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A new melatonin receptor ligand with mt_1-agonist and MT_2-antagonist properties[1]

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Abstract: It has been difficult, so far, to obtain melatonin analogs possessing high selectivity for the respective melatonin receptors, mt_1 and MT_2. In the present work, we report the synthesis and pharmacological characterization of a new compound N-{2-[5-(2-hydroxyethoxy)-1H-indol-3-yl)] ethyl} acetamide or 5-hydroxyethoxy-N-acetyltryptamine (5-HEAT). To assess the activity of the compound, the following tests were performed: affinity determination for the high- and low-affinity receptor states (2-[^{125}I]iodomelatonin binding), potency and intrinsic activity in inducing G protein activation ([^{35}S]GTPγS binding assay). 5-HEAT showed little selectivity for the mt_1 receptor, with pK_i values of 7.77 for mt_1 and 7.12 for the MT_2 receptors, respectively. 5-HEAT was able to differentiate between the high- and the low-affinity receptor states in the mt_1 but not in the MT_2 receptor. 5-HEAT induced a high level of G protein activation when acting through the mt_1 receptor, with a relative intrinsic activity of 0.92. On the contrary, it elicited only minimal MT_2 receptor-mediated G protein activation, with a relative intrinsic activity of 0.16, and was also able to inhibit the melatonin-induced MT_2 receptor-mediated G protein activation, with a pK_B value of 7.4. In conclusion, it appears that 5-HEAT possesses very different efficacies at the two melatonin receptors, behaving as a full melatonin receptor agonist at the mt_1 and as an antagonist/weak partial agonist at the MT_2 receptor. Therefore, it is a promising ligand for use in functional studies aimed at distinguishing between the effects mediated by the different melatonin receptors in the human.

R. Nonno[1], V. Lucini[1], G. Spadoni[2], M. Pannacci[1], A. Croce[1], D. Esposti[3], C. Balsamini[2], G. Tarzia[2], F. Fraschini[1] and B.M. Stankov[1]

[1]Cattedra di Chemioterapia, Dipartimento di Farmacologia, Università degli Studi di Milano, Via Vanvitelli 32, 20129 Milano, Italy; [2]Istituto di Chimica Farmaceutica, Università degli Studi di Urbino, Piazza Rinascimento 6, 61029 Urbino, Italy; [3]Istituto di Fisiologia Umana II, Università degli Studi di Milano, Via Mangiagalli 32, 20131 Milano, Italy

Key words: human melatonin receptors – melatonin analogs – mt_1 melatonin receptor agonist – MT_2 melatonin receptor antagonist – [^{35}S]GTPγS binding

Address reprint requests to Romolo Nonno, Cattedra di Chemioterapia, Dipartimento di Farmacologia, Università degli Studi di Milano, Via Vanvitelli 32, 20129 Milano, Italy

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Introduction

Melatonin is a rhythmic secretory product released from the mammalian pineal gland. It plays a central role in the regulation of circadian and seasonal behavior in mammals [Arendt, 1985]. In the human, melatonin elicits circadian, cardiovascular and hypnotic effects [Dollins et al., 1994; Lewy et al., 1995; Reppert et al., 1996; Cagnacci et al., 1998]. Melatonin appears to evoke its effects through high-affinity, G protein-coupled receptors [Morgan et al., 1994]. Both native and recombinant melatonin receptors are negatively coupled to adenylyl cyclase by pertussis toxin-sensitive G proteins [Morgan et al., 1994; Reppert et al., 1994, 1995]. Currently, two subtypes of human melatonin receptors have been identified: mt_1 (preferentially expressed in the brain) and MT_2 (preferentially expressed in the retina) [Reppert et al., 1994, 1995]. Some recent studies have reported the co-expression of both melatonin receptors in the same tissue: the human fetal kidney [Drew et al., 1998], the human cerebellum [Al-Ghoul et al., 1998] and the mouse suprachiasmatic nucleus [Liu et al., 1997]. These findings highlight the possibility that the melatonin receptors are more widely expressed (and co-expressed) than previously observed, in both the central nervous system and peripheral tissues, and that they could mediate distinct, or even opposite, responses in the same target cell [Liu et al., 1997; Doolen et al., 1998].

Little is known about the relative roles of the two receptors in the transduction of the melatonin signal. A recent study, with mt_1 receptor-deficient

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mice, has suggested that the two effects of melatonin on the mouse suprachiasmatic nuclei, inhibition of neuronal firing and entrainment of the circadian rhythm, could be mediated independently by the two receptors [Liu et al., 1997]. The study of the different roles of the two melatonin receptors is further hindered by the lack of selective melatonin receptor agonists. Some melatonin receptor antagonists (GR128107, 4P-PDOT, 4P-ADOT and 4P-CADOT) have been reported to be highly selective for the human MT_2 receptor [Dubocovich et al., 1997]; one of these melatonin analogs, namely 4P-PDOT, has been successfully used to dissect mt_1 and MT_2 receptor-mediated responses to melatonin in the rat caudal artery [Doolen et al., 1998]. Nevertheless, GR128107 has been reported to behave as a partial agonist in *Xenopous laevis* melanophores and as a full agonist in recombinant human mt_1 and MT_2 receptors [Teh and Sugden, 1999], while 4P-PDOT, 4P-ADOT and 4P-CADOT elicited partial agonist activity on recombinant human MT_2 receptors [Masana et al., 1997; Nonno et al., 1999]. These differences in the measured intrinsic activity could be due to the varying receptor expression level in the different systems or to the assay used for intrinsic activity determination.

Relative intrinsic activity can be measured at a range of points in a signaling cascade. However, due to cross-talk between transduction pathways and varying levels of amplification throughout such cascades, differences in levels of expression of the G protein-coupled receptors and altered R/G protein expression ratios can result in variations in this parameter when using distal points for analysis [MacEwan et al., 1995]. Therefore, a proximal assay point, such as ligand-induced G protein activation, provides a highly appropriate level for such measurements.

Recently, we studied the affinity and efficacy of a number of known melatonin analogs at the mt_1 and MT_2 human melatonin receptor stably expressed in NIH3T3 fibroblast cells, using the agonist-mediated [^{35}S]GTPγS binding method for evaluation of the intrinsic activity [Nonno et al., 1998, 1999]. In the present study, we report that a new melatonin analog, N-{2-[5-(2-hydroxyethoxy)-1H-indol-3-yl)] ethyl} acetamide (we will refer to this ligand hereafter as 5-hydroxyethoxy-N-acetyltryptamine or 5-HEAT), possesses very different intrinsic activities at the human melatonin mt_1 and MT_2 receptors as measured by the agonist-mediated [^{35}S]GTPγS binding method. Thus, 5-HEAT is a new promising tool for the dissection of mt_1 and MT_2 receptor-mediated responses.

Materials and methods

Drugs

2-[^{125}I]iodomelatonin (specific activity ≈ 2,000 Ci/mmol) and [^{35}S]GTPγS (specific activity 1,070 Ci/mmol) were purchased from Amersham (Buckinghamshire, UK). Melatonin, GDP and GTPγS were from Sigma Chemical Co. (St Louis, MO). 2-Iodomelatonin was obtained from RBI (Natick, MA). N-acetyl-5-hydroxytryptamine was purchased from Aldrich Chemical Co. (Milwaukee, WI) Geneticin (G418) was purchased from GIBCO (Grand Island, NY). General laboratory reagents including Tris-HCl, Calf Serum, Dulbecco's Modified Eagle's medium were from Sigma Chemical Co. Filter-Count scintillation fluid was from Packard (Downers Grove, IL).

Preparation of membranes

NIH3T3 cells stably expressing the cloned human mt_1 or MT_2 receptor [Nonno et al., 1998, 1999] and untransfected NIH3T3 cells were grown to confluence. On the day of assay, cells were detached from flasks with 4 mM EDTA in 50 mM Tris-HCl (pH 7.4 at room temperature) and centrifuged at 1,000g for 10 min at 4°C. The cells were then resuspended in 2 mM EDTA/50 mM Tris-HCl, homogenized in 10–15 volumes of ice-cold 2 mM EDTA/50 mM Tris-HCl with an Ultra-Turrax and centrifuged at 50,000g at 4°C for 25 min. The final pellet was then resuspended in ice-cold 50 mM Tris-HCl assay buffer. Membrane protein levels were determined according to the method of Bradford [1976].

2-[^{125}I]iodomelatonin binding

The final membrane concentration was 2–4 mg mL^{-1} and the protein amount was 5–10 μg per tube. The binding conditions were described in detail elsewhere [Stankov et al., 1991]. The incubation time was 90 min. The competition experiments for the study of the high- and the low-affinity states of the receptors were performed in the presence or in the absence of NaCl (700 mM) and GTPγS (100 μM). The total 2-[^{125}I]iodomelatonin concentration was 100 pM in experiments designed for the K_i calculation and 200 pM in competition experiments for the K_{ihigh} and K_{ilow} calculation. Non-specific binding was measured in the presence of 0.1 μM unlabelled 2-iodomelatonin.

[^{35}S]GTPγS binding

Agonist-stimulated [^{35}S]GTPγS binding was performed as previously described [Nonno et al., 1998, 1999]. The final pellet, obtained as described above, was resuspended in ice-cold 50 mM Tris-HCl assay buffer to give a final membrane concentration of 20–30 mg mL^{-1}. The membranes (30–45 μg of protein per tube) were then incubated for 30 min at 30°C, in the presence and in the absence of melatonin analogues, in assay buffer containing 0.3–0.5 nM [^{35}S]GTPγS, 50 μM GDP, 100 mM NaCl and 3 mM $MgCl_2$. To determine the effect of the competition of an antagonist in the melatonin-stimulated [^{35}S]GTPγS binding assay, melatonin (100 nM) and various concentrations of the antagonist were simultaneously added to the membrane preparations.

The final incubation volume was 100 μL. Basal binding was assessed in the absence of drug and non-specific binding was measured in presence of 10 μM GTPγS. The incubation was terminated by adding 1 mL of ice-cold Tris-HCl buffer, pH 7.4, followed by rapid filtration under vacuum through Whatman GF/B glass fiber filters and by three washes with 3 mL of ice-cold Tris-HCl buffer, pH 7.4. Bound radioactivity was determined by liquid scintillation spectrophotometry after overnight extraction in 4 mL Filter-Count scintillation fluid.

Chemistry

The 5-oxyacetic acid methyl ester derivative 2 was prepared in dimethylformamide (DMF) by *O*-alkylation of N-acetyl-5-hydroxytryptamine (1) with methyl chloroacetate, using sodium hydride as a base. Reduction of the ester 2 with $LiAlH_4$ (THF, 0°C, 1.5 hr) gave the 5-hydroxyethoxy melatonin analogue 3 (Fig. 1).

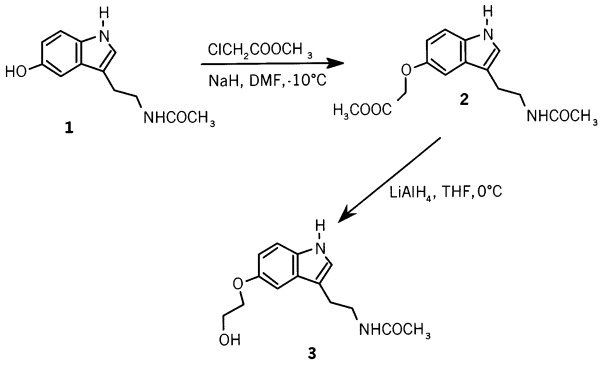

Fig. 1. Scheme of the synthesis of 5-HEAT. 1, N-acetyl-5-hydroxytryptamine; 2, [3-(acetylaminoethyl)-1H-indol-5-yloxy]-acetic acid methyl ester; 3, N-[2-(5-hydroxyethoxy-1H-indol-3-yl)ethyl)]acetamide or 5-HEAT. See the text for details.

Melting points were determined on a Büchi SMP-510 capillary melting point apparatus and are uncorrected. ^{1}H-NMR spectra were recorded on a Bruker AC 200 spectrometer; chemical shifts (δ scale) are reported in parts per million (ppm) relative to the central peak of the solvent. Coupling constants (*J* values) are given in hertz (Hz). EI-MS spectra (70 eV) were taken on a Fisons Trio 1000. Only molecular ions (M^+) and base peaks are given. Infrared spectra were obtained on a Bruker FT-48 spectrometer; absorbances are reported in ν (cm^{-1}). Elemental analyses for C, H and N were performed on a Carlo Erba analyzer, and were within ± 0.4% of theoretical values.

Synthesis of 5-HEAT

[3-(Acetylaminoethyl)-1H-indol-5-yloxy]acetic acid methyl ester (2). N-Acetyl-5-hydroxytryptamine (0.109 g, 0.5 mmol) and methyl chloroacetate (0.054 g, 44 μL, 0.5 mmol) were added to a stirred suspension of sodium hydride (0.016 g of an 80% dispersion in mineral oil, 0.55 mmol) in dry DMF (4 mL) at − 10°C under a N_2 atmosphere, and the resulting mixture was stirred at − 10°C for 2 hr. The mixture was then poured into ice-water (40 g) and extracted with ethyl acetate/*n*-hexane. The organic phase was washed with brine, dried (Na_2SO_4) and concentrated under reduced pressure to give a residue which was purified by chromatography (Ethyl acetate as eluent) and crystallization from ethyl acetate/*n*-hexane. White solid; (0.1 g, 69% yield); mp 128–129°C; MS (EI), *m/z* 290 (M^+), 231 (100); ^{1}H-NMR ($CDCl_3$) δ 1.92 (s, 3H), 2.91 (m, 2H), 3.55 (m, 2H), 3.81 (s, 3H), 4.68 (s, 2H), 5.71 (br s, 1H), 6.91 (dd, 1H, $J = 8.9$ and $J = 2.4$ Hz), 6.99 (m, 1H), 7.03 (d, 1H, $J = 2.4$ Hz), 7.27 (d, 1H, $J = 8.9$ Hz); IR (cm^{-1}, nujol) 3338, 1748, 1651.

N-[2-(5-hydroxyethoxy-1H-indol-3-yl)ethyl)]-acetamide or 5-HEAT (3). A solution of **2** (0.087 g, 0.3 mmol) in dry THF (2 mL) was added dropwise to a stirred suspension of $LiAlH_4$ (0.014 g) in dry THF (2 mL) at 0°C under nitrogen. The mixture was stirred for an additional 1.5 hr at 0°C, and the unreacted $LiAlH_4$ was destroyed by careful addition of water. The resulting mixture was filtered through a Celite® pad and the filtrate was dried over Na_2SO_4. The dried solution was concentrated under reduced pressure to give 0.055 g (70% yield) of a white solid. An analytical sample was obtained by crystallization from dichloromethane. mp 118°C; MS (EI): m/z 262 (M^+), 203 (100); ^{1}H-NMR (acetone-d_6): δ 1.86 (s, 3H), 2.87

Table 1. 2-[^{125}I]iodomelatonin and [^{35}S]GTPγS binding parameters for melatonin and 5-HEAT

		mt_1		MT_2	
		Melatonin	5-HEAT	Melatonin	5-HEAT
2-[^{125}I]Imel binding	pK_I	9.600 ± 0.021	7.770 ± 0.022	9.540 ± 0.018	7.119 ± 0.060
	pK_{ihigh}	9.797 ± 0.012	7.826 ± 0.059	9.739 ± 0.046	7.023 ± 0.033
	pK_{ilow}	8.829 ± 0.086	6.900 ± 0.027	8.709 ± 0.059	6.932 ± 0.031
	pK_{ihigh}-pK_{ilow} difference	0.968	0.926	1.030	0.091
[^{35}S]GTPγS binding	PEC_{50}	9.246 ± 0.062	7.469 ± 0.080	9.197 ± 0.089	7.081 ± 0.075
	Rel. int. act.	1	0.92 ± 0.02	1	0.16 ± 0.02
	pK_B				7.396 ± 0.065

All data are means ± SEM of at least four independent experiments performed in duplicate (2-[^{125}I]Imel binding) or in triplicate ([^{35}S]GTPγS binding). The relative intrinsic activity was determined relative to the maximum stimulation evoked by melatonin in each experiment.

(t, 2H), 3.46 (q, 2H), 3.89 (m, 2H), 4.08 (t, 2H), 6.75 (dd, 1H $J = 2.2$ Hz and $J = 8.79$), 7.12 (d, 1H $J = 2.2$ Hz), 7.14 (d, 1H $J = 2.2$ Hz), 7.26 (d, 1H $J = 8.79$), 8.13 (br s, 1H); IR (cm^{-1}, nujol): 3256, 3114, 1629. Anal. Calcd for C14H18N2O3: C, 64.09; H, 6.93; N, 10.68. Found: C, 63.69; H, 6.86; N, 10.54.

Data analysis

Data reported are the means ± SE of at least four independent experiments that were each performed in duplicate (2-[^{125}I]iodomelatonin binding experiments) or triplicate ([^{35}S]GTPγS binding experiments). The IC_{50}, and EC_{50} values were determined by using nonlinear curve fitting strategies. K_i values were calculated from the IC_{50} values using the Cheng-Prusoff equation [Cheng and Prusoff, 1973]. The K_{ihigh} and K_{ilow} values refer to the K_i values calculated from competition experiments performed in the absence or presence of NaCl (700 mM) and GTPγS (100 μM), respectively. K_B values were calculated using the "functional equivalent" of the Cheng-Prusoff equation, with the agonist EC_{50} value as a functional correlate of the original K_m value [Craig, 1993]:

$$K_B = IC_{50}/1 + (A/EC_{50}).$$

The data from [^{35}S]GTPγS binding experiments are given as percentage of specific basal binding, where the specific basal binding was fixed as 100%. The relative intrinsic activity values are expressed as a fraction of net maximal stimulation induced by melatonin in each experiment.

Results

The results obtained are summarized in Table 1. In competition experiments with a radioligand concentration of 100 pM, 5-HEAT showed a moderate affinity for both receptor subtypes (around 60–260 times less than melatonin) and a low selectivity for the mt_1 receptor.

The optimal binding conditions (incubation in presence of 200 pM 2-[^{125}I]iodomelatonin, 700 mM NaCl and 100 μM GTPγS) for the measurement of the apparent affinity at the low-affinity state of the melatonin receptors expressed in NIH3T3 cells were determined elsewhere [Nonno et al., 1998, 1999]. Here we performed a series of competition experiments in the binding conditions described above in order to calculate the apparent affinity values at the high (pK_{ihigh}) and the low (pK_{ilow}) affinity states for both melatonin and 5-HEAT (see Table 1). The compound 5-HEAT showed a $pK_{ihigh} - pK_{ilow}$ difference of 0.926 (95% of the value obtained with melatonin) in membranes from cells expressing the mt_1 receptor ($NIH3T3_{mt1}$), while the $pK_{ihigh} - pK_{ilow}$ difference was only 0.091 (9% of the value obtained with melatonin) in membranes from cells expressing the MT_2 receptor ($NIH3T3_{MT2}$).

The basal [^{35}S]GTPγS binding to $NIH3T3_{mt1}$ and $NIH3T3_{MT2}$ membranes was 103 ± 4 and 91 ± 6 fmol mg^{-1} protein. Melatonin caused a dose-dependent increase of the basal binding, to reach a plateau at $382 \pm 9\%$ of the basal binding (taken as 100%) with a pEC_{50} value of 9.246 ± 0.062 in $NIH3T3_{mt1}$ membranes, while in $NIH3T3_{MT2}$ membranes the maximum melatonin-stimulated [^{35}S]GTPγS binding was $275 \pm 6\%$ and the melatonin pEC_{50} value was 9.197 ± 0.089. The relative intrinsic activity and the potency of 5-HEAT were then calculated in parallel experiments using both $NIH3T3_{mt1}$ and $NIH3T3_{MT2}$ membranes. As can be seen in Fig. 2, 5-HEAT induced a high G-protein activation in $NIH3T3_{mt1}$ membranes, with a maximum induced [^{35}S]GTPγS binding increase of $359 \pm 9\%$ over basal (not significantly different from the value obtained with

melatonin, t = 1.46, df = 12, $P = 0.085$) and a relative intrinsic activity of 0.92 ± 0.02; on the other hand, it produced very weak G-protein activation in NIH3T3$_{MT2}$ membranes, with a relative intrinsic activity = 0.16 ± 0.02 (Fig. 3). Fig. 3 also shows a representative inhibition curve of the melatonin

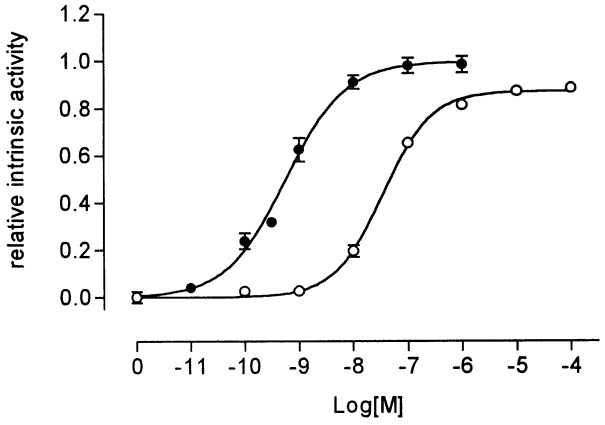

Fig. 2. Comparison of the stimulation of [^{35}S]GTPγS binding to NIH3T3 membranes expressing the mt$_1$ melatonin receptor subtype by melatonin (closed circles) and 5-HEAT (open circles). The data are from a single experiment, representative of at least four independent experiments, with each point determined in triplicate. The experiment was carried out as described in methods, for 30 min at 37°C. Each point represents the mean of triplicate determinations; the error bars indicate the standard error. Values in the y-axis represent the relative intrisic activity, calculated as a fraction of the maximum melatonin-induced [^{35}S]GTPγS binding.

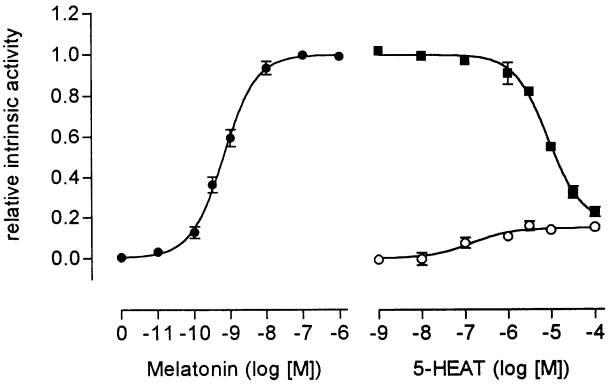

Fig. 3. Stimulation of [^{35}S]GTPγS binding to NIH3T3 membranes expressing the MT$_2$ melatonin receptor subtype by melatonin (closed circles, left x-axis) and 5-HEAT (open circles, right x-axis); on the right x-axis is also shown the inhibition of melatonin (100 nM)-induced [^{35}S]GTPγS binding by 5-HEAT (closed squares). The data are from a single experiment, representative of at least four independent experiments, with each point determined in triplicate. The experiment was carried out as described in materials and methods, for 30 min at 37°C. Each point represents the mean of triplicate determinations; the error bars indicate the SE. Values in the y-axis represent the relative intrinsic activity, calculated as a fraction of the maximum melatonin-induced [^{35}S]GTPγS binding.

(100 nM)-induced [^{35}S]GTPγS binding by 5-HEAT. The maximum [^{35}S]GTPγS binding inhibition by 5-HEAT was 0.16 ± 0.04, relative to the maximal [^{35}S]GTPγS binding induced by melatonin. By using the "functional equivalent" of the Cheng-Prusoff equation, we calculated a 5-HEAT pK_B value of 7.396 ± 0.065. In membranes from native, untransfected NIH3T3 cells, 5-HEAT and melatonin did not induce any change in the level of basal [^{35}S]GTPγS binding.

Discussion

In the present study, we reported the synthesis and pharmacological characterization of a new melatonin ligand, named 5-hydroxyethoxy-N-acetyltryptamine or 5-HEAT, that bears an hydoxyethoxy group on the C5-indole position of melatonin (see Fig. 1).

5-HEAT shows little selectivity for the mt$_1$ receptor, with an affinity around five times higher than for the MT$_2$ receptor. This difference appears to be related to the binding affinity of the compound to the high-affinity state of the receptors: in fact, 5-HEAT binds with the same affinity to the low-affinity state of both receptors (see Table 1). Indeed, 5-HEAT differentiates well between the high- and the low-affinity states of the mt$_1$ receptor, but not of the MT$_2$ receptor (see Table 1).

According to the ternary complex model, the efficacy of a receptor ligand is related to the K_{dlow}/K_{dhigh} ratio [Wreggett and De Lean, 1984]. In this sense, our findings may predict that 5-HEAT possesses different efficacies at the two melatonin receptors. We tested this hypothesis by measuring the intrinsic activity of 5-HEAT in NIH3T3 cell membranes expressing recombinant mt$_1$ or MT$_2$ human melatonin receptors. In both membrane preparations, 5-HEAT responses were directly proportional to receptor stimulation, as shown in Figs. 2 and 3; however, the 5-HEAT G-protein induced activation was nearly maximal at mt$_1$, but very low at MT$_2$ receptor. When applied to melatonin-activated MT$_2$ receptors, 5-HEAT almost completely counteracted the melatonin-induced G protein activation, with a potency value similar to that calculated from binding affinity studies (see Table 1). In particular, at maximal 5-HEAT concentration, both in the presence and in the absence of melatonin (100 nM), the MT$_2$-mediated G protein activation level was only 16% of that induced by melatonin alone, showing that, under our experimental conditions, a complete MT$_2$ receptors occupation by 5-HEAT specifically induces relative intrinsic activity of 0.16.

Previous studies [for a review see Mor et al., 1999] have indicated that compounds showing antagonist activity at the mt_1 and MT_2 melatonin receptors can be obtained by minor structural modification of melatonin agonists. Deletion of the 5-methoxy group, an increase in the overall size of the molecule, modification of the amide function and displacement of the melatonin side chain from C-3 to C-2 indole position were claimed to decrease the intrinsic activity. The present results demonstrate that it is possible to obtain a ligand (5-HEAT) with MT_2 antagonist properties by replacing the 5-methoxy substituent with an hydroxyethoxy group. Surprisingly, 5-HEAT showed agonist properties at the mt_1 receptor.

This is the first case in which a melatonin analog has been found to possess almost opposite efficacies at the two human melatonin receptors, although some melatonin analogs were previously shown to express slight differences in their relative intrinsic activity [Spadoni et al., 1998]. Indeed, we recently characterized some melatonin receptor antagonists by comparing their action at the two human melatonin receptors, revealing that 4P-PDOT and N-[(2-phenyl-1*H*-indol-3-yl)ethyl]cyclobutanecarboxamide behave as antagonists at the mt_1 receptor and as partial agonists at the MT_2 receptor (with relative intrinsic activities of 0.37 and 0.39, respectively) [Nonno et al., 1999]. These results indicate that there are different ligand requirements for antagonist action at the two melatonin receptors, a characteristic that can be useful in distinguishing between the different melatonin actions mediated by the mt_1 and MT_2 receptors, in tissues that naturally co-express these receptors. Recent studies report the co-expression of both melatonin receptors in different human and animal tissues [Liu et al., 1997; Al-Ghoul et al., 1998; Doolen et al., 1998; Drew et al., 1998]. In some of these tissues, a dual effect of melatonin has been reported, and the two melatonin receptors have been shown to be differently involved in mediating the melatonin actions. In the mouse suprachiasmatic nucleus, melatonin appears to elicit acute inhibition of neuronal firing through the mt_1 receptor and phase shifts of the circadian rhythms through the MT_2 receptor [Liu et al., 1997; Dubocovich et al., 1998]. In the rat caudal artery, melatonin potentiates the adrenergic-induced vasoconstriction [Krause et al., 1995]; this effect appears to be the result of opposing contractile responses to melatonin: vasoconstriction is mediated by activation of the mt_1 receptor and vasodilatation by the MT_2 receptor [Doolen et al., 1998]. The physiological role of the MT_2 receptor in mediating the melatonin actions on the vascular system is further supported by the fact that estradiol is able to modulate the vascular responses to melatonin in the rat caudal artery by enhancing the MT_2 melatonin receptor function [Doolen et al., 1999].

The relative intrinsic activity of 5-HEAT, reported in the present study, appears to be directly comparable with the true relative efficacy of the compound, due to the lack of spare receptors in our system, as shown in previous studies [Nonno et al., 1998, 1999]. This suggests that 5-HEAT could behave as a partial agonist, with an intrinsic activity even higher of that reported here, if applied to tissues in which a MT_2 receptor reserve is present; however, up to now no such tissues have been reported. Moreover, in tissues rich in MT_2 melatonin receptors, such as the rabbit retina, the compound 4P-PDOT antagonized the melatonin responses [Dubocovich et al., 1997], although the same compound elicited partial agonist activity on recombinant human MT_2 receptors [Masana et al., 1997; Nonno et al., 1999]. 4P-PDOT has also been successfully used in blocking melatonin MT_2-mediated responses in the mouse suprachiasmatic nuclei [Dubocovich et al., 1998]. These studies suggest that 5-HEAT, which elicits only minimal MT_2-mediated G-protein activation in comparison with 4P-PDOT [relative intrinsic activities of 0.16 and 0.39, respectively, as measured in the present study and in Nonno et al., 1999], could effectively antagonize MT_2-mediated melatonin actions when applied in tissues that naturally express the MT_2 melatonin receptor.

In conclusion, we report the synthesis and characterization of 5-HEAT, a new melatonin analog with mt_1-agonist and MT_2-antagonist/weak partial agonist properties. 5-HEAT could be very useful in the dissection of the melatonin mt_1/MT_2-mediated actions.

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