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Towards the Development of Mixed MT_1-Agonist/MT_2-Antagonist Melatonin Receptor Ligands

Gilberto Spadoni,[a] Annalida Bedini,*[a] Tomaso Guidi,[a] Giorgio Tarzia,[a] Valeria Lucini,[b] Marilou Pannacci,[b] and Franco Fraschini[b]

*Herein we report attempts to optimize the pharmacological properties of 5-(2-hydroxyethoxy)-*N*-acetyltryptamine (5-HEAT), a melatonin receptor ligand previously described by us. Several 5-substituted and 2,5-disubstituted* N*-acyltryptamines were synthesized and evaluated in vitro for the human cloned MT_1 and MT_2 receptors. From this series of* N*-acyltryptamines the 2-bromo derivative (**5c**) retains the interesting efficacy profile of 5-HEAT and shows increased melatonin receptor affinities; it represents one of the first examples of a high-affinity MT_1 agonist/MT_2 antagonist. Some other full agonists for both melatonin receptors which exhibit similar or increased affinity relative to that of melatonin were obtained.*

Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine, MLT, Figure 1) is a neurohormone primarily secreted by the pineal gland at night in all species.[1] The circadian pattern of MLT secretion coupled with the localization of specific MLT binding sites in the brain region associated with the "biological clock" suggests that MLT may play an important role in modulation of the sleep–wake cycle and circadian rhythms in humans.[2] There is evidence that the administration of MLT is of clinical utility in the treatment of various conditions including jet-lag, work-shift syndrome, sleep disorders,[3] neurodegenerative disorders[4] such as Alzheimer's and Parkinson's disease, and neuropsychiatric diseases such as depression[5] and anxiety.[6] Some MLT receptor agonists are currently under clinical evaluation or have been very recently approved. Agomelatine (S-20098),[7] characterized as a melatonin agonist and selective serotonin antagonist, is being evaluated as an antidepressant drug;[8,9] ramelteon (TAK 375)[10] has been approved, and β-methyl-6-chloromelatonin (LY 156735)[11] is under investigation for the treatment of insomnia.[12]

Other effects of MLT described in the literature include its anti-inflammatory,[13] pain modulatory,[14] retinal,[15] vascular,[16] antitumor,[17] and antioxidant[18] properties. It has also been highlighted as having a marked efficacy in animal models of focal cerebral ischemia, suggesting the hormone as a candidate neuroprotective drug for human stroke.[19]

Most physiological MLT effects result from the activation of high-affinity G-protein-coupled receptors, two of which (MT_1 and MT_2)[20–22] have been found in mammals, including humans, and subsequently cloned. A third subtype (Mel_{1c}), first cloned from *Xenopus laevis*, has been found only in non-mammalians. In addition to these high-affinity MLT receptors ($K_i \approx 0.1$ nM), another low-affinity MLT binding site, termed MT_3, ($K_i \approx 60$ nM) has recently been characterized as a melatonin-sensitive form of the human enzyme quinone reductase 2.[23]

Whereas it is known that the receptors MT_1 and MT_2 are expressed both centrally (suprachiasmatic nucleus, cortex, *pars tuberalis*, etc.) and peripherally (kidney, adipocytes, retina, blood vessels, etc.),[24] the distinct function of these receptors has yet to be clearly defined. There is evidence that MT_1 receptors might be implicated in the sleep-promoting effects of MLT[25] and in mediating vasoconstriction,[26] whereas MT_2 receptors appear to play a major role in the resynchronizing activity of MLT[27,28] and in mediating vasodilation.[26] Another experimentally complex aspect of MLT pharmacology is that in vivo studies of MLT-mediated responses are dependent on the diurnal–nocturnal cycle of the experimental animals. Throughout the 24-hour cycle, the sensitivity of the receptors to specific cues such as MLT itself, photoperiod, and estradiol fluctuates. Studies using knockout MT_1 and/or MT_2 mice suggest a significant degree of redundancy, as these two receptors appear to compensate for one another.[29] To understand the complex pharmacophysiology of MLT, it is important to study the specific functional responses in target tissues linked to each MLT receptor subtype in depth, to facilitate the design and development of novel therapeutic agents.

To date, information about MT_1 and MT_2 melatonin receptor function in target tissues is derived primarily from pharmacological studies with two melatonin receptor ligands: luzindole

[a] Prof. G. Spadoni, Dr. A. Bedini, Dr. T. Guidi, Prof. G. Tarzia
Istituto di Chimica Farmaceutica e Tossicologica
Università degli Studi di Urbino "Carlo Bo"
piazza Rinascimento 6, 61029 Urbino (Italy)
Fax: (+39) 0722-2737
E-mail: annalida@uniurb.it

[b] Dr. V. Lucini, Dr. M. Pannacci, Prof. F. Fraschini
Dipartimento di Farmacologia, Chemioterapia e Tossicologia Medica
Università degli Studi di Milano
Via Vanvitelli 32, 20129 Milano (Italy)

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(a nonselective MT_1/MT_2 antagonist/partial agonist) and 4P-PDOT (a selective MT_2 antagonist/partial agonist) (Figure 1);[23–25] luzindole and 4P-PDOT are also MT_1 antagonists at 300 nM and higher concentrations.[29] Functional characterization of the MT_1 receptor is hampered by the paucity of MT_1-selective ligands; none of these MT_1 ligands have been tested in tissues expressing native melatonin receptors.

We previously reported the discovery and binding characteristics of a novel MLT analogue, 5-hydroxyethoxy-*N*-acetyltryptamine (5-HEAT, Figure 1), a ligand of human MT_1 and MT_2 melatonin receptors[30] which was obtained by replacing the 5-methoxy substituent of MLT with a 2-hydroxyethoxy group. Although this ligand binds to h-MT_1 and h-MT_2 receptors with lower affinity than MLT, it presents an interesting pharmacological profile. 5-HEAT behaves as a full agonist at h-MT_1 and as an antagonist/weak partial agonist at the h-MT_2 receptor subtype. Therefore, it can be considered a promising ligand to be used in functional studies aimed at distinguishing the effects mediated by the different melatonin receptor subtypes in native tissues. Recently, another compound with an MT_1-agonist/MT_2-antagonist profile (S 24268, Figure 1)[31] was reported, but it binds the MT_2 receptor with low affinity. Herein we present our attempts to ameliorate the binding affinity of 5-HEAT while maintaining its very interesting efficacy profile. More specifically, the synthesis and biological activity of 5-HEAT analogues bearing a C2-phenyl, C2-Br, or N1-benzyl substituent are discussed.

A second objective of this work was to clarify the structural requirements that influence the intrinsic activity at both receptor subtypes. For this purpose, new 5-substituted MLT derivatives were synthesized and pharmacologically characterized.

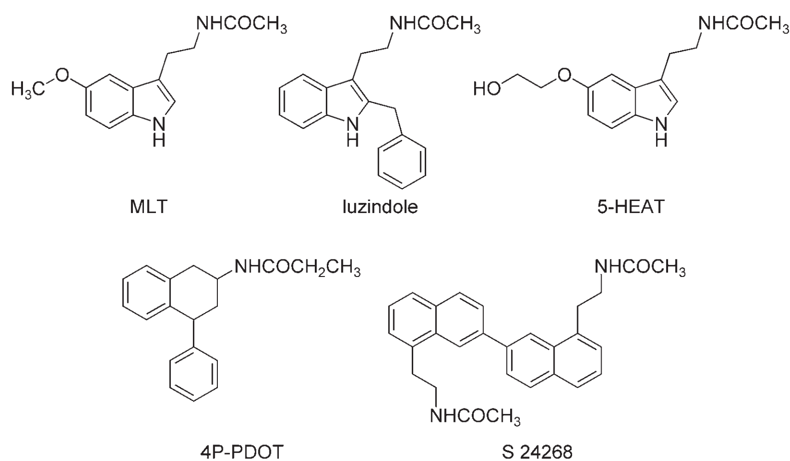
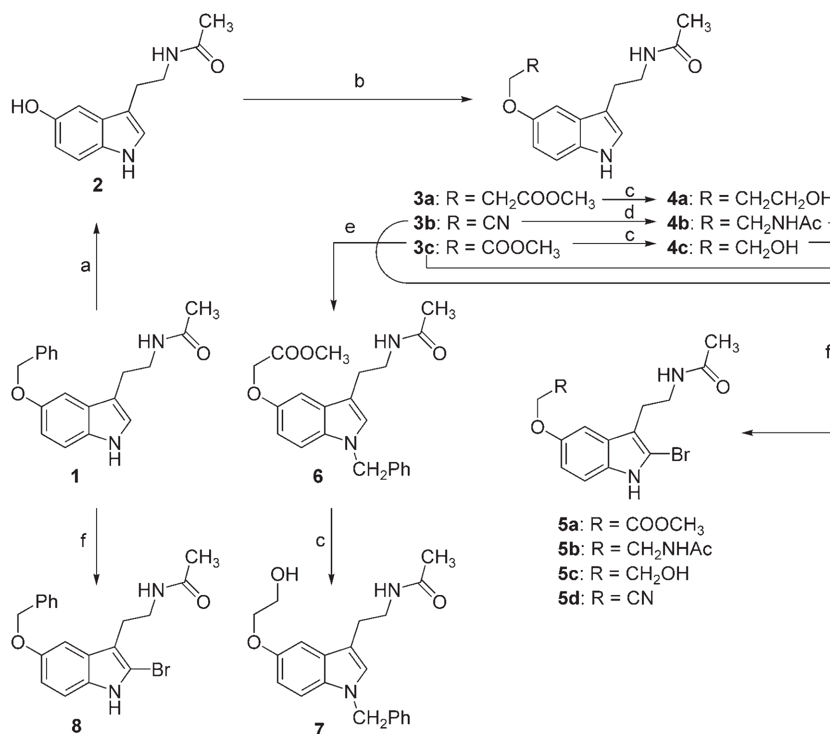


Figure 1. Structures of MLT and some MLT receptor ligands.

Chemistry

The syntheses of 5-substituted-*N*-acyltryptamines **3b**,**c**, **4a**–**c**, **5a**–**d**, **7**, **8**, and **12** were carried out according to the synthetic pathways depicted in Schemes 1 and 2, following known chemical procedures. *N*-Acetylserotonin **2**, the common starting material for most of the target compounds, was prepared by hydrogenolysis of *N*-[2-(5-benzyloxy-1*H*-indol-3-yl)ethyl]acetamide **1**[32] over 10% Pd-C (Scheme 1).

Compounds **3a**, **3b**, and **3c**[30] were prepared by O-alkylation of *N*-acetylserotonin **2** with methyl acrylate, chloroacetonitrile, or methyl chloroacetate re-



Scheme 1. Reagents and conditions: a) H_2, Pd-C (10%), 400 kPa, MeOH, RT; b) NaH, methyl acrylate for **3a**, chloroacetonitrile for **3b**, and methyl chloroacetate for **3c**, *N*,*N*-dimethylformamide (DMF); c) $LiAlH_4$, THF, 0°C; d) H_2, Raney-Ni, 400 kPa, Ac_2O, THF, 50°C; e) NaH, benzyl bromide, DMF, RT; f) phenyltrimethylammonium tribromide, THF, RT.

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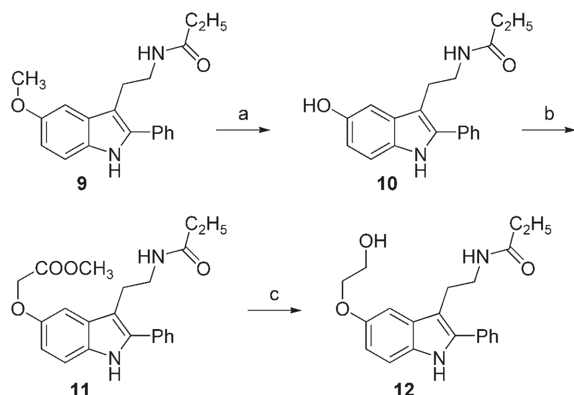
spectively, in the presence of sodium hydride as a base. The methyl ester derivatives **3a** and **3c** were converted into the corresponding alcohols **4a** and **4c** (5-HEAT) by reduction with $LiAlH_4$. Hydrogenation of the cyanomethoxytryptamine derivative **3b** over Raney nickel and concomitant N-acylation with acetic anhydride gave the bis(acetylaminoethyl)indole **4b**. Indole N1 alkylation of **3c** with benzyl bromide afforded the *N*-benzyl derivative **6** which was converted into the corresponding (2-hydroxyethoxy)indole **7** by treatment with $LiAlH_4$.

The 2-bromo derivatives **8** and **5a–d** were synthesized by direct bromination of **1**, **3b**,**c**, and **4b**,**c** with 1 equiv phenyltrimethylammonium tribromide (in THF at room temperature) according to a recently described procedure that was used to synthesize 2-bromomelatonin from melatonin.[33]

5-Hydroxy-2-phenyl-*N*-propionyltryptamine **10** was obtained by O-demethylation of the corresponding 5-methoxy analogue **9**[34] with BBr_3 (Scheme 2). O-Alkylation of **10** with methyl chloroacetate in the presence of sodium hydride in DMF afforded **11**, which was converted into the target compound **12** by the usual $LiAlH_4$-mediated reduction.



Scheme 2. Reagents and conditions: a) BBr_3, CH_2Cl_2, RT; b) NaH, methyl chloroacetate, DMF; c) $LiAlH_4$, THF, 0 °C.

Binding studies

The new derivatives **3b**,**c**, **4a–c**, **5a–d**, **7**, **8**, and **12** were evaluated as potential melatonin receptor ligands through binding experiments. Binding affinity was assessed in competition experiments by using 2-[^{125}I]iodomelatonin as the labeled ligand on cloned human MT_1 and MT_2 receptors expressed in NIH3T3 rat fibroblast cells. The relative intrinsic activity (IA_r) was determined with the GTPγS test by measuring the direct activation of the G protein after binding of the tested compound to the cloned human MT_1 or MT_2 receptor.

Results and Discussion

We previously reported that replacement of the 5-methoxy substituent of MLT with a 5-(2-hydroxyethoxy) group results in an MLT analogue (5-HEAT) with low MLT receptor affinity and opposing intrinsic activity at the two MLT receptor subtypes (MT_1 agonist and MT_2 antagonist). The goal of the present study was to prepare new MLT receptor ligands that have the same interesting efficacy profile of 5-HEAT, but with improved h-MT_1 and/or h-MT_2 receptor binding affinity. The binding results for h-MT_1 and h-MT_2 receptor subtypes as well as the induced G-protein activation of the synthesized compounds **3b**,**c**, **4a–c**, **5a–d**, **7**, **8**, and **12** are reported in Table 1.

In the course of our studies, various modifications of the MLT structure have been examined[35–38] for the purpose of determining which structural features are required for receptor affinity, intrinsic activity, and subtype selectivity. For example, we demonstrated that the introduction of electron-withdrawing substituents with an optimal lipophilicity (π values around 1) at the indole C2 position of MLT leads to a substantial increase in MT_1 and MT_2 receptor affinity.[39]

In our attempt to improve the receptor affinity of 5-HEAT (**4c**), we first synthesized 5-HEAT analogues substituted at the indole C2 position with bromine (compound **5c**) or phenyl (compound **12**). As shown in Table 1, C2 halogenation (**5c** vs. **4c**) or C2 substitution with a phenyl ring (**12** vs. **4c**) led to a substantial increase in binding affinity for both MT_1 and MT_2 receptors; **5c** also displays modest selectivity for the MT_1 subtype (pK_i MT_1: 8.84, pK_i MT_2: 7.70).

However, whereas the 2-bromo derivative **5c** retains approximately the same particular efficacy profile of 5-HEAT ($IA_r = 0.82$ for MT_1 and $IA_r = 0.29$ for MT_2), the intrinsic activity of the 2-phenyl analogue **12** varied greatly, and both MT_1- and MT_2-agonist properties were observed. Another modification concerned the introduction of a benzyl group at the indole N1 position of 5-HEAT. In fact, it has been previously reported that variations in size and shape of the substituent at the indole nitrogen atom of MLT analogues can modulate binding affinity and intrinsic activity toward the two MT_1/MT_2 receptor subtypes.[40] However, indole N1 benzylation of 5-HEAT was found to be detrimental to both MT_1 and MT_2 receptor affinity (**7** vs. **4c**).

There is evidence that structural modifications in the region corresponding to position 5 of MLT influence the binding to MT_1 and MT_2 receptors in different ways. For example, it seems that MT_1 selectivity is favored by replacement of the 5-methoxy group with a larger substituent.[31,41,42]

Both site-directed mutagenesis experiments[43–46] and work with chimeric receptors[47,48] have been employed to investigate the structure–function relationships for MT_1 and MT_2 receptors. Unfortunately, no clear information has been obtained about which amino acids are essential for ligand binding, but there is evidence that agonist and antagonist binding sites are not identical. The most relevant information is that for both MT_1 and MT_2 receptors, the substitution of a histidine residue in transmembrane α helix 5 (TM5; His 195 in MT_1 and His 208 in MT_2) led to a four- to eightfold decrease in binding affinity for MLT receptor agonists bearing a methoxy substituent (such as MLT and S-20098), whereas the potency of antagonists without the methoxy group (such as luzindole and 4P-PDOT) was slightly influenced.[43,46] Ser 110 and Ser 114 in TM3 of MT_1 seem to be involved in agonist binding only, as their mutation did not affect the affinity for the antagonist luzindole.[49]

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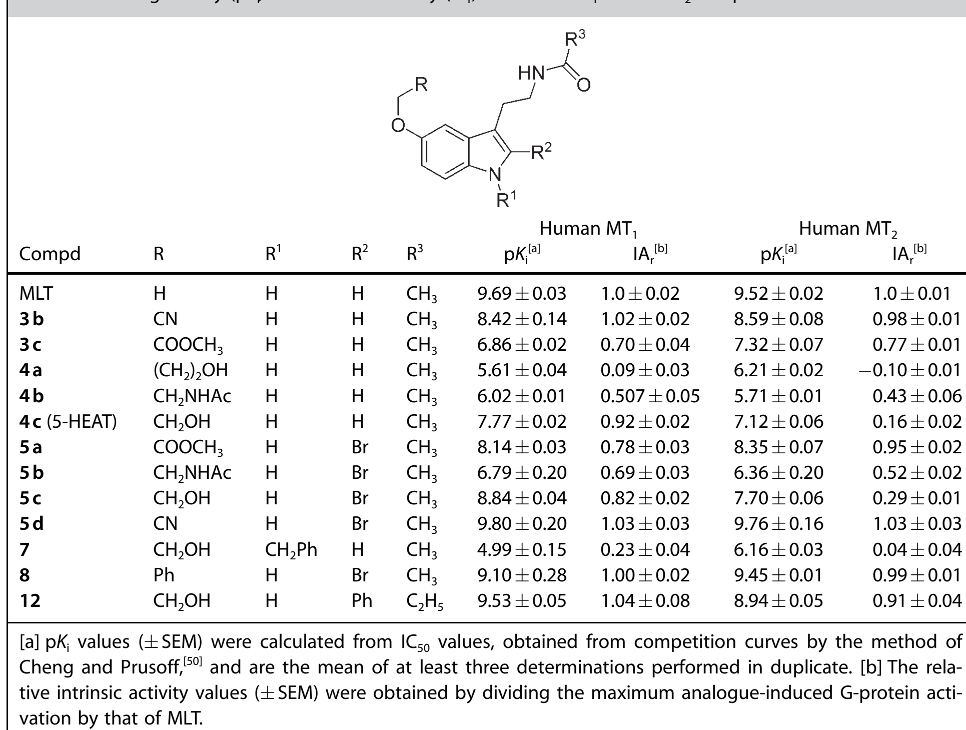
Table 1. Binding affinity (pK_i) and intrinsic activity (IA_r) toward h-MT_1 and h-MT_2 receptors.



Compd	R	R1	R2	R3	Human MT1 pK_i[a]	Human MT1 IA_r[b]	Human MT2 pK_i[a]	Human MT2 IA_r[b]
MLT	H	H	H	CH_3	9.69±0.03	1.0±0.02	9.52±0.02	1.0±0.01
3b	CN	H	H	CH_3	8.42±0.14	1.02±0.02	8.59±0.08	0.98±0.01
3c	$COOCH_3$	H	H	CH_3	6.86±0.02	0.70±0.04	7.32±0.07	0.77±0.01
4a	$(CH_2)_2OH$	H	H	CH_3	5.61±0.04	0.09±0.03	6.21±0.02	−0.10±0.01
4b	CH_2NHAc	H	H	CH_3	6.02±0.01	0.507±0.05	5.71±0.01	0.43±0.06
4c (5-HEAT)	CH_2OH	H	H	CH_3	7.77±0.02	0.92±0.02	7.12±0.06	0.16±0.02
5a	$COOCH_3$	H	Br	CH_3	8.14±0.03	0.78±0.03	8.35±0.07	0.95±0.02
5b	CH_2NHAc	H	Br	CH_3	6.79±0.20	0.69±0.03	6.36±0.20	0.52±0.02
5c	CH_2OH	H	Br	CH_3	8.84±0.04	0.82±0.02	7.70±0.06	0.29±0.01
5d	CN	H	Br	CH_3	9.80±0.20	1.03±0.03	9.76±0.16	1.03±0.03
7	CH_2OH	CH_2Ph	H	CH_3	4.99±0.15	0.23±0.04	6.16±0.03	0.04±0.04
8	Ph	H	Br	CH_3	9.10±0.28	1.00±0.02	9.45±0.01	0.99±0.01
12	CH_2OH	H	Ph	C_2H_5	9.53±0.05	1.04±0.08	8.94±0.05	0.91±0.04

[a] pK_i values (±SEM) were calculated from IC_{50} values, obtained from competition curves by the method of Cheng and Prusoff,[50] and are the mean of at least three determinations performed in duplicate. [b] The relative intrinsic activity values (±SEM) were obtained by dividing the maximum analogue-induced G-protein activation by that of MLT.

Taking into account these results, we hypothesized that the introduction of suitable substituents at the indole 5 position of MLT could contribute to differentiate the MT_1/MT_2 pharmacological profile of the resulting ligands by establishing additional interactions with each binding pocket. Therefore, a second screening campaign was performed with the *N*-acetyltryptamine or *N*-acetyl-2-bromotryptamine scaffold and various substitutions at the indole 5 position. As shown in Table 1, the replacement of the 2-hydroxyethyl group of 5-HEAT with methyl acetate (compound **3c**) did not alter MT_2 affinity and resulted in a modest decrease in MT_1 affinity; **3c** behaves as partial agonist at both receptor subtypes.

Homologation of the 2-hydroxyethyloxy chain to a 3-hydroxypropyloxy group (compound **4a**) resulted in a considerable loss in binding affinity at both MLT receptors, and the resulting compound **4a** behaved as antagonist. Replacement of the 2-hydroxyethyl group of 5-HEAT with a cyanomethyl moiety (compound **3b**) results in an approximate 10- to 15-fold increase in MLT receptor affinity, and full MT_1 and MT_2 agonist behavior was observed.

By hydrogenation of the cyano group of **3b** and contemporary *N*-acetylation, we obtained the 5-acetylaminoethoxy derivative **4b**, which binds MLT receptors with severalfold lower affinity than its parent compound; the intrinsic activity of **4b** shifted toward partial agonism. The data in Table 1 also indicate that there is a trend in which the 2-bromo derivatives **5a**–**d** and **8** have about 10- to 15-fold higher MT_1/MT_2 affinity relative to the corresponding C2-unsubstituted analogues.

The 2-bromo-5-benzyloxy derivative **8** and the 2-bromo-5-cyanomethoxy derivative **5d** bind to MT_1 and MT_2 receptors with similar or modestly increased affinity relative to MLT. Unfortunately these latter derivatives, which act as full MT_1/MT_2 agonists, did not preserve the interesting efficacy profile of our lead compound 5-HEAT.

Conclusions

The results of this study are consistent with our initial hypothesis that it could be possible to improve the binding affinity of 5-HEAT and they extend what had been presented so far on the structural requirements for affinity and intrinsic activity at the h-MT_1 and h-MT_2 receptors. High-affinity MLT receptor agonists were obtained by replacing the 5-methoxy substituent with a 5-benzyloxy (in **8**) or a 5-cyanomethoxy (in **5d**) moiety. The new 5-hydroxyethoxy compounds synthesized respond to structural modifications similarly to what was expected for the 5-methoxy analogues; thus substitution at C2 with bromine or phenyl groups enhances affinity. However, this type of substitution greatly influenced the intrinsic activity of the resulting compounds toward MT_2; whereas C2-phenyl substitution changed the pharmacological profile from antagonist/partial agonist to agonist ($IA_r=0.91$, **12** vs. **4c**), C2 halogenation (compound **5c**) induced only minimal MT_2 receptor-mediated G-protein activation ($IA_r=0.29$). By virtue of its high affinity and its particular intrinsic activity profile, **5c** is currently the best mixed MT_1 agonist/MT_2 antagonist and it could be a valuable tool for determining whether specific melatonin effects are mediated by MT_1 or MT_2, thus facilitating the elucidation of the role of the MLT complex system.

Metabolic stability of 5-HEAT and congeners was not evaluated, but the compounds are expected to be at the best as stable as MLT.

Experimental Section

Chemistry: Melting points were determined on a Büchi SMP-510 capillary melting point apparatus and are uncorrected. ^1H NMR spectra were recorded on a Bruker AC 200 spectrometer; chemical shifts (δ) are reported in ppm relative to the central peak of the solvent. Coupling constants (*J* values) are given in Hz. EIMS data (70 eV) were taken on a Fisons Trio 1000 instrument. Only molecular ions [M^+] and base peaks are given. IR spectra were obtained on a Nicolet Avatar 360 FTIR spectrometer; absorbance values are reported in $\tilde{\nu}$ (cm^{-1}). The elemental analyses for C, H, and N are within 0.4% of the calculated values. Column chromatography purifications were performed under "flash" conditions using Merck 230–400 mesh silica gel. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel 60 F_{254} plates. The two ra-

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dioligands 2-[^{125}I]iodomelatonin (specific activity 2000 Ci mmol^{-1}) and [^{35}S]GTPγS ([^{35}S]guanosine-5′-*O*-(γ-thiotriphosphate); specific activity 1000 Ci mmol^{-1}) were purchased from Amersham Pharmacia Biotech (Italy).

***N*-[2-(5-Hydroxy-1*H*-indol-3-yl)ethyl]acetamide (2)**: A solution of *N*-[2-(5-benzyloxy-1*H*-indol-3-yl)ethyl]-acetamide **1** (0.11 g, 0.36 mmol)[32] in dry MeOH (6 mL) was hydrogenated over Pd-C (10%, 0.026 g) at 400 kPa H_2 for 5 h at room temperature. The catalyst was filtered on celite, the filtrate was evaporated under reduced pressure to give the desired oily product **2**, which was used without further purification (88% yield). Analytical data are in agreement with those reported.[51] MS (EI): *m/z* 218 [M^+], 159 (100).

Methyl-3-[3-(2-acetylaminoethyl)-1*H*-indol-5-yloxy]propionate (3a): Sodium hydride (80% in mineral oil, 0.06 g, 2 mmol) and methyl acrylate (0.3 mL, 3.32 mmol) were added to a solution of **2** (0.36 g, 1.65 mmol) in dry DMF (6.5 mL) at −15 °C under nitrogen atmosphere. The reaction mixture was stirred at room temperature for 2 h, poured into ice-cooled water and extracted twice with CH_2Cl_2. The combined organic phases were washed with water and dried (Na_2SO_4). The organic solvent was evaporated to dryness, and the residue purified by flash chromatography (CH_2Cl_2/MeOH 95:5 as eluent) to give an amorphous solid (20% yield); MS (EI): *m/z* 304 [M^+], 232 (100); ^{1}H NMR ($CDCl_3$): $\delta = 1.93$ (s, 3H), 2.76 (t, 2H, $J = 6.7$), 2.82 (t, 2H, $J = 6.7$), 3.50 (q, 2H, $J = 6.7$), 3.63 (s, 3H), 4.32 (t, 2H, $J = 6.7$), 6.02 (br t, 1H), 6.84 (dd, 1H, $J = 2.4$ and 8.8), 6.88 (br s, 1H), 7.03 (d, 1H, $J = 2.4$), 7.13 (d, 1H, $J = 8.8$), 8.00 (br s, 1H); IR ($CHCl_3$): $\tilde{\nu} = 3603$, 3450, 1736, 1659 cm^{-1}; anal. ($C_{16}H_{20}N_2O_4$) C, H, N.

***N*-[2-(5-Cyanomethoxy-1*H*-indol-3-yl)ethyl]acetamide (3b)**: Sodium hydride (80% in mineral oil, 0.12 g, 4 mmol) and chloroacetonitrile (0.235 mL, 3.72 mmol), were added to a solution of **2** (0.6 g, 2.75 mmol) in dry DMF (10 mL) at −15 °C under nitrogen atmosphere. The reaction mixture was stirred at that temperature for 4 h, poured into ice-cooled water and extracted three times with EtOAc. The organic phases were combined, dried (Na_2SO_4), and evaporated to give a crude residue, which was purified by flash chromatography (CH_2Cl_2/MeOH 95:5 as eluent) and triturated with diethyl ether (53% yield). White solid, mp: 107–8 °C; MS (EI): *m/z* 257 [M^+], 198 (100); ^{1}H NMR ($CDCl_3$): $\delta = 1.96$ (s, 3H), 2.94 (t, 2H, $J = 6.6$), 3.60 (q, 2H, $J = 6.6$), 4.82 (s, 2H), 5.66 (br s, 1H), 6.95 (dd, 1H, $J = 2.5$ and 8.8), 7.09 (d, 1H, $J = 1.9$), 7.20 (d, 1H, $J = 2.5$), 7.34 (d, 1H, $J = 8.8$), 8.12 (br s, 1H); IR (Nujol): $\tilde{\nu} = 3331$, 3265, 1629 cm^{-1}; anal. ($C_{14}H_{15}N_3O_2$) C, H, N.

Methyl[3-(2-acetylaminoethyl)-1*H*-indol-5-yloxy]acetate (3c): The title compound was prepared according to published procedures.[30]

***N*-{2-[5-(3-Hydroxypropoxy)-1*H*-indol-3-yl]ethyl}acetamide (4a)**: A suspension of **3a** (0.185 g, 0.6 mmol) in dry THF (4 mL) was added dropwise to a stirred ice-cooled suspension of $LiAlH_4$ (0.043 g, 1.13 mmol) in dry THF (4 mL) under nitrogen. Upon completion of the addition, the mixture was stirred at 0 °C for 2 h. The unreacted $LiAlH_4$ was destroyed by careful addition of water at 0 °C, and the resulting mixture was filtered through a celite pad. The filtrate was concentrated in vacuo, and the residue was partitioned between EtOAc and water. The organic phases were combined and washed once with brine, dried (Na_2SO_4), and evaporated to yield the crude desired product, which was purified by flash chromatography (CH_2Cl_2/MeOH 9:1 as eluent); oil (45% yield); MS (EI): *m/z* 276 [M^+], 160 (100); ^{1}H NMR ([D$_6$]acetone): $\delta = 1.86$ (s, 3H), 1.94 (m, 2H), 2.80 (t, 2H, $J = 7.4$), 3.37–3.55 (m, 4H), 4.18 (t, 2H, $J = 6.8$), 6.72 (dd, 1H, $J = 2.0$ and 8.8), 6.97 (d, 1H, $J = 2.1$), 7.03 (br s, 1H), 7.18 (br s, 1H), 7.21 (d, 1H, $J = 8.8$), 7.78 (br s, 1H); anal. ($C_{15}H_{20}N_2O_3$) C, H, N.

***N*-{2-[5-(2-Acetylaminoethoxy)-1*H*-indol-3-yl]ethyl}acetamide (4b)**: A solution of **3b** (0.593 g, 2.3 mmol) in THF (15 mL) and acetic anhydride (3.56 mL, 37.8 mmol) was hydrogenated over Raney nickel at 400 kPa H_2 for 5 h at 50 °C. The catalyst was filtered on celite and washed with MeOH; the filtrate was concentrated in vacuo, and the residue was partitioned between ethyl acetate and 2 N NaOH. The organic layer was washed with brine, dried (Na_2SO_4), and evaporated under reduced pressure to give a crude residue, which was purified by flash chromatography (EtOAc/MeOH 9:1 as eluent); oil (49% yield); MS (EI): *m/z* 303 [M^+], 159 (100); ^{1}H NMR ($CDCl_3$): $\delta = 1.95$ (s, 3H), 2.03 (s, 3H), 2.93 (t, 2H, $J = 6.9$), 3.57 (q, 2H, $J = 6.9$), 3.67 (q, 2H, $J = 5.4$), 4.09 (t, 2H, $J = 5.4$), 5.74 (br s, 1H), 6.20 (br s, 1H), 6.85 (dd, 1H, $J = 2.4$ and 8.8), 7.03 (d, 1H, $J = 2.3$), 7.08 (d, 1H, $J = 2.4$), 7.28 (d, 1H, $J = 8.8$), 8.21 (br s, 1H); IR ($CHCl_3$): $\tilde{\nu} = 3479$, 3452, 1665 cm^{-1}; anal. ($C_{16}H_{21}N_3O_3 \cdot 0.04$ EtOAc) C, H, N.

***N*-{2-[5-(2-Hydroxyethoxy)-1*H*-indol-3-yl]ethyl}acetamide (4c, 5-HEAT)**: The title compound was prepared according to published procedures.[30]

Methyl-[1-benzyl-3-(2-acetylaminoethyl)-1*H*-indol-5-yl]oxyacetate (6): A solution of **3c** (0.29 g, 1 mmol) in dry DMF (3 mL) was added to a suspension of sodium hydride (60% in mineral oil, 0.052 g, 1.3 mmol) in dry DMF (3 mL) at 0 °C. After stirring for 30 min, benzyl bromide (0.13 mL, 1.1 mmol) was added to the reaction mixture, and stirring was continued for 20 h at room temperature. The resulting mixture was poured into ice-cooled water and extracted four times with EtOAc. The organic phases were combined, washed once with brine, dried (Na_2SO_4), and evaporated to give the crude desired product, which was purified by flash chromatography (cyclohexane/EtOAc 2:8 as eluent) and crystallization (75% yield). Beige solid, mp: 114–5 °C dec (EtOAc/hexanes); MS (EI): *m/z* 380 [M^+], 91 (100); ^{1}H NMR ($CDCl_3$): $\delta = 1.93$ (s, 3H), 2.92 (t, 2H, $J = 6.8$), 3.56 (q, 2H, $J = 6.8$), 3.82 (s, 3H), 4.69 (s, 2H), 5.25 (s, 2H), 5.53 (br s, 1H), 6.92 (dd, 1H, $J = 2.5$ and 8.8), 6.96 (br s, 1H), 7.05 (d, 1H, $J = 2.5$), 7.10 (m, 2H), 7.19 (d, 1H, $J = 8.8$), 7.30 (m, 3H); IR (Nujol): $\tilde{\nu} = 3292$, 1749, 1641 cm^{-1}; anal. ($C_{22}H_{24}N_2O_4$) C, H, N.

***N*-{2-[1-Benzyl-5-(2-hydroxyethoxy)-1*H*-indol-3-yl]ethyl}acetamide (7)**: The title compound was prepared according to the procedure described above for **4a** starting from **6**. The product was purified by flash chromatography (EtOAc as eluent) and crystallization (41% yield). White solid, mp: 140 °C (EtOAc/hexanes); MS (EI): *m/z* 352 [M^+], 91 (100); ^{1}H NMR ($CDCl_3$): $\delta = 1.94$ (s, 3H), 2.94 (t, 2H, $J = 6.7$), 3.57 (q, 2H, $J = 6.7$), 3.98 (m, 2H), 4.15 (m, 2H), 5.25 (s, 2H), 5.79 (br s, 1H), 6.87 (dd, 1H, $J = 2.4$ and 8.8), 6.95 (br s, 1H), 7.10 (m, 3H), 7.17 (d, 1H, $J = 8.8$), 7.28 (m, 3H); IR ($CDCl_3$): $\tilde{\nu} = 3450$, 1665 cm^{-1}; anal. ($C_{21}H_{24}N_2O_3$) C, H, N.

General procedure for the indole C2 bromination of 3-(2-acetylaminoethyl)indole derivatives: Phenyltrimethylammonium tribromide (1 mmol) was added to a stirred solution of the appropriate *N*-acetyltryptamine (**1**, **3b,c**, **4b,c**, 1 mmol) in THF (20 mL) under nitrogen. The reaction mixture was stirred for 30 min, then the solvent was evaporated under reduced pressure, and the residue partitioned between water and EtOAc. The organic phase was washed with brine, dried (Na_2SO_4), and evaporated to give a crude residue, which was purified by flash chromatography and crystallization.

Methyl[3-(2-acetylaminoethyl)-2-bromo-1*H*-indol-5-yloxy]acetate (5a): Starting from **3c**, flash chromatography, eluent: EtOAc. White

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solid (54% yield), mp: 152–3 °C dec (EtOAc-petroleum ether); MS (EI): *m/z* 368–370 [M^+], 309–311 (100); ^{1}H NMR ($CDCl_3$): δ = 1.94 (s, 3H), 2.90 (t, 2H, J = 6.4), 3.52 (q, 2H, J = 6.4), 3.83 (s, 3H), 4.69 (s, 2H), 5.56 (brs, 1H), 6.91 (dd, 1H, J = 2.4 and 8.8), 7.00 (d, 1H, J = 2.4), 7.22 (d, 1H, J = 8.8), 8.11 (brs, 1H); IR (Nujol): $\tilde{\nu}$ = 3340, 3135, 1755, 1618 cm^{-1}; anal. ($C_{15}H_{17}BrN_2O_4$) C, H, N.

***N*-{2-[5-(2-Acetylaminoethoxy)-2-bromo-1*H*-indol-3-yl]ethyl}acetamide (5b)**: Starting from **4b**, flash chromatography, eluent: Et_2O/MeOH (95:5). Amorphous solid (9% yield); MS (EI): *m/z* 86 (100); ^{1}H NMR ([D$_6$]acetone): δ = 1.87 (s, 3H), 1.91 (s, 3H), 2.85 (t, 2H, J = 7.4), 3.40 (q, 2H, J = 7.4), 3.56 (q, 2H, J = 5.8), 4.06 (t, 2H, J = 5.8), 6.76 (dd, 1H, J = 2.5 and 8.8), 7.18 (d, 1H, J = 2.5), 7.23 (d, 1H, J = 8.8), 7.28 (brs, 1H), 7.51 (brs, 1H); IR ($CHCl_3$): $\tilde{\nu}$ = 3457, 1665 cm^{-1}; anal. ($C_{16}H_{20}BrN_3O_3$) C, H, N.

***N*-{2-[2-Bromo-5-(2-hydroxyethoxy)-1*H*-indol-3-yl]ethyl}acetamide (5c)**: Starting from **4c**, flash chromatography, eluent: EtOAc. White solid (40% yield), mp: 151–152 °C (MeOH/EtOAc/petroleum ether); MS (EI): *m/z* 340–342 [M^+], 281–283 (100); ^{1}H NMR ([D$_6$]DMSO): δ = 1.83 (s, 3H), 2.76 (t, 2H, J = 7.1), 3.28 (m, 2H), 3.78 (m, 2H), 3.97 (m, 2H), 6.70 (dd, 1H, J = 2.4 and 8.8), 6.92 (s, 1H), 7.10 (d, 1H, J = 8.8), 7.32 (brs, 1H), 10.88 (s, 1H); IR (Nujol): $\tilde{\nu}$ = 3332, 3304, 1621 cm^{-1}; anal. ($C_{14}H_{17}BrN_2O_3$) C, H, N.

***N*-[2-(2-Bromo-5-cyanomethoxy-1*H*-indol-3-yl)ethyl]acetamide (5d)**: Starting from **3b**, flash chromatography, eluent: EtOAc. Beige solid (49% yield), mp: 122–3 °C (EtOAc/petroleum ether); MS (EI): *m/z* 335–337 [M^+], 276–278 (100); ^{1}H NMR ([D$_6$]acetone): δ = 1.86 (s, 3H), 2.86 (m, 2H), 3.41 (q, 2H, J = 6.9), 5.09 (s, 2H), 6.91 (dd, 1H, J = 2.5 and 8.7), 7.18 (brs, 1H), 7.30–7.36 (m, 2H), 10.67 (brs, 1H); IR (Nujol): $\tilde{\nu}$ = 3172, 3112, 2257, 1607 cm^{-1}; anal. ($C_{14}H_{14}BrN_3O_2$) C, H, N.

***N*-[2-(5-Benzyloxy-2-bromo-1*H*-indol-3-yl)ethyl]acetamide (8)**: Starting from **1**, flash chromatography, eluent: cyclohexane/EtOAc (3:7). Beige solid (62% yield), mp: 111–2 °C (EtOAc/petroleum ether); MS (EI): *m/z* 386–388 [M^+], 91 (100); ^{1}H NMR ($CDCl_3$): δ = 1.92 (s, 3H), 2.90 (t, 2H, J = 6.4), 3.51 (q, 2H, J = 6.4), 5.06 (s, 2H), 3.85 (s, 3H), 5.72 (brt, 1H), 6.90 (dd, 1H, J = 2.4 and 8.7), 7.06 (d, 1H, J = 2.14), 7.20 (d, 1H, J = 8.7), 7.31–7.50 (m, 5H), 8.86 (brs, 1H); IR (Nujol): $\tilde{\nu}$ = 3188, 1643 cm^{-1}; anal. ($C_{19}H_{19}BrN_2O_2$) C, H, N.

***N*-[2-(5-Hydroxy-2-phenyl-1*H*-indol-3-yl)ethyl]propanamide (10)**: A solution of BBr_3 (1 M in CH_2Cl_2, 5 mL) diluted with dry CH_2Cl_2 (5 mL) was added dropwise to a solution of **9**[34] (0.322 g, 1 mmol) in dry CH_2Cl_2 (13 mL) at 0 °C, and the resulting suspension was stirred at room temperature for 3 h. The reaction mixture was neutralized with a aqueous solution of 2 N Na_2CO_3 and extracted with EtOAc, trying to dissolve all the sticky solid. The organic phases were combined, dried (Na_2SO_4), and concentrated under reduced pressure. The crude residue was purified by flash chromatography (gradient cyclohexane/EtOAc ratio from 6:4 to 3:7) and crystallization (65% yield). White solid, mp: 201–2 °C (CH_2Cl_2/hexanes); MS (EI): *m/z* 308 [M^+], 249 (100); ^{1}H NMR ([D$_6$]DMSO): δ = 0.98 (t, 3H, J = 7.7), 2.05 (q, 2H, J = 7.7), 2.85 (m, 2H), 3.28 (m, 2H), 6.62 (dd, 1H, J = 2.4 and 8.8), 6.90 (d, 1H, J = 2.4), 7.14 (d, 1H, J = 8.8), 7.31–7.65 (m, 5H), 7.98 (brt, 1H), 8.69 (s, 1H), 10.87 (s, 1H); IR ([D$_6$]DMSO): $\tilde{\nu}$ = 3482, 3252, 1620 cm^{-1}; anal. ($C_{19}H_{20}N_2O_2$) C, H, N.

Methyl[(2-phenyl-3-propanoylaminoethyl)-1*H*-indol-5-yl]oxacetate (11): The title compound was obtained following the procedure above described for **3b** starting from **10** using methyl chloroacetate instead of chloroacetonitrile. The product was purified by flash chromatography (cyclohexane/EtOAc 3:7 as eluent) and crystallization (68% yield). Yellowish solid, mp: 185–195 °C dec (EtOAc/hexanes); MS (EI): *m/z* 380 [M^+], 294 (100); ^{1}H NMR ([D$_6$]DMSO): δ = 0.96 (t, 3H, J = 7.8), 2.03 (q, 2H, J = 7.8), 2.91 (m, 2H), 3.29 (m, 2H), 3.71 (s, 3H), 4.77 (s, 2H), 6.79 (dd, 1H, J = 2.4 and 8.8), 7.11 (d, 1H, J = 2.4), 7.26 (d, 1H, J = 8.8), 7.34–7.67 (m, 5H), 7.95 (brt, 1H), 11.08 (s, 1H); IR (Nujol): $\tilde{\nu}$ = 3327, 3279, 1755, 1648 cm^{-1}; anal. ($C_{22}H_{24}N_2O_4$) C, H, N.

***N*-[2-(5-Hydroxyethyloxy-2-phenyl-1*H*-indol-3-yl)ethyl]propanamide (12)**: The title compound was obtained following the reduction procedure above described for **4a** starting from **11**. The product was purified by flash chromatography (EtOAc as eluent) and crystallization (43% yield). White solid, mp: 174–5 °C (hot EtOAc/petroleum ether); MS (EI): *m/z* 352 [M^+], 266 (100); ^{1}H NMR ([D$_6$]DMSO): δ = 0.97 (t, 3H, J = 7.4), 2.04 (q, 2H, J = 7.4), 2.90 (m, 2H), 3.30 (m, 2H), 3.74 (m, 2H), 4.02 (m, 2H), 4.87 (t, 1H, J = 5.4), 6.77 (dd, 1H, J = 2.4 and 8.6), 7.11 (d, 1H, J = 1.7), 7.24 (d, 1H, J = 8.6), 7.32–7.66 (m, 5H), 7.98 (brt, 1H), 11.02 (s, 1H); IR (Nujol): $\tilde{\nu}$ = 3442, 3252, 1620 cm^{-1}; anal. ($C_{21}H_{24}N_2O_3$) C, H, N.

Receptor binding experiments: Binding affinities of compounds were determined by using 2-[^{125}I]iodomelatonin as the labeled ligand in competition experiments on cloned human MT_1 and MT_2 receptors expressed in NIH3T3 rat fibroblast cells. The characterization of NIH3T3 MT_1 and MT_2 cells has already been described in detail.[52,53] Membranes were incubated for 90 min at 37 °C in binding buffer (Tris HCl 50 mM, pH 7.4). The final membrane concentration was 5–10 µg protein per tube. The membrane protein level was determined in accordance with a previously reported method.[54] 2-[^{125}I]Iodomelatonin (100 pM) and various concentrations of the new compounds were incubated with the receptor preparation for 90 min at 37 °C. Nonspecific binding was assessed with 10 µM MLT; IC_{50} values were determined by nonlinear fitting strategies with the program PRISM (GraphPad Software Inc., San Diego, USA). The pK_i values were calculated from the IC_{50} values in accordance with the Cheng–Prusoff equation.[50] The pK_i values are the mean of at least three independent determinations performed in duplicate.

The intrinsic activity of the compounds was evaluated through [^{35}S]guanosine-5′-*O*-(γ-thiotriphosphate) ([^{35}S]GTPγS) binding in NIH3T3 cells stably transfected with human MT_1 or MT_2 receptors, as previously described.[52,53,55] Nonspecific binding was defined using GTPγS (10 µM). The data of [^{35}S]GTPγS binding experiments are given as the percentage of basal binding (basal stimulation is the amount of [^{35}S]GTPγS specifically bound in the absence of the compounds); basal binding was fixed at 100%. Under optimized conditions,[52,53] in cell lines expressing human MT_1 or MT_2 receptors, MLT (100 nM) produced a concentration-dependent stimulation of basal [^{35}S]GTPγS binding, with a maximal stimulation above basal levels of 370% and 250% in MT_1 and MT_2, respectively.

Full agonists increased the basal [^{35}S]GTPγS binding in a concentration-dependent manner, like the natural ligand MLT, whereas partial agonists increased it to a much lesser extent; antagonists are without effect. To optimize [^{35}S]GTPγS binding and measure without uncertainty the percent variation in [^{35}S]GTPγS binding evoked by the test compounds relative to MLT, compounds were added at three different concentrations (one concentration was equivalent to 100 nM MLT, another was 10-fold less, and a third, 10-fold greater). The equivalent concentration was estimated on the basis of the ratio of the affinity of the test compound over that of MLT. It was assumed that at the equivalent concentration the test compound occupies the same number of receptors as MLT at 100 nM. All measurements were performed in triplicate. The relative intrinsic activity values (IA_r) were determined by dividing the maximum

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net stimulation of [^{35}S]GTPγS binding of a test compound by that of MLT, as measured in the same experiment.

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