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Radioligand binding affinity and biological activity of the enantiomers of a chiral melatonin analogue

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Abstract

Melatonin, a hormone secreted by the pineal gland, can act on the central circadian oscillator in the suprachiasmatic nucleus of the hypothalamus. It has been proposed that melatonin or its analogues may be useful in restoring disturbed circadian rhythms in jet-lag, shift-work and some blind subjects, and as sleep-promoting agents. In the present study, the (−)- and (+)-enantiomers of *N*-acetyl-4-aminomethyl-6-methoxy-9-methyl-1,2,3,4-tetrahydrocarbazole (AMMTC) were separated and tested. The affinity of the enantiomers at the specific 2-[^{125}I]iodomelatonin binding site in chick brain membranes was compared in competition assays, and their biological activity in a specific melatonin receptor bioassay, aggregation of pigment granules in *Xenopus laevis* melanophores. The (−)-enantiomer of AMMTC was 130-fold and 230-fold more potent than the (+)-enantiomer in competition radioligand binding assays and melanophores, respectively. Both enantiomers are melatonin receptor agonists; (−)-AMMTC is slightly more potent than melatonin itself. As the tetrahydrocarbazole nucleus holds the C-3 amido side-chain of AMMTC in a restricted conformation, the analogues will be useful in modelling the melatonin receptor binding site.

Keywords: Melatonin; Chiral analog; Binding site; Melanophore, *Xenopus laevis*

1. Introduction

In the last few years, high affinity, G-protein-coupled binding sites for the pineal hormone, melatonin have been found in membranes prepared from various brian regions, and, more recently, in several peripheral tissues (for reviews see Morgan et al., 1994; Krause and Dubocovich, 1991). In some tissues these binding sites have been shown to mediate distinct biochemical and cellular responses (for review see Sugden, 1994a). One of the earliest melatonin responses discovered was its ability to trigger a redistribution of pigment granules to the centre of amphibian melanophores (Lerner et al., 1958); a response which is part of the physiological mechanisms which regulate skin colouration (for review see Rollag, 1988). Recently, the melatonin receptor has been cloned from *Xenopus laevis* dermal melanophores (Ebisawa et al., 1994). The sheep and human melatonin receptors, which mediate the effects of melatonin on seasonal reproduction and circadian rhythms, have subsequently been cloned and characterized (Reppert et al., 1994). Melatonin receptors are members of a new receptor group distinct from others in the G protein-coupled receptor family (Reppert et al., 1994).

The prospect that therapeutic agents based on analogues of melatonin might be used to treat disorders of circadian rhythms in old age and the blind, and manage sleep-wake distubances caused by shift-work and jet-lag has stimulated interest in the pharmacology of this hormone (Guardiola-Lemaitre, 1991). We have synthesized a number of analogues of melatonin to define the functional groups needed for biological activity (Garratt et al., 1994a,b, 1995). The C-3 amidoethane side-chain of melatonin is essential but is very flexible, being able to rotate with little hinderance from the rest of the molecule. To determine the conformation the C-3 side-chain adopts on binding to the active site of the receptor we have synthesized analogues in which the conformation of the side-chain is restricted by incorporation into a ring (Garratt et al.,

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1994b). As these analogues are chiral, we now report the separation of the enantiomers of one of these analogues and an evaluation of the ability of the enantiomers to bind to and activate the melatonin receptor.

2. Materials and methods

2.1. Chemistry

N-Acetyl-4-aminomethyl-6-methoxy-9-methyl-1,2,3,4-tetrahydrocarbazole (AMMTC; Fig. 1) was prepared as described previously (Garratt et al., 1994b). Racemic AMMTC was dissolved in ethanol (10 mg/ml) and injected in 1 ml aliquots onto a Chiracell AD preparative high performance liquid chromatography column (23 × 2 cm). The column was eluted with hexane:ethanol (85:15 v/v) and the eluant monitored at 280 nm. The two enantiomers eluted with baseline separation, the (+)-enantiomer eluting first. Analytical high performance liquid chromatography studies on a Chiracell column showed that neither sample contained the other enantiomer. (−)-AMMTC $[\alpha]_D^{20} = -29.0°$ (1.0 mg/ml ethanol); (+)-AMMTC $[\alpha]_D^{20} = +28.5°$ (1.0 mg/ml ethanol). Circular dichroism spectra were acquired on a Jasco J600 spectrometer in ethanol. Both compounds showed absorption maxima at 211.0, 232.0, 263.4 and 287.0 nm, the spectrum of one enantiomer being the equal and opposite of the other. *N*-Cyclobutanecarbonyl 2-phenyltryptamine was prepared as described previously (Garratt et al., 1995).

2.2. 2-[^{125}I]Iodomelatonin binding in chicken brain membranes

White Leghorn chicks (*Gallus domesticus*, Orchard Farms, Buckinghamshire, UK) were purchased at 1-day of age and housed for two weeks under controlled lighting conditions (12L:12D, lights on at 06:00 h) in a temperature-controlled room (28 ± 2°C). Chicks were decapitated between 14:00 and 15:00 h and the whole brain rapidly removed and frozen in liquid nitrogen. Whole brain membranes were prepared as described previously (Sugden and Chong, 1991), suspended in Tris-HCl (50 mM, pH 7.4) containing phenylmethylsulphonyl fluoride (1 mM), leupeptin (50 μg/ml) and EGTA (1 mM) and aliquots stored in liquid nitrogen. In competition experiments, duplicate membrane aliquots were incubated (25°C, 60 min) with competing drugs and 70–80 pM of 2-[^{125}I]iodomelatonin (2200 Ci/mmol, DuPont UK, Stevenage, UK). Non-specific binding was defined using cold melatonin (1 μM; Sigma, Poole, Dorset, UK).

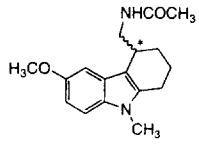

Fig. 1. Structure of *N*-acetyl-4-aminomethyl-6-methoxy-9-methyl-1,2,3,4-tetrahydrocarbazole (AMMTC).

2.3. Pigment aggregation responses in Xenopus laevis melanophores

Xenopus laevis melanophores were prepared from stage 20 embryos as decribed previously (Messenger and Warner, 1977; Sugden and Rowe, 1992), and were maintained in Leibovitz L-15 medium (ICN, Thame, UK) diluted 1 to 1 with deionized water containing 10% foetal calf serum (Imperial Laboratories, Andover, UK), penicillin (200 i.u./ml), streptomycin (200 μg/ml), amphotericin B (2.5 μg/ml) and α-melanocyte stimulating hormone (α-MSH, 30 nM). The response of melanophores to addition of drugs was determined in randomly chosen individual cells as described previously (Sugden, 1991; Sugden, 1994b) by measuring the area occupied by pigment before and after drug addition. Alternatively, the number of cells within a colony showing distinct pigment granule aggregation in response to a given concentration of agonist was counted. The mean percentage change in pigmented area or the proportion of cells showing clear pigment granule aggregation was calculated. Drugs were prepared as stock solutions (10 mM) in methanol and stored at −20°C in the dark. Drugs were diluted with deionized water immediately before use and added to cultures from 100 × stock solutions. Cumulative additions were made to construct concentration-response curves.

2.4. Data analysis

IC_{50} values were determined by fitting a four-parameter logistic equation to the radioligand binding data from competition assays using the ALLFIT programme (DeLean et al., 1978). Inhibition constants (K_i) were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). For experiments on melanophores, the concentration of agonist producing a distinct pigment aggregation response in 50% of tested cells was defined as the EC_{50}. The concentration-response curves were plotted and EC_{50} determined using ALLFIT.

3. Results

In chick brain membranes, 2-[^{125}I]iodomelatonin binding was saturable with an equilibrium dissociation

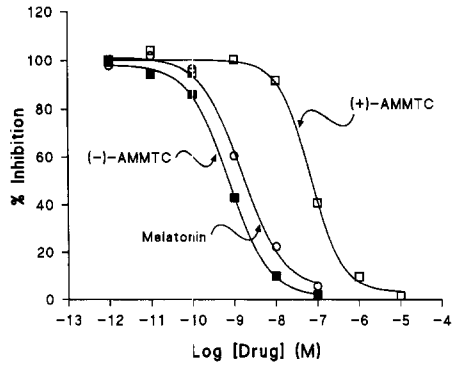

Fig. 2. Inhibition of 2-[^{125}I]iodomelatonin binding in chicken brain membranes by melatonin and the (−)- and (+)-enantiomers of AMMTC. Each point is the average of duplicate determinations. The results of a typical experiment are shown.

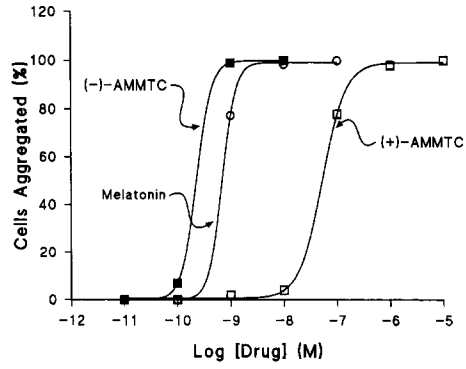

Fig. 3. Pigment granule aggregation concentration-response curves for melatonin, (−)-AMMTC and (+)-AMMTC. The percentage of melanophores showing clear pigment granule aggregation in response to cumulative additions of the concentrations of the compounds shown is plotted. The number of cells examined was: melatonin, 62 cells; (−)-AMMTC, 81 cells; (+)-AMMTC, 61 cells.

constant (K_d) of 32.3 ± 2.3 pM and a maximal number of binding sites (B_{max}) of 19.6 ± 0.5 fmol/mg protein (errors given are the standard errors of the computer-derived estimates).

Melatonin, (−)-and (+)-AMMTC competitively inhibited 2-[^{125}I]iodomelatonin binding to chick brain membranes with pseudo Hill coefficients close to unity (range 0.89–1.11) suggesting that these analogues interact with a single binding site (Fig. 2; Table 1). The affinity of (−)-AMMTC at the 2-[^{125}I]iodomelatonin binding site was 372 ± 53 pM (n = 3), 130-fold higher than the affinity of (+)-AMMTC (K_i 48.4 ± 8.1 nM; n = 3). The racemate had an affinity of 0.97 ± 0.20 nM (data not shown), close to what would be expected for a 50:50 mixture of the two enantiomers. The affinity of melatonin (K_i 487 ± 120 pM; mean ± S.E.M, n = 3) was similar to that reported previously (Sugden and Chong, 1991; Sugden and Rowe, 1994).

In *Xenopus laevis* melanophores, all three compounds were agonists; the proportion of cells aggregating was related to the concentration of agonist added (Fig. 3). At a maximal concentration of each compound, complete pigment aggregation was observed, indicating that all of these compounds were full agonists (Fig. 4). (−)-AMMTC (EC_{50} 230 pM) was ~ 230-fold more potent than (+)-AMMTC (EC_{50} 52.3 nM; Fig. 3; Table 1). The potency of melatonin (EC_{50} 690 pM) was similar to that previously reported under these assay conditions (Sugden and Rowe, 1994).

Pigment granule aggregation induced by melatonin can be prevented and reversed by melatonin receptor antagonists, such as luzindole (*N*-acetyl 2-benzyltryptamine; Sugden, 1992). In the present study, a new antagonist, *N*-cyclobutanecarbonyl 2-phenyltryptamine (Garratt et al., 1995) produced a significant reversal of aggregation induced by melatonin and each of the enantiomers of AMMTC (Fig. 4). Luzindole (10^{-5} M) also significantly antagonized pigment aggregation induced by the enantiomers of AMMTC and melatonin (data not shown).

Table 1
Inhibition constants (K_i) and potency (EC_{50}) of melatonin, and (−)- and (+)-enantiomers of AMMTC

Compound	2-[^{125}I]Iodomelatonin binding		Melanophore
	K_i ± S.E.M. (pM)	Slope factor ± S.E.M.	EC_{50} (pM)
Melatonin	487 ± 120	0.91 ± 0.04	690
(−)-AMMTC	372 ± 53	1.00 ± 0.11	230
(+)-AMMTC	48400 ± 8100	0.90 ± 0.08	52300

K_i values were calculated using the Cheng-Prusoff equation from IC_{50} values determined in three competition experiments as described in Materials and methods. The potency of each compound in aggregating pigment granules in *Xenopus laevis* melanophores (EC_{50}) was defined as the concentration producing readily detectable aggregation in 50% of the melanophores tested (Fig. 2).

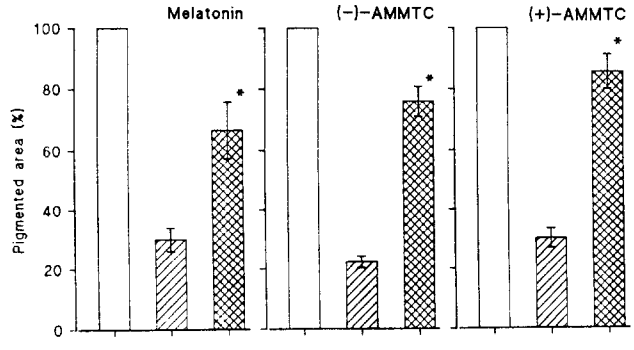

Fig. 4. The melatonin receptor antagonist, *N*-cyclobutanecarbonyl 2-phenyltryptamine reverses melatonin, (−)- and (+)-AMMTC-induced pigment aggregation in melanophores. Data were obtained by measuring the area occupied by pigment in individual cells before treatment (clear bars), 15 min after agonist addition (melatonin 10^{-8} M, (−)-AMMTC 10^{-8} M or (+)-AMMTC 10^{-6} M; hatched bars) and 30 min after subsequent addition of *N*-cyclobutanecarbonyl 2-phenyltryptamine (10^{-5}M, double hatched bars). Results indicate the means ± S.E.M. pigment areas of eight melanophores; $^{*}P < 0.05$ compared to agonist alone.

4. Discussion

This is the first report to describe the biological activity of an enantioselective melatonin receptor ligand. We recently synthesized and tested a series of analogues of melatonin which have restricted conformations of the C-3 amidoethane side-chain, an essential part of the melatonin molecule for receptor binding (Garratt et al., 1994b). In the present experiments, the racemate of one of these chiral tetrahydrocarbazoles, AMMTC, was resolved by high performance liquid chromatography on a chiral preparative column. In competition assays on chick brain membranes using 2-[^{125}I]iodomelatonin, (−)-AMMTC had ~130-fold greater affinity than the (+)-enantiomer. In the specific melatonin bioassay, pigment aggregation in *Xenopus* melanophores, (−)-AMMTC was ~230-fold more potent than the (+)-enantiomer. In both assays (−)-AMMTC was slightly more potent than melatonin itself. Both enantiomers of AMMTC are full agonists and probably produce pigment granule aggregation in melanophores by an action at the melatonin receptor as the melatonin receptor antagonists, *N*-cyclobutanecarbonyl 2-phenyltryptamine (Garratt et al., 1995) and luzindole (Dubocovich, 1988) significantly reversed AMMTC-induced aggregation.

As the C-3 amido side-chain of tetrahydrocarbazole analogues such as AMMTC is conformationally restricted, compared to the amidoethane side-chain of melatonin, and the two enantiomers have such a large difference in receptor affinity and biological potency, the enantiomers will be valuable tools in molecular modelling studies which aim to define the conformation of melatonin when it binds to the active site of the receptor. The finding that (−)-AMMTC was as potent as melatonin itself suggests that the conformation of (−)-AMMTC is very similar to that adopted by melatonin when it interacts with the binding site. Recent molecular modelling studies have proposed a model of the receptor active site (Sugden et al., 1995) based on a quantitative structure activity relationship analysis, the predicted amino acid sequence of the cloned *Xenopus* melanophore receptor (Ebisawa et al., 1994) and homology with other G-protein-coupled receptor models. This model suggests that melatonin binding may involve hydrogen bonding between 5-methoxyl and amide moieties and complementary residues within putative transmembrane domains II and IV, and charge transfer interactions between the indole ring and a tryptophan residue in transmembrane domain VI. The constraints introduced by the rigid tricyclic ring on the side-chain of AMMTC will allow this model to be critically tested. We are currently attempting to determine the absolute configuration of the enantiomers of AMMTC.

It will be interesting also to test the enantiomers of AMMTC on other established models of melatonin action, such as the inhibition of dopamine release from the retina (Dubocovich, 1985), inhibition of cyclic AMP synthesis in pars tuberalis (Morgan et al., 1989; Carlson et al., 1989), inhibition of gonadotropin-releasing hormone-induced luteinizing hormone release and cyclic AMP synthesis in neonatal rat anterior pituitary (Martin and Klein, 1976; Vanecek and Vollrath, 1989) and spontaneous firing of suprachiasmatic neurones in hypothalamic slices (Mason and Brooks, 1988). Limited pharmacological evidence, obtained with the relatively small number of melatonin analogues so far available, has suggested that the high affinity receptor mediating melatonin's effects in these models is similar. The enantiomers of AMMTC may also be useful in assessing the role of specific melatonin receptors in several other biological actions attributed to melatonin in recent years. Such actions include an inhibitory effect on oestrogen receptor expression and proliferation in MCF-7 cells (Hill and Blask, 1988; Molis et al., 1994), activation of human monocytes (Morrey et al., 1994) and vasoconstrictor activity in isolated pressurized segments of rat caudal artery (Evans et al., 1992).

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