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Original article

Design of subtype selective melatonin receptor agonists and antagonists

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Abstract — Studies of the physiological actions of melatonin have been hindered by the lack of specific, potent and subtype selective agonists and antagonists. In the present study, we describe the utility of a melanophore cell line from *Xenopus laevis* for exploring structure–activity relationships among novel melatonin analogues and report a novel MT_2-selective agonist (IIK7) and MT_2-selective receptor antagonist (K185). IIK7 is a potent melatonin receptor agonist in the melanophore model, and in NIH3T3 cells expressing human mt_1 and MT_2 receptor subtypes. In radioligand binding experiments IIK7 is 90-fold selective for the MT_2 subtype. K185 is devoid of agonist activity, but acts as a competitive melatonin antagonist in melanophores. A low concentration (10^{-9} M) antagonizes melatonin inhibition of forskolin stimulation of cyclic AMP in NIH3T3 cells expressing human MT_2 receptors, but has no effect in cells expressing mt_1 receptors. In binding assays, K185 is 140-fold selective for the MT_2 subtype. © Inra/Elsevier, Paris

MT_2 selective agonist / MT_2 selective antagonist / melatonin receptor subtypes / *Xenopus* / melanophores

Résumé — Étude d'agonistes et d'antagonistes sélectifs de sous-types de récepteurs à la mélatonine. Les études des effets physiologiques de la mélatonie ont été freinées par le manque d'agonistes et d'antagonistes spécifiques, puissants et sélectifs de sous-types de récepteurs. Dans la présente étude, nous décrivons l'utilisation d'une lignée cellulaire de mélanophores issue de *Xenopus laevis* pour l'évaluation des relations structure–activité de nouveaux analogues de la mélatonine et nous présentons un agoniste (IIK7) et un antagoniste (K185) nouveaux, sélectifs du récepteur MT_2. IIK7 est un puissant agoniste du récepteur de la mélatonine dans le modèle mélanophore et dans les cellules, NIH3T3, qui expriment les sous-types des récepteurs humains mt_1 et MT_2. Dans les expériences de liaison, IIK7 présente une sélectivité de 90 fois en faveur du sous-type MT_2. K185 n'a pas d'activité agoniste, mais il agit comme un anagoniste compétitif de la mélatonine sur les mélanophores. Une concentration faible (10^{-9} M) empêche l'inhibition par la mélatonine, de la stimulation de l'APM

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cyclique par la forskoline sur les cellules NIH3T3 exprimant le sous-type MT_2 humain, mais n'a aucun effet sur les cellules exprimant le récepteur mt_1. Dans les expériences de liaison, K185 présente une sélectivté de 140 fois en faveur du sous-type MT_2. © Inra/Elsevier, Paris

agoniste sélectif MT_2 / antagoniste sélectif MT_2 / sous-types de récepteurs à la mélatonine / *Xenopus* / mélanophores

1. INTRODUCTION

There are few in vitro model systems utilizing native receptors which are suitable for high throughput screening for novel melatonin ligands and which can be used to explore the structure–activity relationships of the receptor. One such system uses the pigment aggregation response of melanophores obtained from *Xenopus laevis* – the first physiological response to melatonin ever discovered [15]. The development of methods for obtaining pure cultures of melanophores which can be maintained indefinitely [18, 21], has allowed us to use these melatonin-responsive cells to investigate the structural features of the melatonin molecule necessary for binding to and activation of its receptor [3, 7–10]. Responses to melatonin in melanophores can be measured by manual scoring of the distribution of melanosomes within the cell using the melanophore index [22], which, although subjective, is direct and simple and useful for studies on small numbers of cells. The quantitation of changes in pigment area in individual melanophores using computer-assisted analysis of digitized images eliminates some of the tedium of manual scoring, and has been used extensively to determine the effects of melatonin and the potency of melatonin analogues [17, 23–25]. Another technique, more suited to the rapid analysis of ligand potency, depends upon the use of a microtitre plate reader to measure the absorbance (or transmittance) of light (wavelength 630 nm) through a monolayer of melanophores grown in a 96-well plate. When pigment is dispersed in melanophores absorbance of light is high, but when pigment aggregates absorbance decreases. This technique has been developed and used most ingeniously and successfully by Lerner's group [12, 14, 16, 18], and can also be applied to the discovery of novel melatonin ligands.

In the present study, we describe the characteristics of two novel melatonin analogues on the pigment response in melanophores. The affinity of the analogues on mammalian receptors was also determined on human mt_1 and MT_2 receptor subtypes (previously termed Mel_{1a} and Mel_{1b}) expressed in NIH3T3 cells using radioligand binding studies, and their potency at these recombinant subtypes was evaluated by measuring changes in intracellular cyclic AMP accumulation [19, 20].

2. MATERIALS AND METHODS

2.1. Cell culture

A clonal line of *Xenopus laevis* melanophores (kindly provided by Dr Michael Lerner, University of Texas, Dallas, USA), was grown in diluted (0.7×) L-15 medium (Sigma Chemical Co., Poole, Dorset, UK) containing 15 % heat-inactivated foetal calf serum (GIBCO/BRL), penicillin (100 i.u.·mL^{-1}) and streptomycin (100 μg·mL^{-1}) as previously described [2, 26]. NIH3T3 cells expressing recombinant human mt_1 or MT_2 melatonin receptor subtypes were provided by Dr Steven Reppert (Harvard Medical School, Boston, Massachusetts, USA), and were grown in complete Dulbecco's Eagle medium (cDMEM) containing penicillin (100 i.u.·mL^{-1}), streptomycin (100 μg·mL^{-1}), L-glutamine (4 mM), 10 % foetal bovine serum (Imperial Laboratories, UK) and Geneticin (G418; 1 mg·mL^{-1}; GIBCO/BRL) in humidified 5 % CO_2/95 % air at 37 °C.

2.2. Measurement of pigment aggregation

Flat-bottomed 96-well cell culture plates containing approximately 6–8 × 10^3 melanophores/well were used for pigment aggregation experiments. One hour prior to all concentration–response experiments, growth medium in each well was aspirated and replaced with 0.7× L-15 medium (containing 1 mg·mL^{-1} bovine albumin). In 0.7× L-15 medium pigment remained fully dispersed throughout the cells. The change in distribution of pigment granules within melanophores (*figure 1*) was quantitated using a Bio-Tek microtitre plate reader (model EL3115, Anachem, Luton, UK) by measuring the change in absorbance (630 nm), before and after drug treatment (*figure 2*). The fractional change in absorbance, $1 - (A_f/A_i)$ where A_i is the initial absorbance before drug treatment and A_f is the final absorbance, was calculated. All drugs were freshly prepared from 10^{-2} M stock solutions in methanol or DMSO kept at –20 °C. The maximal concentration of solvent was 1 % v/v which did not cause pigment redistribution in melanophores (data not shown). Antagonists were incubated with cells for 60 min before the addition of melatonin. Antagonist potency (pK_B) was estimated by constructing dose–response curves to melatonin in the absence and presence of a single concentration of antagonist (10^{-5} M). Estimated pK_B values were calculated from the equation log (concentration ratio – 1) – log [antagonist].

2.3. Membrane preparation and 2-[^{125}I]-iodomelatonin binding assays

NIH3T3 cells expressing recombinant mt_1 and MT_2 receptors were harvested and lysed as described previously [26]. Protein was measured using a dye binding method with bovine serum albumin as a standard [1] and aliquots were stored in liquid nitrogen until use. Radioligand binding saturation assays were performed as previously described [26]. Competition assays using recombinant human mt_1 or MT_2 receptors used 17 and 32 µg of homogenate protein, respectively. Samples were incubated at 37 °C for 90 min. Preliminary studies showed that equilibrium is reached under these conditions (data not shown). Specific binding was calculated by subtracting non-specific binding (defined using 1 µM melatonin) from total binding. For competition assays, 80–100 pM 2-[^{125}I]-iodomelatonin was used. Binding assays were terminated by the addition of 2 mL ice-cold buffer A to each tube and immediate filtration through 1 % v/v polyethylenimine pre-treated glass fibre filters (GF/C, Whatman Ltd, Maidstone, Kent, UK). Each tube was then rinsed with a further 2 mL of buffer and each filter was washed twice with 5 mL of buffer. 2-[^{125}I]-iodomelatonin trapped on the filter was counted (Cobra II Auto-Gamma, Packard). All assays were performed on duplicate homogenate aliquots and were repeated at least three times.

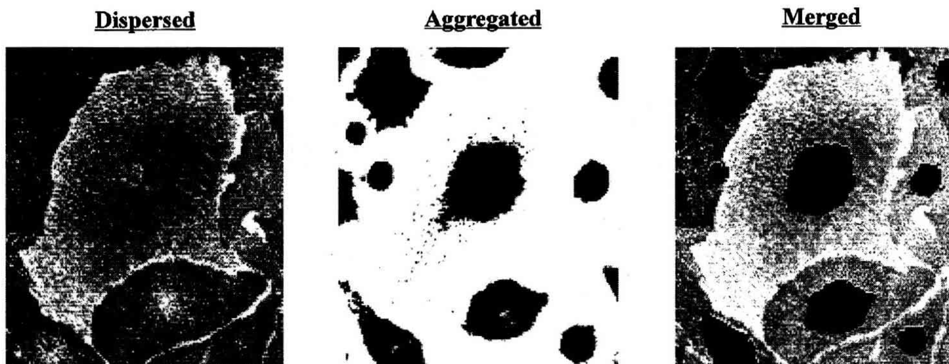

Figure 1. Bright field images of *Xenopus leavis* melanophores with dispersed pigment (left) with pigment granules aggregated around the nucleus after melatonin-treatment (10^{-9} M, 60 min; centre), and a merged picture of these two images (right).

2.4. Determination of cyclic AMP

NIH3T3 cells expressing recombinant human mt_1 or MT_2 receptors were seeded into 24-well plates and cultured for 48 h until they reached confluence (3–4 × 10^4 cells/well). After washing twice with serum-free DMEM, IBMX (2.5 × 10^{-4} M) was added for 10 min at 37 °C before the addition of forskolin (10^{-5} M) in the absence or presence of melatonin or the melatonin analogues IIK7 or K185. All drugs were diluted in DMEM containing IBMX as described above. After 10 min incubation at 37 °C, the medium in each well was aspirated and 300 µL ice-cold acetic acid (50 mM) were added and cells were scraped from the plate. The lysate was then collected and boiled for 3 min. All samples were kept at –20 °C until the concentration of intracellular cyclic AMP was determined in duplicate by RIA following acetylation as described previously [13, 29].

2.5. Data analysis

The four parameter logistic equation was used for curve-fitting concentration–response data to obtain the concentration of analogues producing 50 % inhibition of the specific 2-[^{125}I]-iodomelatonin binding (for determination of K_i), or 50 % of maximal pigment aggregation (EC_{50}) or inhibition of forskolin-stimulated cyclic AMP (IC_{50}).

2.6. Materials

Melatonin and all cell culture media and reagents were purchased from Sigma Chemical Co. (Poole, UK) unless indicated. Luzindole (*N*-acetyl-2-benzyltryptamine) and 4-phenyl-2-propionamidotetralin (4-P-PDOT) were obtained from Tocris Cookson (Bristol, UK). 2-[^{125}I]-iodomelatonin and cyclic AMP 2'-*O*-succinyl [^{125}I]-tyrosine methyl ester (specific activity 2 200 Ci/mol) were from DuPont UK Ltd (Stevenage, UK). GR135533 (3-(-*N*-propyl-2-pyrrolidine)-5-methoxy-indole) was generously provided by Dr I.J.M. Beresford (Glaxo, Stevenage, UK), and S20929 (*N*-[2-napth-1-yl-ethyl]cyclopropyl carboxamide) by Dr B. Guardiola (Institut de recherches internationales Servier, France). *N*-butanoyl-2-(2-methoxy-6*H*-isoindolo[2,1-a]indol-11-yl)ethanamine (IIK7) and *N*-butanoyl-2-(5,6,7-trihydro-11-methoxybenzo[3,4]cyclohept[2,1-a]indol-13-yl)ethanamine (K185) were synthesized at University College London, UK.

3. RESULTS

Figure 1 shows a bright-field image of several melanophores in control, dispersed conditions and after treatment with melatonin (10^{-9} M, 60 min). Melatonin treatment has resulted in the aggregation of pigment granules (melanosomes) at the centre of each cell, around the nucleus, which is clearly

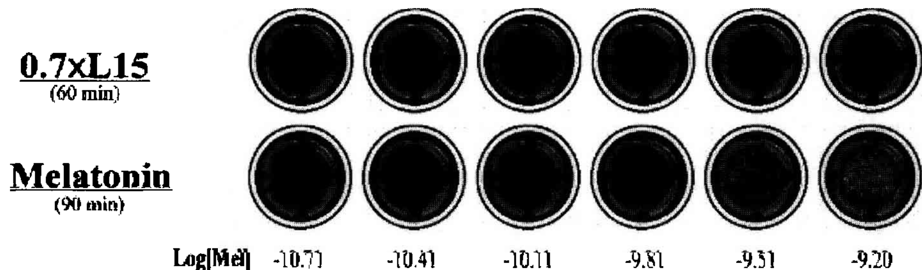

Figure 2. Example of quantitative analysis of pigment movement by absorbance measurement. Individual wells from a 96-well microtitre plate treated with vehicle or the melatonin concentrations indicated are shown. The change in absorbance ($1 - A_{f\,=\,final} - A_{i\,=\,initial}$) at each concentration is shown.

visible in some melanophores. The right-hand figure shows a merged image illustrating the substantial change in the area occupied by pigment granules.

Figure 2 shows the appearance of melanophores cultured on a 96-well plate treated with vehicle or differing concentrations of melatonin. Measurement of changes in melanophore absorbance in the 96-well format is very rapid, allows the use of multiple replicates and concentrations, and reflects changes in several thousand pigment cells. This method is particularly useful for the determination of concentration–response curves and the precise measurement of the potency of potential melatonin receptor agonists and antagonists (*figure 3*).

Figure 3 shows the concentration–response curves for melatonin-induced pigment aggregation in the absence and presence of a single concentration (10^{-5} M) of the melatonin receptor antagonists luzindole [5], GR135533 and 4-P-PDOT (4-phenyl-2-propionamido-tetralin) [6] and S20929 [4, 28]. All four antagonists produced a rightward shift in the melatonin concentration–response curve, giving pK_B values of luzindole, 6.53; GR135533, 6.31; 4-P-PDOT, 6.86; S20929, 6.62. None of the four antagonists triggered pigment granule aggregation when added to melanophores alone (data not shown) indicating that they lack any agonist or partial agonist activity. pA_2 values for antagonists can also be determined using multiple concentrations of antagonist [e.g. 26].

In a series of recent studies we have sought to understand how melatonin binds to and activates its receptor by investigating

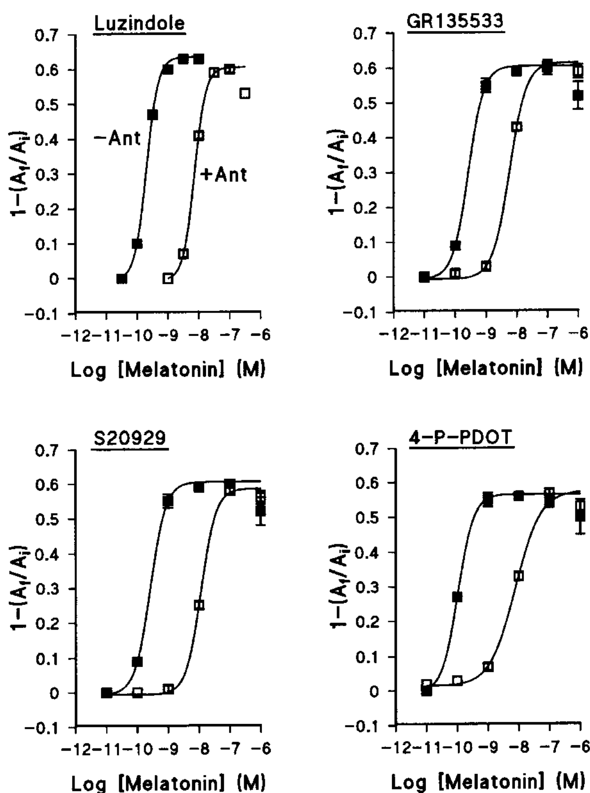

Figure 3. Concentration–response curves for melanophore pigment granule aggregation to melatonin in the absence and presence of the melatonin receptor antagonists, luzindole, GR135533, S20929 and 4-P-PDOT. Each data point represents mean ± SEM of triplicate samples.

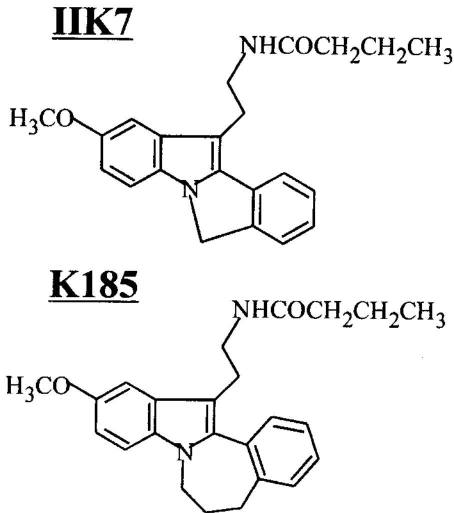

Figure 4. Chemical structures of IIK7 and K185.

the structure–affinity and structure–activity relationships of several different series of analogues which we have made [3, 7–11]. The information gained from these studies has led us to design and synthesize a number of novel melatonin receptor ligands. In the present study, two novel analogues, IIK7 and K185 (*figure 4*) have been examined using radioligand binding experiments on recombinant mt_1 and MT_2 receptors. Both analogues display substantial (90–130-fold) MT_2 subtype selectivity (*figure 5*). The pK_i for IIK7 at the mt_1 subtype is 8.35, and at the MT_2 subtype 10.30; for K185, the pK_i at the mt_1 subtype is 7.18, and at the MT_2 subtype 9.30. IIK7 acts as an agonist on *Xenopus* melanophores (–log pEC_{50} 8.24, *figure 6A*) and is 40-fold more potent at inhibiting forskolin stimulation of cyclic AMP in cells expressing MT_2 receptors than in cells expressing mt_1 receptors (*table I*). K185, on the other hand, has no agonist activity on melanophores (*figure 6A*) or on NIH3T3 cells expressing mt_1 or MT_2 receptor subtypes (data not shown). It does, however, antagonize melatonin-induced pigment aggregation (pK_B 7.06 ± 0.04; *figure 6B*). Furthermore, a low concentration (10^{-9} M) significantly blocks melatonin inhibition of forskolin stimulation of cyclic AMP accumulation in NIH3T3 cells expressing MT_2 receptors, but does not do so in cells expressing the mt_1 subtype (*figure 7*).

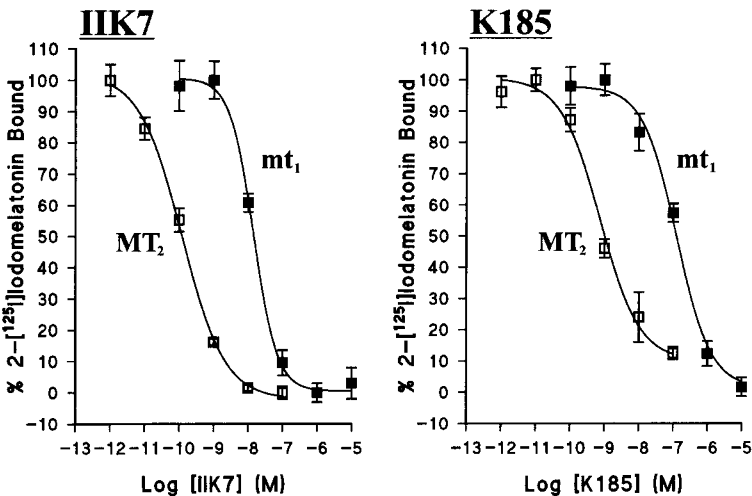

Figure 5. Competition radioligand binding curves for IIK7 and K185 on recombinant human mt_1 and MT_2 receptor subtypes. Each data point represents a mean ± SEM of duplicate samples.

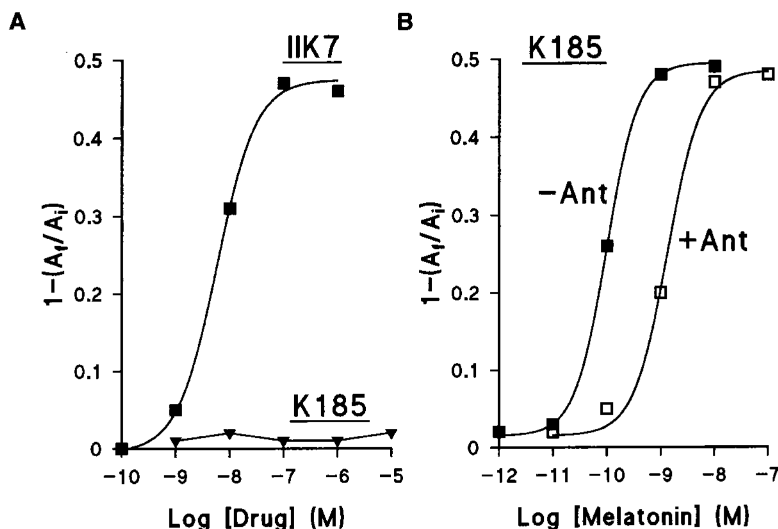

Figure 6. Effect of IIK7 and K185 on pigment movement in melanophores. **A**) IIK7 (like melatonin) acted as a full agonist, producing concentration-related pigment granule aggregation. K185 had no agonist activity (even at 10^{-5} M). **B**) K185 shifted the concentration–response curve for melatonin to the right thus showing antagonist activity. Each data point represents a mean ± SEM of triplicate samples.

Table I. Potency of melatonin and IIK7 for inhibition of forskolin-stimulated cyclic AMP in NIH3T3 cells expressing recombinant human mt_1 and MT_2 clones (pIC_{50} ± SEM; $n = 4$).

Species	mt_1	MT_2
Melatonin	10.10 ± 0.22	9.81 ± 0.07
IIK7	8.66 ± 0.16	10.26 ± 0.10

4. DISCUSSION

Melanophores have proven to be a useful model system for the rapid assessment of the potential agonist and antagonist activity of novel analogues of melatonin. The high density of melatonin receptors in melanophores makes it likely that any partial agonist activity of receptor antagonists is revealed. Model systems in which the number of melatonin receptors is few or limiting (e.g. dopamine release in rabbit retina) may not reveal intrinsic agonist activ-

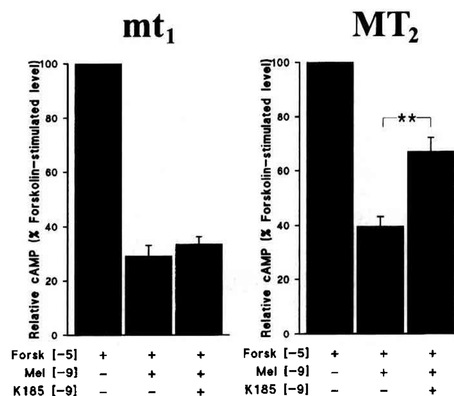

Figure 7. Effect of K185 on melatonin inhibition of forskolin stimulation of cyclic AMP accumulation in NIH3T3 cells expressing human mt_1 or MT_2 receptors. Bars represent a mean ± SEM of duplicate determinations of four samples. ** $P < 0.01$, by Student's t-test.

ity of novel, putative antagonist ligands. For example, GR128107 a competitive antagonist in the rabbit retina model [6], was revealed to have partial agonist activity on *Xenopus* melanophores and in NIH3T3 cells expressing recombinant mt_1 and MT_2 receptors [27].

The compounds described in the present report, IIK7 and K185, are members of a large series of tetracyclic compounds which we have recently synthesized [11]. These compounds can be viewed as analogues of *N*-pentanoyl 2-benzyltryptamine (DH97), a selective MT_2 receptor antagonist [26] and congener of luzindole, in which the benzyl moiety is linked to the indole nitrogen by one (IIK7) or three (K185) carbon atoms. Remarkably altering this link from one carbon atom to three, changes the biological activity of the compound from an agonist (IIK7) to an antagonist (K185). This was true not only for native receptors in *Xenopus* melanophores, a model in which the melatonin receptor mediating the biological response has not been formerly defined, but also for both recombinant human receptor subtypes. For the agonist IIK7, annelation of the 2-benzyl group to the indole nitrogen with a single carbon improved MT_2 binding affinity (7-fold) relative to melatonin, but reduced mt_1 binding affinity (~7-fold). In these radioligand binding experiments, IIK7 is ~90-fold selective for the MT_2 receptor subtype. Measurement of cyclic AMP in NIH3T3 cells expressing the recombinant human subtypes indicated that MT_2 potency (relative to melatonin) and selectivity were similarly improved.

Annelation of the 2-benzyl group of DH97 to the indole nitrogen with three carbon atoms to give K185, improved both mt_1 and MT_2 binding affinity (15- to 20-fold relative to DH97) [26], and MT_2 selectivity slightly (from 90- to 130-fold). In melanophores, K185 acted as a competitive melatonin receptor antagonist and was slightly more potent (2–6-fold) than the other antagonists tested in this model (luzindole, GR135533, 4-P-PDOT and S20929; *figure 3*) and DH97 [26]. An antagonist action of K185 at the mammalian MT_2 receptor subtype was confirmed in functional studies, which showed that a low concentration (1 nM) significantly antagonized melatonin inhibition of forskolin stimulation of cyclic AMP in cells expressing MT_2 receptors, but was without effect on cells expressing the mt_1 subtype. Importantly, K185 had no agonist action in these studies, even at the highest concentration used (10^{-5} M).

IIK7 and K185 differ only in the number of carbon atoms bridging the indole and phenyl rings (*figure 4*). Energy minimized structures (not shown) indicate that the changes in ring size that this brings about, results in a conformational change between the phenyl ring and indole part of the molecule. Yet methoxyl and ethanamide sidechains, previously shown to be important sites of attachment between ligand and receptor, retain a similar relationship to one another. Presumably, the altered relationship between the phenyl and indole rings is the key change switching activity from the agonist to the antagonist. The improvement in MT_2 receptor selectivity of IIK7 and K185 is consistent with our earlier findings that the structural requirements at the binding pocket of the MT_2 receptor subtype are less stringent than at the mt_1 subtype [25]. Selective and potent MT_2 receptor agonists and antagonists, such as IIK7 and K185, will be useful tools in defining the physiological role of the MT_2 receptor subtype. The design of mt_1 subtype selective ligands remains an important future challenge.

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Wellcome) and Dr B. Guardiola (Servier) for supplying GR135533 and S20929, respectively, and to Professor Peter Garratt (University College London) for helpful discussions.

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