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Synthesis and biological activity of new melatonin dimeric derivatives

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Abstract—A new series of melatonin (MLT) dimers were obtained by linking together two melatonin units with a linear alkyl chain through the MLT acetamido group or through a C-2 carboxyalkyl function. The binding properties of these ligands were evaluated in *in vivo* experiments on cloned human MT_1 and MT_2 receptors expressed in NIH3T3 rat fibroblast cells. The class of 2-carboxyalkyl dimers was the most interesting one with compounds having good MT_1/MT_2 nanomolar affinity. The data obtained suggest that the spacer length is crucial for optimal interaction at both receptor subtypes as well as to determine functional activity of the resulting dimers.

1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine, MLT, Chart 1) is a tryptophan-derived hormone mainly synthesized and secreted by the pineal gland following a circadian rhythm.[1–4] Extensive studies have demonstrated the role of MLT in many physiological processes, such as the regulation of circadian[5] and seasonal rhythms,[6] immune functions,[7] and retinal physiology.[8] Different potential therapeutic applications have been proposed for MLT and its derivatives.[9] They comprise the regulation of disrupted circadian rhythms and of sleep disorders,[10,11] the treatment of depression,[12] headache,[13] and neurodegenerative pathologies, such as Alzheimer's disease,[14] as well as the application as anticancer agents.[15] However, in humans, appropriate clinical trials have confirmed the efficacy of melatonergic ligands only in circadian-rhythm disfunctions[11] and affective disorders.[12] Besides the free radical scavenging and antioxidant properties[16,17] recognized for this endogenous hormone, MLT exerts many of its effects activating two high-affinity G-protein coupled receptors (MT_1 and MT_2),[18–20] localized in the central nervous system and in peripheral tissues.

A growing number of observations suggest that most G protein coupled receptors (GPCRs) form homodimers and heterodimers with other member of the same receptor superfamily.[21–23] Recently, constitutive homo and heterodimerization among MT_1 and MT_2 receptors were observed in HEK 293 cells, expressing physiological levels of these receptors, using co-immunoprecipitation and bioluminescence resonance energy transfer (BRET) experiments.[24,25]

In particular, it has been reported that MT_1 homodimers and MT_1/MT_2 heterodimers constitute a significant proportion of all dimers, whereas the MT_2 homodimer formation is 3- to 4-fold lower.[24] GPCR dimers are potentially composed of two closely related binding sites. Whether both sites are indeed functional and whether ligand binding to both sites is necessary for receptor activation are critical questions to understand the activation mechanism of GPCRs. In this context, bivalent ligands, molecules containing two pharmacophoric groups linked through an appropriate spacer, could represent a valuable pharmacological tool to address these and other key questions. For example, they could be useful to understand if neighboring receptor sites are allosterically coupled and to calculate, by docking procedures, intersite distances in order to identify possible dimerization interfaces.[26,27]

Keywords: Melatonin; MLT receptors; Biligands; Melatonin dimers.
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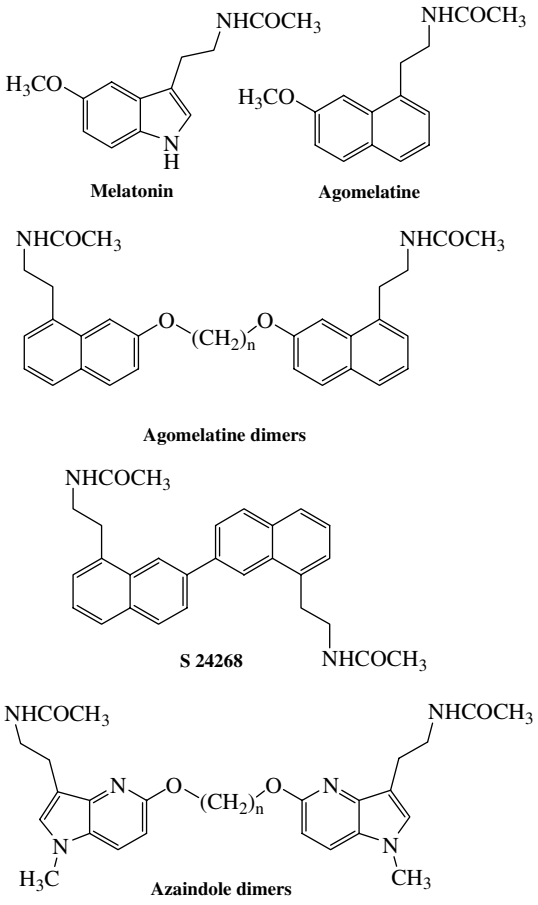


Chart 1. Melatonergic dimers.

Since the pioneering work of Portoghese and his collaborators in the field of opioid research,[26] the 'biligand approach' has been applied to develop ligands able to target many different GPCRs such as muscarinic,[28] norepinephrine,[29] dopamine,[30] and serotonin receptors.[27,31] To date, only few reports on dimeric molecules designed to target MLT-receptors appeared in the literature. The first dimeric melatonergic ligands described were developed from agomelatine (Chart 1), a potent bioisostere of MLT, linking two agomelatine units through their methoxy substituents by polymethylene chains[32] or directly via the C-6 aromatic carbon atoms as in **S-24268** (Chart 1).[33] Within these series of compounds, potent and MT_1 selective ligands were obtained. Azaindole dimers with bisalkoxyalkyl spacers were also reported (Chart 1), but they showed lower affinity and weaker selectivity compared to the agomelatine dimers.[34] On the basis of these findings, we looked for alternative melatonergic biligands. In particular, we synthesized and tested on MT_1 and MT_2 receptors dimeric molecules (**5a–g** Scheme 1; **8a** and **8b** Scheme 2) obtained by coupling two melatonin units through anchor points alternative to the methoxy group, such as the acetamido group or the C-2 indole position. Polymethylene chains of variable length were used as spacers. The monovalent ligands **6** and **7** (Scheme 1) were also synthesized and their affinity and intrinsic activity on MLT receptors established for a comparison with their bivalent counterpart.

2. Results and discussion

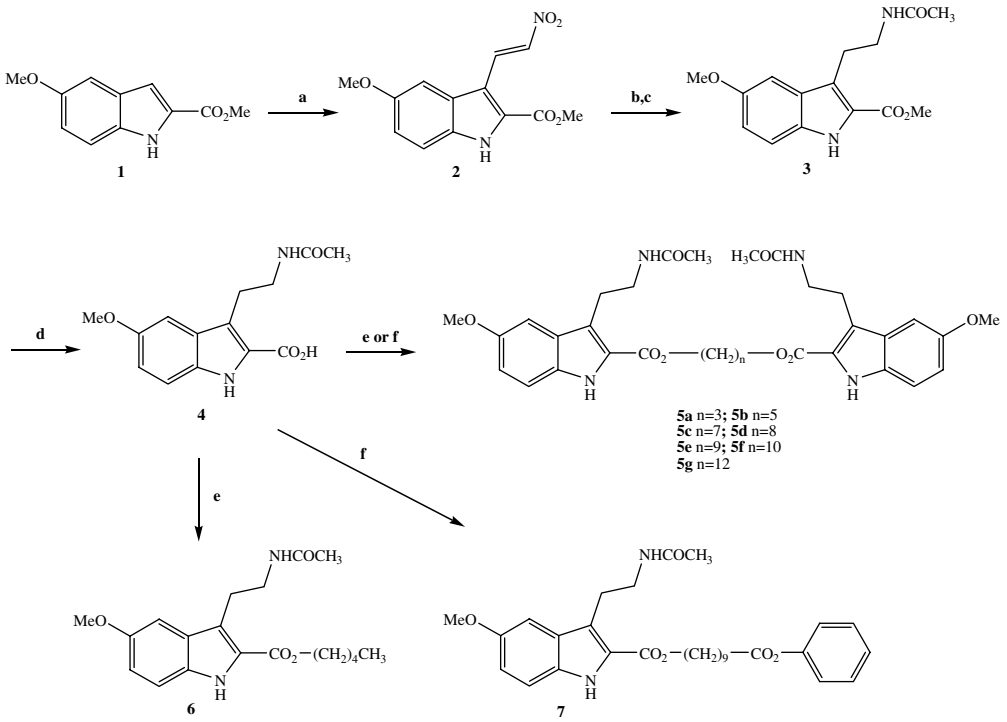
2.1. Chemistry

The synthesis of dimers **5a–g** is summarized in Scheme 1. According to a reported procedure, commercially available 5-methoxy-2-carboxymethylindole was reacted with 1-(dimethylamino)-2-nitroethylene in trifluoroacetic acid to afford 52% of nitroethene **2**.[35] This compound was converted to the acetamido derivative **3** by treatment with $NaBH_4$ followed by hydrogenation over Ni-Raney (50 psi, 60 °C, THF) and concomitant N-acylation with acetic anhydride. Attempts to obtain **3** in a single step reaction by hydrogenation of **2** over Pd–C 10% in the presence of acetic anhydride resulted in a dirty reaction, a troublesome purification procedure, and a lower yield. The 2-carboxylic acid derivative **4**, obtained by alkaline ester hydrolysis of **3**, was coupled with the appropriate hydroxy derivative, in the presence of dicyclohexylcarbodiimide (DCC) and a catalytic amount of 4-dimethylaminopyridine (DMAP) to yield compounds **5b–g** and **6** (Scheme 1). Compounds **5a** and **7** were obtained by treatment of a solution of **4** in DMF with *N*,*N*-carbonyldiimidazole (CDI), a catalytic amount of DMAP, and 1,3-propanediol or 9-hydroxynonylbenzoate,[36,37] respectively (Scheme 1). Finally, the two dimers **8a** and **8b** were synthesized, by reaction of commercially available 5-methoxytryptamine with pimeloylchloride and sebacoylchloride, respectively (Scheme 2).

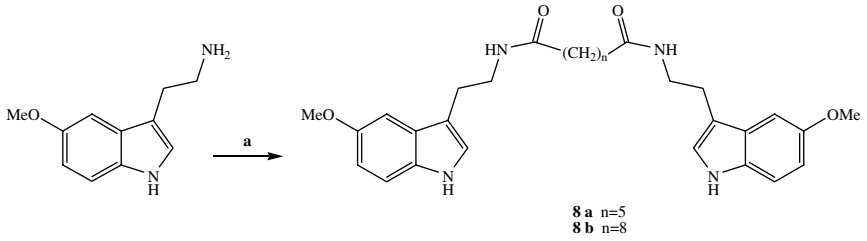
2.2. Pharmacology

The new compounds **5a–g**, **6**, **7**, and **8a** and **8b** 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. The data obtained from *in vivo* pharmacological assays, performed on the new derivatives, are summarized in Table 1.

Comparison of the relative MT_1 and MT_2 binding affinity (Table 1) shows that the dimers obtained by linking the C-2 indole position (**5a–g**) have higher affinity than those connected through the acetamido substituent (**8a** and **b**). These findings are in agreement with previously described SAR reporting that the increase in the size of the alkyl substituent attached to the amide carbonyl group, larger than a propyl, leads to a decreased binding affinity, whereas the introduction of a lipophilic substituent at C-2 gives high-affinity compounds.[38] On this base, we thought that it would not be worthwhile to further explore the acetamidic series of dimers. Among the class of dimeric ligands **5a–g**, polymethylene chains of variable length were employed to find the optimal separation of the two headgroups. Initially, in order to get some hints, we synthesized compounds **5a**, **5b**, and **5d** which, respectively, possess three, five and eight methyl-



Scheme 1. Reagents and conditions: (a) 1-(dimethylamino)-2-nitroethylene, CF_3CO_2H, 24 h, 0 °C–rt; (b) $NaBH_4$, MeOH, 1 h, rt; (c) H_2, Ni-Raney, THF, Ac_2O, 50 psi, 24 h, 60 °C; (d) 3 M KOH, THF/EtOH, 24 h, rt; (e) CH_3–$(CH_2)_4$–OH or HO–$(CH_2)_n$–OH, CH_2Cl_2, DCC, DMAP, 48 h, 0 °C–rt; (f) HO–$(CH_2)_3$–OH or $C_6H_5CO_2$–$(CH_2)_9$–OH, DMF, CDI, DMAP.



Scheme 2. Reagents and conditions: (a) ClCO–$(CH_2)_n$–COCl, Et_3N, dry THF, 1 h, rt.

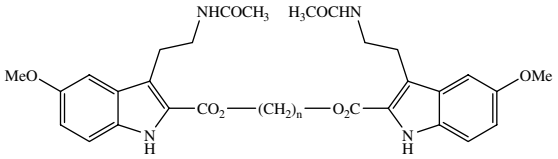
ene units. Compound **5d** displayed the highest affinity on both MLT receptors therefore, by assuming that longer spacers would have been suitable to bridge two neighboring receptors, we decided to address our efforts toward the optimization of the eight methylene spacer. For this purpose we prepared the 'shorter' dimer **5c** and the longer dimers **5e–g**. The resulting affinity values for these compounds clearly indicated the eight methylene spacer as the optimal one for interaction at both MLT receptor subtypes.

In detail, the compounds with spacer of three (**5a**) or eight (**5d**) methylene units showed higher affinity and similar agonistic properties on MT_1 receptor, while, on the MT_2 subtype, **5d** and **5e**, with eight and nine methylene units, respectively, displayed the best affinity but opposite intrinsic activity. These data seem to indicate that spacers length and their points of attachment within the melatonin scaffold are crucial for affinity and intrinsic activity on both receptor subtypes.

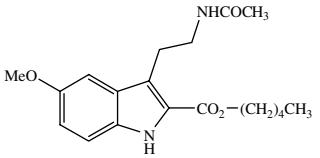
All tested compounds displayed lower affinity compared to melatonin and only few of them showed a moderate selectivity for the MT_2 receptor (**5e** and **6**). Nevertheless, we cannot ignore the progressive increase in hydrophobicity due to the homologation of the alkyl spacer employed. For this reason, we cannot just compare the binding affinity of the dimeric molecules with that of melatonin. Thus, in order to evaluate the influence of the spacer itself to the binding, the monovalent ligands **6** and **7** were synthesized and tested. Compound **6** was chosen because its C-2 pentylester substituent retains a lipophilicity representative, on the average, of that of the spacers used. Moreover, compound **7** was designed to clarify the contribution to the binding and intrinsic activity of the methoxy and ethylacetamido substituents on the second headgroup of compound **5e**.

As shown in Table 1, both compounds **6** and **7** display among the lowest affinity for the MT_1 receptor, compared with that of the C-2 spaced dimers (**5a–g**), suggesting a negative contribution of the spacers to the

Table 1. Binding affinity[a] and intrinsic activity[b] (IA_r) of new compounds for the human MT_1 and MT_2 receptors stably expressed in NIH3T3 cells



5a-g



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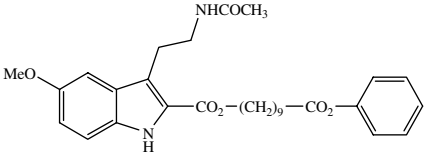

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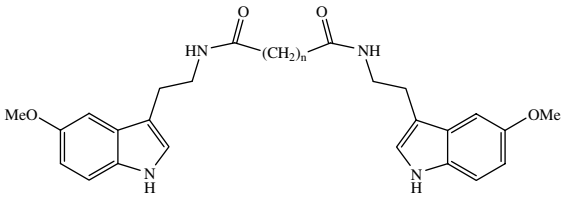

8 a, b

Compound	*n*	Human MT_1		Human MT_2	
		K_i[c] (nM)	$IA_r \pm SEM$	K_i[c] (nM)	$IA_r \pm SEM$
MLT		0.20	1.00 ± 0.01	0.30	1.00 ± 0.01
5a	3	9.50	0.77 ± 0.01	14.30	0.80 ± 0.02
5b	5	20.10	1.00 ± 0.01	62.40	0.93 ± 0.04
5c	7	214.50	0.35 ± 0.01	65.02	0.08 ± 0.07
5d	8	7.91	1.00 ± 0.02	7.92	0.90 ± 0.03
5e	9	59.80	0.33 ±0.02	7.75	−0.17 ± 0.02
5f	10	24.17	0.79 ± 0.04	15.43	0.65 ± 0.17
5g	12	25.45	0.72 ± 0.03	44.01	0.64 ± 0.07
6		70.10	0.06 ± 0.05	4.80	0.23 ± 0.03
7		83.00	0.75 ± 0.03	20.00	0.38 ± 0.05
8a	5	6470.00	0.35 ± 0.03	7290.00	0.26 ± 0.02
8b	8	387.00	0.81 ± 0.01	573.00	0.79 ± 0.02

[a] K_i values were calculated from IC_{50} values, obtained from competition curves by the method of Cheng and Prusoff,[40] and are the mean of at least three independent determinations performed in duplicate.
[b] The relative intrinsic activity values were obtained by dividing the maximal analogue-induced G-protein activation by that of MLT.
[c] SEM of K_i values were lower than ±0.8.

binding. The presence of the second pharmacophore in compound **5d** clearly counteracts the binding decrease observed in **6**. On the other side, the K_i values of the dimers with greatest affinity for the MT_2 receptor, **5d** and **5e**, are close to that of monomers **6** indicating that the presence of the second pharmacophore does not greatly influence the MT_2-binding. However, if we just compare the behavior of the monomer **7** with that of its closest dimeric derivative **5e**, we observe an increase in binding affinity of about three fold. In brief, the binding data relative to **6** and **7** as well as the differences in the K_i values among the series of dimer, lead us to suggest that the higher affinity of **5d** and **5e** can be the consequence of the simultaneous interaction of both headgroups with two distinct binding sites. Despite this, we are unable to exactly locate the second binding site within hypothetical dimeric forms of the MLT receptors. Indeed, this second site could be placed in a region close to the main ligand binding pocket, in one of the extracellular loops within the same receptor, or inside the ligand binding pocket of the neighboring receptor. Interactions with adjacent sites are most likely to occur with shorter linked dimers such as compound **5a**, while, interaction of the second headgroup with extracellular loops or with a fused receptor can only be possible with the longer linkers used in our study as in **5d** and **5e**. The latter possibility has been found in studies of bivalent pharmacophores on other GPCR systems.[26,39]

Finally, we underline that the highest affinity compounds here described (**5d** and **e**) are characterized by spacer lengths similar in size to that of the most potent MT_1/MT_2 agomelatine biligand previously reported.[32]

3. Conclusions

A series of bivalent ligands consisting of two melatonin residues linked through spacers of variable length were synthesized and evaluated by radioligand binding assay and GTPγS test. The dimeric compound **5d** was one of the most interesting substances with good nanomolar affinity for both MLT receptors and a full agonist behavior. Elongation of the spacer by a methylene unit (**5e**) confers a modest MT_2 selectivity and a different intrinsic activity profile; compound **5e** behaved as MT_2 partial inverse agonist (K_i = 7.75 nM).

The biological properties of compounds **5d** and **5e** could reflect specific interaction of both pharmacophores with binding sites within homodimeric form of the MT_1 or MT_2 receptors (these studies were performed on cells

expressing supraphysiological levels of only one receptor subtype thus, the probability that heterodimers are formed is negligible). This dual binding mode hypothesis is supported by the affinity data of the two monovalent ligands **6** and **7**, structurally related to the dimers **5d** and **5e**.

The development and the study of diverse dimeric ligands, obtained by varying the linker and the headgroups, may lead to a better comprehension of the biligands behavior and of the MLT receptors oligomerization phenomena.

4. Experimental

4.1. Chemistry

4.1.1. General. Proton NMR spectra were recorded on a Bruker Avance 200 spectrometer (200 MHz). Chemical shifts were reported in parts per million (ppm) relative to the internal standard TMS (tetramethylsilane). Coupling constants (*J* values) are given in hertz (Hz). Melting points were determined on a Buchi SMP-510 capillary melting point apparatus and are uncorrected. EI-MS spectra (70 eV) were taken on a Fisons Trio 1000 spectrometer. Only molecular ions (M^+) and base peaks are given. ESI-MS spectra were taken on a Waters Micromass ZQ spectrometer. Infrared spectra were obtained on a Nicolet Avatar 360 FT-IR spectrometer, transmittance is reported in cm^{-1}. Elemental analyses of new compounds (C, H, and N) were measured on a Carlo Erba analyzer and were within ±0.4% of theoretical 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 F254 plates. All chemicals were purchased from commercial suppliers and used directly without any further purification. The two radioligands 2-[^{125}I]iodomelatonin (specific activity, 2000 Ci/mmol) and [^{35}S]GTPγS ([^{35}S]-guanosine-5′-*O*-(3-thiotriphosphate); specific activity, 1000 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Italy).

4.1.2. 5-Methoxy-3-(2-nitrovinyl)-1*H*-indole-2-carboxylic acid methyl ester (2). Trifluoroacetic acid (2 mL) was added dropwise to 1-(dimethylamino)-2-nitroethylene (0.32 g, 2.76 mmol) at 0 °C. Subsequently, 5-methoxy-2-carboxymethylindole (0.5 g, 2.44 mmol) was added portionwise to the stirred mixture, the temperature was allowed to rise to room temperature and the stirring continued for 24 h. The reaction mixture was poured into a saturated solution of $NaHCO_3$ (50 mL)/crushed ice and the aqueous phase extracted 3× with $CHCl_3$. The organic phases were combined, sequentially washed with 2 N HCl, H_2O, brine, and dried (Na_2SO_4). The solvent was evaporated under reduced pressure to give 0.55 g of crude product which was purified by crystallization from EtOAc/cyclohexane (0.25 g). Purification of the mother liquors deriving from the crystallization by flash-chromatography (eluent: EtOAc/cyclohexane 1:1) yielded further 0.1 g of product (52% overall yield). Mp 233–235 °C; MS (EI): *m*/*z* 276 (M^+), 230 (100); IR (Nujol) cm^{-1}: 3307, 1670, 1629; ^{1}H NMR ($CDCl_3$) δ: 9.38 (br s, 1H), 9.01 (d, 1H, *J* = 13.9), 7.91 (d, 1H, *J* = 13.9), 7.42 (d, 1H, *J* = 8.8), 7.14 (m, 2H), 4.07 (s, 3H), 3.93 (s, 3H).

4.1.3. 3-(2-Acetamidoethyl)-5-methoxy-1*H*-indole-2-carboxylic acid methyl ester (3). Sodium borohydride (0.72 g, 19 mmol) was added portionwise to a stirred suspension of **2** (0.35 g, 1.3 mmol) in MeOH (20 mL) at room temperature. When gas evolution ceased (1 h), the reaction was quenched by addition of glacial acetic acid to adjust the pH value to 6. The reaction mixture was concentrated under *vacuum* and the residue partitioned between EtOAc and H_2O. The organic phase was sequentially washed with saturated solution of Na_2CO_3, H_2O, and brine, dried (Na_2SO_4) and evaporated to obtain the crude 5-methoxy-3-(2-nitroethyl)-1*H*-indole-2-carboxylic acid methyl ester which was employed for the following reaction without any further purification (0.344 g, 98% yield). MS (EI): *m*/*z* 278 (M^+), 199 (100).

A solution of crude 5-methoxy-3-(2-nitroethyl)-1*H*-indole-2-carboxylic acid methyl ester (0.34 g, 1.2 mmol) in THF (10 mL) and acetic anhydride (2.3 mL) was hydrogenated over Ni-Raney at 50 psi of H_2 for 24 h at 60 °C. The catalyst was filtered on celite and the filtrate was concentrated in vacuo. The residue was partitioned between CH_2Cl_2 and H_2O, and the organic phase was sequentially washed with 2 N NaOH, H_2O, and brine. The organic solution was dried (Na_2SO_4), and evaporated under reduced pressure and the crude product purified by flash-chromatography (eluent: EtOAc) to obtain 0.174 g of solid (49% yield). Mp 160–161 °C; MS (EI): *m*/*z* 290 (M^+), 186 (100); IR (Nujol) cm^{-1}: 3275, 1707, 1624; ^{1}H NMR ($CDCl_3$) δ: 8.61 (br s, 1H), 7.21 (d, 1H, *J* = 8.9), 6.95 (m, 2H), 5.89 (br s, 1H), 3.89 (s, 3H), 3.79 (s, 3H), 3.49 (m, 2H), 3.23 (t, 2H), 1.83 (s, 3H).

4.1.4. 3-(2-Acetamidoethyl)-5-methoxy-1*H*-indole-2-carboxylic acid (4). A solution of aqueous 3 M KOH (5.75 mL, 17.25 mmol) was added to a stirring solution of **3** (2 g, 6.9 mmol) in THF (19 mL) and EtOH (19 mL). The reaction mixture was stirred at room temperature for 24 h and then concentrated under reduced pressure to give a residue which was taken up with H_2O, cooled to 0 °C, and acidified with 2 N HCl. The precipitate was filtered, washed with H_2O, and dried under *vacuum* to obtain 1.8 g of desired product (94% yield). Mp 240–242 °C; MS (EI): *m*/*z* 276 (M^+), 160 (100); IR (Nujol) cm^{-1}: 3343, 3300, 1676, 1646; ^{1}H NMR (DMSO-d_6) δ: 11.29 (br s, 1H), 7.92 (br t, 1H), 7.27 (d, 1H, *J* = 9.2), 7.10 (d, 1H, *J* = 2.4), 6.88 (dd, 1H, *J* = 9.2 and 2.4), 3.76 (s, 3H), 3.25 (m, 2H), 3.13 (m, 2H), 1.74 (s, 3H).

4.1.5. 3-(3-(2-Acetamidoethyl)-5-methoxy-1*H*-indole-2-carbonyloxy)propyl 3-(2-acetamidoethyl)-5-methoxy-1*H*-indole-2-carboxylate (5a). *N*,*N*-carbonyldiimidazole (0.035 g, 0.22 mmol) was added to a solution of **4** (0.05 g, 0.18 mmol) in dry DMF (0.5 mL) and the resulting mixture was stirred at room temperature under

nitrogen atmosphere. After stirring for 3 h, 1,3-propandiol (0,007 mL, 0.09 mmol) and a catalytic amount of DMAP were added to the mixture which was stirred and heated at 40 °C for 2 h, and then left at room temperature for 3 days. Et_2O was added to the reaction mixture, the precipitate was crushed with EtOAc and EtOH, filtered, washed first with EtOH then with acetone, and finally dried under *vacuum* to yield 0.03 g of solid (28% yield). Mp 212–214 °C; MS (EI): *m/z* 592 (M^+), 159 (100); IR (Nujol) cm^{-1}: 3386, 3345, 1686, 1651; ^1H NMR (DMSO-d_6) δ: 11.46 (br s, 2H), 7.94 (br t, 2H), 7.30 (d, 2H, $J = 9.1$), 7.11 (d, 2H, $J = 1.8$), 6.91 (dd, 2H, $J = 9.1$ and 1.8), 4.46 (m, 4H), 3.77 (s, 6H), 3.32 (m, 4H), 3.19 (m, 4H), 2.27 (m, 2H), 1.76 (s, 6H). Anal. ($C_{31}H_{36}N_4O_8$) C, H, N.

4.1.6. General procedure for the synthesis of derivatives 5b–g and 6. Dicyclohexylcarbodiimide (1 mmol), the appropriate alcohol (0.3 mmol), and a catalytic amount of DMAP were added to a suspension of **4** (0.65 mmol) in dry CH_2Cl_2 (9 mL) at 0 °C and the resulting mixture was stirred for 2 days at room temperature. The solvent was removed under reduced pressure, the residue was crushed with hot EtOAc and filtered. The product was purified by flash-chromatography and crystallization.

4.1.7. 5-(3-(2-Acetamidoethyl)-5-methoxy-1*H*-indole-2-carbonyloxy)pentyl 3-(2-acetamidoethyl)-5-methoxy-1*H*-indole-2-carboxylate (5b). Flash-chromatography eluent: EtOAc/MeOH/conc. NH_4OH 95:5:2 dried over Na_2SO_4. The solid obtained was further purified by crystallization from EtOAc/Et_2O (25% yield). Mp 218–219 °C; MS [(+)-ESI]: *m/z* 621 $[M+H]^+$; IR (Nujol) cm^{-1}: 3344, 3269, 1676, 1629; ^1H NMR ($CDCl_3$) δ: 9.05 (br s, 2H), 7.26 (d, 2H, $J = 9.4$), 6.93 (m, 4H), 6.03 (br t, 2H), 4.43 (t, 4H), 3.58 (s, 6H), 3.55 (m, 4H), 3.26 (t, 4H), 1.90 (m, 4H), 1.89 (s, 6H), 1.68 (m, 2H). Anal. ($C_{33}H_{40}N_4O_8$) C, H, N.

4.1.8. 7-(3-(2-Acetamidoethyl)-5-methoxy-1*H*-indole-2-carbonyloxy)heptyl 3-(2-acetamidoethyl)-5-methoxy-1*H*-indole-2-carboxylate (5c). Flash-chromatography eluent: EtOAc and then EtOAc/MeOH 97:3. The product was further purified by crystallization from CH_2Cl_2/cyclohexane (14% yield). Mp 185–186 °C; MS [(+)-ESI]: *m/z* 649 $[M+H]^+$; IR (Nujol) cm^{-1}: 3347, 1686, 1657; ^1H NMR ($CDCl_3$) δ: 9.04 (br s, 2H), 7.29 (m hidden by the solvent peak, 2H), 6.99 (m, 4H), 6.03 (br t, 2H), 4.38 (t, 4H), 3.86 (s, 6H), 3.60 (m, 4H), 3.29 (t, 4H), 1.90 (m, 4H), 1.90 (s, 6H), 1.82 (m, 4H), 1.51 (m, 6H). Anal. ($C_{35}H_{44}N_4O_8$) C, H, N.

4.1.9. 8-(3-(2-Acetamidoethyl)-5-methoxy-1*H*-indole-2-carbonyloxy)octyl 3-(2-acetamidoethyl)-5-methoxy-1*H*-indole-2-carboxylate (5d). Flash-chromatography eluent: EtOAc. The product was further purified by crystallization from MeOH/Et_2O (16% yield). Mp 224–226 °C; MS [(+)-ESI]: *m/z* 663 $[M+H]^+$; IR (Nujol) cm^{-1}: 3372, 3256, 1685, 1653; ^1H NMR (DMSO-d_6) δ: 11.40 (br s, 2H), 7.81 (br t, 2H), 7.29 (d, 2H, $J = 8.9$), 7.10 (d, 2H, $J = 2.1$), 6.89 (dd, 2H, $J = 8.9$ and 2.1), 4.26 (t, 4H), 3.76 (s, 6H), 3.22 (m, 4H), 3.13 (m, 4H), 1.73 (s, 6H), 1.72 (m, 4H), 1.37 (m, 8H). Anal. ($C_{36}H_{46}N_4O_8$) C, H, N.

4.1.10. 9-(3-(2-Acetamidoethyl)-5-methoxy-1*H*-indole-2-carbonyloxy)nonyl 3-(2-acetamidoethyl)-5-methoxy-1*H*-indole-2-carboxylate (5e). Flash-chromatography eluent: EtOAc and then EtOAc/MeOH 97:3. The product was further purified by crystallization from MeOH (15% yield). Mp 115–116 °C; MS [(+)-ESI]: *m/z* 677 $[M+H]^+$; IR (Nujol) cm^{-1}: 3379, 1685, 1648; ^1H NMR (DMSO-d_6) δ: 11.40 (br s, 2H), 7.92 (br t, 2H), 7.29 (d, 2H, $J = 8.9$), 7.10 (d, 2H, J=2.2), 6.90 (dd, 2H, $J = 8.9$ and 2.2), 4.26 (t, 4H), 3.76 (s, 6H), 3.23 (m, 4H), 3.12 (m, 4H), 1.71 (s, 6H), 1.70 (m, 4H), 1.35 (m, 10H). Anal. ($C_{37}H_{48}N_4O_8$) C, H, N.

4.1.11. 10-(3-(2-Acetamidoethyl)-5-methoxy-1*H*-indole-2-carbonyloxy)decyl 3-(2-acetamidoethyl)-5-methoxy-1*H*-indole-2-carboxylate (5f). Flash-chromatography eluent: EtOAc and then EtOAc/MeOH 97:3. The product was further purified by crystallization from MeOH (13% yield). Mp 179–180 °C; MS [(+)-ESI]: *m/z* 691 $[M+H]^+$; IR (Nujol) cm^{-1}: 3385, 1683, 1653; ^1H NMR (DMSO-d_6) δ: 11.40 (br s, 2H), 7.92 (br t, 2H), 7.30 (d, 2H, $J = 8.8$), 7.10 (d, 2H, J=2.2), 6.90 (dd, 2H, $J = 8.8$, and 2.2), 4.26 (t, 4H), 3.76 (s, 6H), 3.22 (m, 4H), 3.14 (m, 4H), 1.72 (s, 6H), 1.70 (m, 4H), 1.31 (m, 12H). Anal. ($C_{38}H_{50}N_4O_8$) C, H, N.

4.1.12. 12-(3-(2-Acetamidoethyl)-5-methoxy-1*H*-indole-2-carbonyloxy)dodecyl 3-(2-acetamidoethyl)-5-methoxy-1*H*-indole-2-carboxylate (5g). Flash-chromatography eluent: EtOAc and then EtOAc/MeOH 97:3. The product was further purified by crystallization from MeOH (15% yield). Mp 160–161 °C; MS [(+)-ESI]: *m/z* 719 $[M+H]^+$; IR (Nujol) cm^{-1}: 3372, 1682, 1650; ^1H NMR (DMSO-d_6) δ: 11.39 (br s, 2H), 7.91 (br t, 2H), 7.30 (d, 2H, $J = 8.9$), 7.11 (d, 2H, $J = 1.9$), 6.90 (dd, 2H, $J = 8.9$ and 1.9), 4.26 (t, 4H), 3.76 (s, 6H), 3.23 (m, 4H), 3.13 (m, 4H), 1.72 (s, 6H), 1.70 (m, 4H), 1.45–1.22 (m, 16H). Anal. ($C_{40}H_{54}N_4O_8$) C, H, N.

4.1.13. Pentyl 3-(2-acetamidoethyl)-5-methoxy-1*H*-indole-2-carboxylate (6). Flash-chromatography eluent: EtOAc. The product was further purified by crystallization from EtOAc/cyclohexane (45% yield). Mp 144–145 °C; MS (EI): *m/z* 346 (M^+), 199 (100); IR (film) cm^{-1}: 3339, 1651; ^1H NMR ($CDCl_3$) δ: 8.65 (br s, 1H), 7.30 (d, 1H, $J = 9.6$), 7.02 (m, 2H), 6.34 (br s, 1H), 4.36 (t, 2H), 3.87 (s, 3H), 3.59 (m, 2H), 3.31 (t, 2H), 1.95 (s, 3H), 1.81 (m, 2H), 1.44 (m, 4H), 0.95 (t, 3H). Anal. ($C_{19}H_{26}N_2O_4$) C, H, N.

4.1.14. 9-(Benzoyloxy)nonyl 3-(2-acetamidoethyl)-5-methoxy-1*H*-indole-2-carboxylate (7). *N*,*N*-carbonyldiimidazole (0.035 g, 0.22 mmol) was added to a solution of **4** (0.050 g, 0.18 mmol) in dry DMF (0.5 mL) and the mixture stirred at room temperature under nitrogen atmosphere. After stirring for 3 h, 9-hydroxynonylbenzoate (0.057 g, 0.22 mmol) and a catalytic amount of DMAP were added to the reaction mixture and the stirring continued for 24 h. The mixture was partitioned between CH_2Cl_2 and H_2O and the organic phase was washed several times with water (5×), dried (Na_2SO_4) and evaporated. The crude product was purified by flash-chromatography (eluent: EtOAc) to obtain

15 mg of sticky solid (16% yield). MS (EI): *m/z* 522 (M^+), 105 (100); IR (neat) cm^{-1}: 3343, 1703, 1655; ^1H NMR ($CDCl_3$) δ: 8.76 (br s, 1H), 8.09 (m, 2H), 7.51 (m, 3H), 7.30 (d, 1H, $J = 9.5$), 7.01 (m, 2H), 6.23 (br s, 1H), 4.33 (m, 4H), 3.87 (s, 3H), 3.62 (m, 2H), 3.30 (t, 2H), 1.92 (s, 3H), 1.78 (m, 4H), 1.48 (m, 10H). Anal. ($C_{30}H_{38}N_2O_6$) C, H, N.

4.1.15. N^1,N^7-bis(2-(5- methoxy-1*H*-indol-3-yl)ethyl)heptanediamide (8a). A solution of pimeloylchloride (0.04 mL, 0.21 mmol) in dry THF (2 mL) was added dropwise to a solution of 5-methoxytryptamine (0.080 g, 0.42 mmol) in dry THF (2 mL) and Et_3N (0.07 mL, 0.6 mmol). The resulting mixture was stirred at room temperature for 1 h under nitrogen and then concentrated under *vacuum* and the residue partitioned between CH_2Cl_2 and H_2O. The organic phase was sequentially washed with 2 N HCl, saturated solution of Na_2CO_3, H_2O, and brine, dried (Na_2SO_4) and evaporated. The crude product was purified by flash-chromatography (eluent: $CHCl_3$/MeOH 3%) to obtain 85 mg of a gummy solid (80% yield). MS (EI): *m/z* 504 (M^+), 173 (100); IR (Nujol) cm^{-1}: 3280, 1701; ^1H NMR ($CDCl_3$) δ: 8.26 (br s, 2H), 7.26 (d, 2H, $J = 8.8$), 7.01 (m, 4H), 6.86 (dd, 2H, $J = 8.8$ and 2.2), 5.55 (bt, 2H), 3.86 (s, 6H), 3.60 (m, 4H), 2.95 (t, 4H), 2.05 (t, 4H), 1.49 (m, 4H), 1.21 (m, 2H). Anal. ($C_{29}H_{36}N_4O_4$) C, H, N.

4.1.16. N^1,N^{10}-bis(2-(5- methoxy-1*H*-indol-3-yl)ethyl)decanediamide (8b). This compound was synthesized following the same procedure reported for compound **8a**, by condensation of 5-methoxytryptamine (0.08 g, 0.42 mmol) with sebacoylchloride (0.05 mL, 0.21 mmol). Purification by flash-chromatography (eluent: $CHCl_3$/MeOH 3%) yielded 95 mg of resinous solid (82% yield). MS (EI): *m/z* 546 (M^+), 173 (100); IR (CH_2Cl_2) cm^{-1}: 3276, 1687; ^1H NMR ($CDCl_3$) δ: 8.53 (br s, 2H), 7.28 (d, 2H, $J = 8.8$), 7.01 (m, 4H), 6.85 (dd, 2H, $J = 8.8$ and 2.4) 5.60 (br t, 2H), 3.85 (s, 6H), 3.62 (m, 4H), 2.95 (t, 4H), 2.10 (t, 4H), 1.52 (m, 4H), 1.21 (m, 8H). Anal. ($C_{32}H_{42}N_4O_4$) C, H, N.

4.2. Pharmacology

4.2.1. Receptor binding experiments. Binding affinities of compounds at each receptor were determined 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.[41,42] 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 of protein per tube. Membrane protein level was determined in accordance with a previously reported method.[43] 2-[^{125}I]iodomelatonin (100 pM) and different 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 K_i values were calculated from the IC_{50} values in accordance with the Cheng–Prusoff equation[40] and are the mean of at least three independent determinations performed in duplicate; SEM of K_i values were lower than ±0.87.

4.2.2. Intrinsic activity determination. The intrinsic activity of the new compounds at each melatonin receptor subtype was evaluated on [^{35}S]guanosine-5′-*O*-(3-thiotriphosphate) ([^{35}S]GTPγS) binding in NIH3T3 cells stably transfected with human MT_1 or MT_2 receptors as previously described.[41,42,44] Nonspecific binding was defined using GTPγS (10 μM). In cell lines expressing human MT_1 or MT_2 receptors, MLT produced a concentration-dependent stimulation of basal [^{35}S]GTPγS binding. The maximal G-protein activation was measured in each experiment by using MLT (100 nM). 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 than that of MLT, antagonists are without effect, and inverse agonists decrease the basal [^{35}S]GTPγS binding. Compounds were added at three different concentrations (one concentration was equivalent to 100 nM MLT, a second one 10 times smaller, and a third-one 10 times larger), and the percent stimulation above basal was determined. All of the measurements were performed in triplicate. The relative intrinsic activity values (IA_r) were obtained by dividing the maximum ligand-induced stimulation of [^{35}S]GTPγS binding by that of MLT, as measured in the same experiment.

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