetyl groups is most similar to that of melatonin, produced complete pigment aggregation. This compound was ≈40 fold less potent than melatonin with an EC_{50} values of 27 nM. Compounds **15** and **17** in which the position of the methoxy group is different to that of melatonin had little (<70% aggregation) or no agonist activity even at 10^{-5} M.

Radioligand binding studies

Saturation studies with 2-[^{125}I]-iodomelatonin revealed a single population of saturable, high affinity melatonin binding sites in both chicken retina and sheep PT membranes (data not shown), with K_D values of 36.6±2.8 (retina) and 37.3±4.3 pM (PT), and a maximal number of binding sites (B_{max}) of 16.6±0.5 (retina) and 40.1± 1.7 fmol mg^{-1} protein (PT; errors given are computer derived estimates). Competition assays (curves not shown) gave monophasic inhibition curves and pseudo Hill coefficients close to unity (0.8–1.2). The K_i values of all analogues tested in the retina and PT, and the EC_{50} value from the pigment aggregation studies are presented in Table 1. The correlation of the K_i values obtained in the radioligand binding studies in chicken retina and sheep PT, and the EC_{50} values from the pigment aggregation assay were highly significant in each case ($r \geq 0.97$, $P < 0.001$, Figure 4a and b).

Discussion

The ability of melatonin to produce pigment aggregation in amphibian melanophores has long been recognized (for review see Rollag, 1998). In the present study, *Xenopus* melanophores have been used as one model to determine the potency and structure-activity relationships of a series of melatonin analogues. In addition to this, the affinity of these analogues has been determined in two tissues known to mediate a physiological response of melatonin, the chicken retina and sheep PT. Saturation studies in chicken retina and sheep PT membrane preparations revealed a single, saturable site, with an affinity (<60 pM) comparable to that obtained previously for Mel_1 sites (Morgan *et al.*, 1994; Dubocovich, 1995), and competition studies produced monophasic inhibition curves for all of the analogues tested. In contrast to Howell *et al.* (1994), who suggested that sheep PT expressed two melatonin receptors of differing affinity, or a single site with multiple affinity states, we found no evidence of biphasic inhibition curves in the PT. Further evidence that the binding site identified in sheep PT and chicken retina in the present study is a Mel_1 receptor

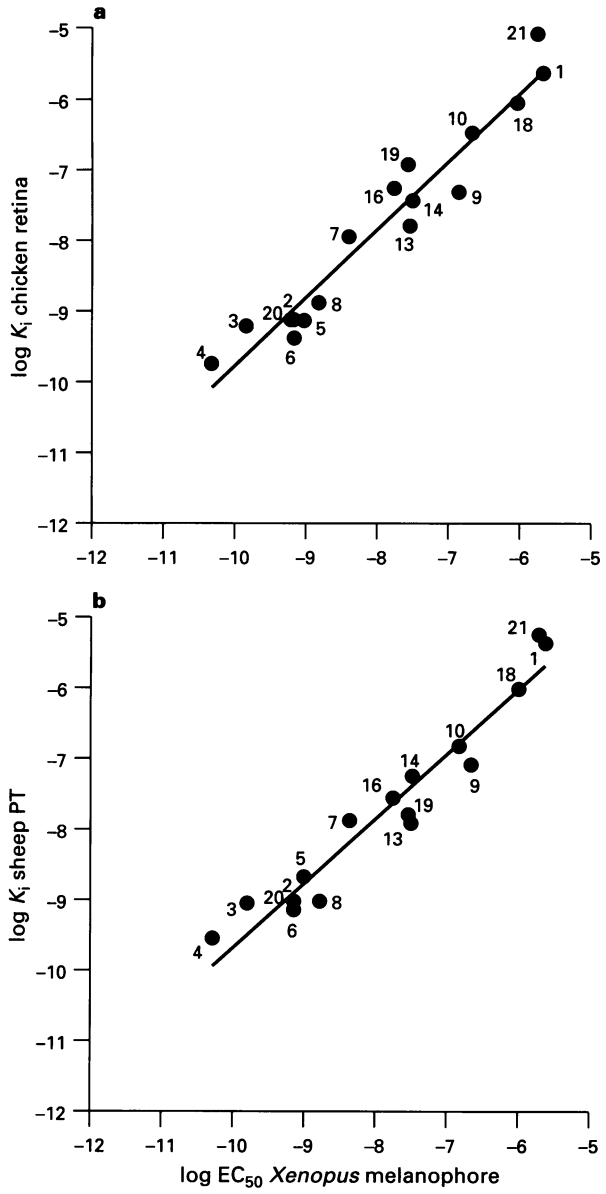


Figure 4 Comparison of the log of the potency (EC_{50}) of compounds in the *Xenopus* pigment aggregation assay and the log of their affinity (K_i) for 2-[^{125}I]-iodomelatonin binding sites in the chicken retina (**a**) and sheep pars tuberalis (PT) (**b**). K_i and EC_{50} values were determined as described in the Methods. Linear regression of the data in (**a**) yielded a slope of 0.98 and a correlation coefficient (r) of 0.97 ($P<0.001$, $n=17$), and in (**b**) a slope of 0.92 and $r=0.97$ ($P<0.001$, $n=17$). Correlation of K_i values for sheep PT with chicken retina yielded a slope of 1.05 and $r=0.98$ ($P<0.001$, $n=21$, data not shown). For compound identity see Table 1.

comes from the observation that the melatonin precursor N-acetylserotonin (**11**, $K_i \approx 1$ μM) had a much lower affinity than melatonin itself (**2**, K_i = 0.60–0.75 nM). Previous studies have identified a low affinity melatonin binding site, which has a similar affinity for N-acetylserotonin and melatonin (Duncan *et al.*, 1989).

Comparison of the affinity of the melatonin analogues tested in the radioligand binding studies, and their potency in the melanophore pigment aggregation assay produced a highly significant correlation (Figure 4a and b). This indicates that the analogues tested cannot discriminate between the melatonin binding site in each of the tissues. In *Xenopus* melanophores the receptor mediating pigment aggregation may be the Mel_{1c} subtype. Indeed the Mel_{1c} receptor was first cloned by use of an expression strategy from an immortalized clonal melanophore cell line (Ebisawa *et al.*, 1994). However, reverse transcription-polymerase chain reaction (RT-PCR) analysis has also detected receptor fragments in *Xenopus laevis* homologous to Mel_{1a} and Mel_{1b} subtypes, and it is possible that Mel_{1a} and Mel_{1b} receptors are also expressed in melanophores. In addition, in the present study a primary culture of melanophores, which may not express the same complement of receptor subtypes, was used rather than the clonal melanophore cell line.

In the sheep PT, the Mel_{1a} subtype has been detected, but as yet neither *in situ* hybridization nor RT-PCR analysis has detected Mel_{1b} transcripts in this tissue. It has been suggested that the Mel_{1a} receptor expressed in the PT mediates the reproductive actions of melatonin (Reppert *et al.*, 1994; Lincoln & Clark, 1994). However, as a Mel_{1b} receptor has not yet been cloned in sheep it is possible that primers based on the Mel_{1b} sequence of other species are sufficiently mismatched that amplification fails, and that the receptor is present and functional at very low levels (Reppert *et al.*, 1995). Cloning experiments have, so far, failed to detect the Mel_{1c} receptor mRNA in mammals (Reppert *et al.*, 1994).

In chicken retina both the Mel_{1a} and Mel_{1c} mRNA is expressed, and recently a fragment of receptor cDNA has been isolated which is most closely related to the Mel_{1b} subtype (Liu *et al*, 1995). The chicken Mel_{1b} subtype mRNA is expressed in brain and peripheral tissues, but the retina was not examined (Liu *et al.*, 1995). It is not yet clear which receptor subtype regulates melatonin-induced inhibition of Ca^{2+}-dependent dopamine release (Dubocovich, 1985), and inhibition of forskolin stimulated cyclic AMP synthesis in the chicken retina (Iuvone & Gan, 1994).

The analogues tested were unable to distinguish the melatonin binding sites in the sheep PT, chicken retina and *Xenopus* melanophore, which is surprising considering the large variation in the affinity/potency of the analogues ($\approx$5 log units), and their structural diversity. The largest difference in affinity/potency was a 12 fold selectivity of N-acetyl 3-amino-6-methoxy-1,3,4,5-methyltetrahydrocarbazole (**19**) for the PT over the retina (Table 1). This compound is one of a series of tricyclic analogues synthesized recently (Garratt *et al.*, 1994b), and may provide a lead for the synthesis of subtype specific agents.

One of the earliest studies of melatonin pharmacology suggested that the 5-methoxy group of the indole ring conferred agonist activity (Heward & Hadley, 1975). N-acetyltryptamine (**12**) (H-substitution at the 5-position) produced very little pigment aggregation in melanophores, and also had very low affinity ($K_i \approx 1$ μM) in the radioligand binding assays. However, addition of a bromo (**14**) or phenyl (**13**) group at the 2-position of the indole nucleus restored agonist activity, even in the absence of a 5-methoxy group, and dramatically increased the affinity in both the chicken retina and sheep PT. This confirms a previous study (Garratt *et al.*, 1994a), which suggested that although the 5-methoxy group makes a substantial contribution to binding affinity, it is not an essential requirement for agonist activity.

Lengthening the N-acyl side chain caused a progressive increase both in the affinity and the biological potency of melatonin analogues, in agreement with previous studies (Sugden & Chong, 1991; Sugden, 1991; Howell *et al.*, 1994). Replacement of the linear N-acyl side chain (N-butanoyl, **4**) with a cyclic group (N-cyclopropanecarbonyl, **5**) reduced both potency and affinity. This contrasts with previous binding studies in sheep PT, in which the cyclopropanecarbonyl analogue (**5**) was 3000 fold more potent than melatonin (Howell *et al.*, 1991; Yous *et al.*, 1992). Another study in which quail brain membranes were used also described a reduction in affinity following N-cyclopropanecarbonyl substitution (Spadoni *et al.*, 1993).

Analogues with 6-position substituents are of interest as studies on the cloned human Mel_{1a} and Mel_{1b} receptors expressed in COS-7 cells show that 6-chloromelatonin has a 10 fold selectivity for Mel_{1b} over Mel_{1a} receptors (Reppert *et al.*,

1995a). In the present study 6-chloromelatonin (**8**) had a potency and affinity almost equal to melatonin, whereas 6-hydroxymelatonin (**7**) showed a reduced potency and affinity. This agrees with the pharmacological characteristics of the cloned sheep and chicken Mel_{1a} and *Xenopus* and chicken Mel_{1c} receptors expressed in COS-7 cells (Ebisawa *et al.*, 1994; Reppert *et al.*, 1994; 1995b) and suggests that the selectivity of 6-chloromelatonin may be particular to the cloned human Mel_{1b} human receptor subtype.

Compounds **18–21** have an alternate heterocyclic system in place of the indole nucleus. All of these compounds were agonists in the pigment aggregation assay, indicating that the indole nucleus is not vital for biological activity. In all of these analogues, the flexible ethanamide side chain of melatonin is held in a rigid conformation (Garratt *et al.*, 1994b). N-Acetyl 4-aminomethyl-6-methoxy-9-methyl-1,2,3,4-tetrahydrocarbazole (**20**) had an affinity and potency equal to that of melatonin, suggesting that this molecule holds the ethanamide side chain in a position similar to that adopted by melatonin when it interacts with the ligand binding site of the receptor (Sugden *et al.*, 1995). The other rigid analogues had reduced potency and affinity indicating that these conformations of the side chain diminished the analogue's ability to fit the ligand binding site.

Compounds **15–17** are methoxy substituted phenylalkylamides (Garratt *et al.*, 1996). Only N-acetyl 3-methoxy-1-(3-aminopropyl)benzene (**16**), a direct analogue of melatonin, in which the N- and C-1-carbon atoms of the indole ring have been removed, possessed any agonist activity, and had the highest affinity of the three analogues in the binding assays. The reduced affinity of **15** and **17** suggests that the relative positions of the methoxy and amide groups of these analogues do not allow both groups to bind simultaneously to the receptor.

Conceivably, analogues based on a tetralin or naphthalene nucleus – not tested in the present study – may be able to discriminate Mel_1 receptor subtypes (Yous *et al.*, 1992; Copinga *et al.*, 1993). Alternatively, the Mel_1 receptor subtypes may exhibit very similar or identical pharmacological specificity, differing perhaps, in their distribution, regulation or coupling to intracellular second messenger systems. Further detailed pharmacological studies are needed to compare melatonin receptors in native tissues and cells where melatonin has a physiological action with the cloned receptor subtypes expressed in transfected cell lines.

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