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Bivalent ligand approach on *N*-{2-[(3-methoxyphenyl)methylamino]ethyl}-acetamide: Synthesis, binding affinity and intrinsic activity for MT_1 and MT_2 melatonin receptors

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ABSTRACT

We report the synthesis, binding properties and intrinsic activity at MT_1 and MT_2 melatonin receptors of new dimeric melatonin receptor ligands in which two units of the monomeric agonist *N*-{2-[(3-methoxyphenyl)methylamino]ethyl}acetamide (**1**) are linked together through different anchor points. Dimerization of compound **1** through the methoxy substituent leads to a substantial improvement in selectivity for the MT_1 receptor, and to a partial agonist behavior. Compound **3a**, with a trimethylene linker, was the most selective for the MT_1 subtype (112-fold selectivity) and compound **3d**, characterized by a hexamethylene spacer, had the highest MT_1 binding affinity ($pK_{iMT1} = 8.47$) and 54-fold MT_1-selectivity. Dimerization through the aniline nitrogen of **1** abolished MT_1 selectivity, leading to compounds with either a full agonist or an antagonist behavior depending on the nature of the linker.

1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine, MLT, Fig. 1) is a neurohormone primarily secreted by the pineal gland at night in all species.[1] The ability of MLT to synchronize the 'circadian biological clock', by its direct action on the suprachiasmatic nucleus has led to the investigation of MLT and its analogs as a remedy for treating disordered circadian rhythms that occur in jet lag, shift work, certain types of insomnia, and some neuropsychiatric diseases. Some MLT receptor agonists are currently under clinical evaluation or have been very recently approved. The MT_1/MT_2 melatonin receptor agonist ramelteon (Rozerem®) was approved and launched in 2005 in the U.S. for the treatment of primary insomnia,[2] and other compounds,[3] such as Neu-P11,[4] TIK-301[5] or tasimelteon[6] are undergoing evaluation in clinical trials for their hypnotic properties. Moreover, the naphthalenic MLT bioisostere agomelatine is a novel antidepressant with an innovative pharmacological profile (MT_1/MT_2 agonist and $5HT_{2c}$ antagonist) which was recently approved by the EU-EMA for the treatment of major depressive disorders and is available in several European countries.[7–9] In mammals, two melatonin receptors, MT_1 and MT_2, have been identified. They belong to the G-protein-coupled receptor superfamily and exhibit subnanomolar affinity for MLT.[10] Elucidation of the distinct functions of MT_1 and MT_2 receptors in many target tissues is still under investigation and requires a continual development of specific and selective affinity ligands. Whereas some selective MT_2 receptor ligands have been recently described,[11,12] the limited availability of MT_1 subtype-selective ligands has hampered an exhaustive elucidation of the MT_1 receptor patho/physiological role. Although, a few monomeric ligands displaying moderate MT_1-selectivity were reported,[13] the most applied approach for the design of MT_1 selective receptor ligands relies in the preparation of symmetric dimers, by coupling two moieties deriving from known MLT receptor ligands.

Accumulating evidence indicates that most GPCRs (classically considered to function as monomers) exist as functional dimers or higher oligomeric units.[14] Oligomerization may occur in native tissues and may have important consequences on receptor function. As evidenced for several other GPCRs the formation of MT_1 and MT_2 homodimers and MT_1/MT_2 heterodimers has been shown in heterologous expression systems at physiological expression levels.[15,16] Although MT_1/MT_2 heterodimers remain to be identified in native tissues, their formation has to be taken into account by virtue of the documented co-expression of MT_1 and MT_2 receptors in many melatonin-sensitive tissues, such as the hypothalamic suprachiasmatic nucleus, retina, arteries, and adipose tissue.[17,18]

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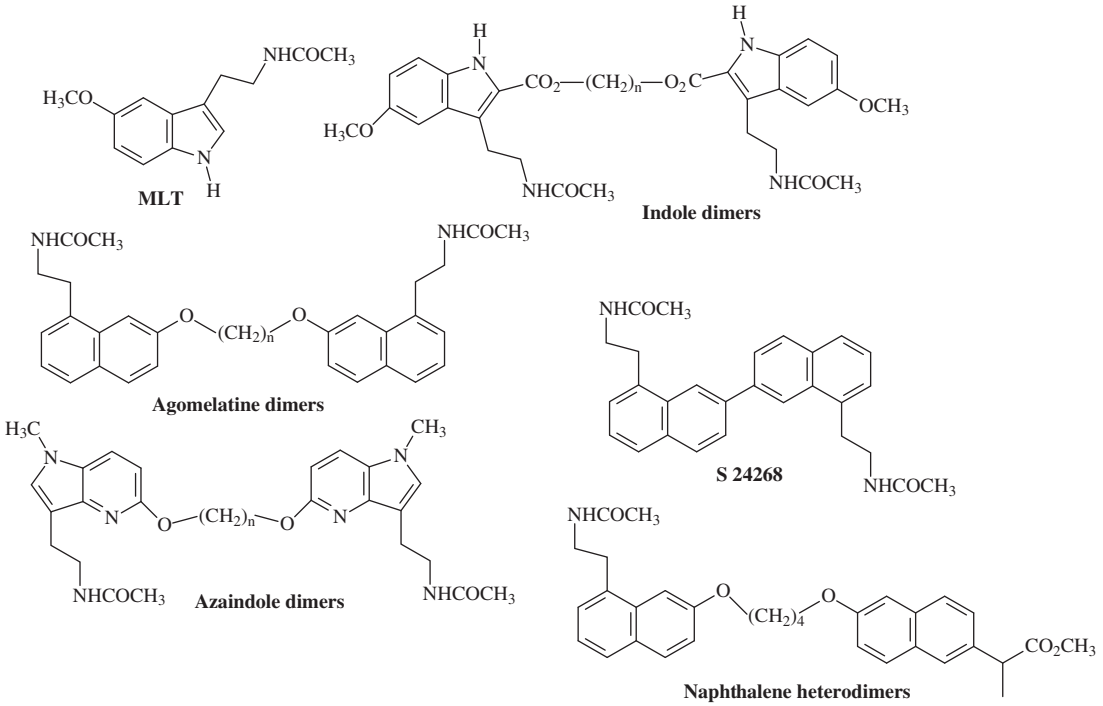


Figure 1. Melatonin and dimeric melatonin receptor ligands.

Homodimerization and heterodimerization processes could generate receptors with novel characteristics, and pharmacological properties different from any of the monomers, providing new opportunities for rational drug design and discovery.[19] Different strategies have been developed to specifically target GPCR dimers. Bivalent ligands, which are composed of two functional pharmacophores linked by a spacer, are among the most promising approaches.

It was postulated that these ligands would have distinct properties, such as increased selectivity, potency and different efficacy, when compared to the activity of each monomer; therefore, there seems to be a great potential in developing new leads compounds by linking two single chemical entities to generate bivalent ligands. To date, only few reports on dimeric molecules designed to target MLT-receptors appeared in the literature. Dimers of melatonin[20] and agomelatine[21] were prepared and tested for their activity on MT_1 and MT_2 melatonin receptors, showing interesting pharmacological profiles. Melatonin dimers linked through position 2 of the indole ring showed greater MT_1 and MT_2 binding affinity than dimers linked through the acylamino side chain. The most potent derivatives displayed nanomolar affinity for MT_1 and MT_2 receptors, while the intrinsic activity strongly varied with the linker length. Agomelatine dimers are characterized by a polymethylene chain connecting the oxygen atoms in position 7 on the naphthalenic nucleus. These derivatives showed a moderate to good MT_1-selectivity, with an antagonist behavior reported for the compound with a trimethylene linker. Other azaindoles[22] or naphthalene[23] dimeric melatonin receptor ligands were described, but they showed lower affinity and weaker selectivity when compared to the agomelatine dimers (Fig. 1). Following the same 'bivalent ligands' approach, a series of novel asymmetric heterodimers was recently reported to be selective partial agonists (K_i MT_2/MT_1 = 70–90) with subnanomolar affinity (Fig. 1).[24]

Recently, we reported a new class of high affinity melatonin receptor ligands, structurally characterized by a *N*-(substituted-anilinoethyl)amide scaffold (**A**, Fig. 2).[25] We decided to apply the 'bivalent ligand' approach to this new scaffold by synthesizing the new dimeric melatonin receptor ligands highlighted in Figure 2. We linked two units of the monomeric agonist (Fig. 2, R^1 = OMe, R^2 = Me)[25] through different anchor points, in order to evaluate the effects of the length and position of the spacers on their ability to bind to and activate MT_1 and MT_2 receptors.

2. Chemistry

The synthesis of the new compounds is described in Schemes 1 and 2. Key intermediate in the synthesis of the target compounds **3a–f** and **4** is the phenol derivative **2**, obtained from the previously described methoxy analog **1**[25] by cleavage of the methyl ether using boron tribromide. Homodimers **3a–f** were prepared by reaction of the phenol derivative **2** with 0.5 equiv of the appropriate dibromoalkane in the presence of K_2CO_3 in acetonitrile. Small amounts (ca. 10–12%) of a by-product (*N*-[2-(3-allyoxyphenyl)methylamino)ethyl]acetamide) resulting from HBr elimination of the monoalkylated starting material **2**, was also observed during the homodimerization. The monovalent ligand **4** was obtained by O-alkylation of the phenol **2** with 6-bromo-1-phenoxyhexane[26] in the presence of sodium hydride as a base.

The dimeric ligands linked through the aniline nitrogen (**5a–b** and **6**) were synthesized by N-alkylation, or N-acylation, of *N*-[2-(3-methoxyphenylamino)ethyl]acetamide[25] with 0.5 equiv of the opportune dibromoalkane or adipoyl chloride, respectively (Scheme 2).

3. Biological results and discussion

The chemical structures, binding affinities at MT_1 and MT_2 receptors and the intrinsic activity of the new compounds **3a–f**, **4**, **5a–b** and **6** are reported in the Table 1. Length, position and chemical properties of the spacer connecting each pharmacophore significantly affect affinity and activity at melatonin receptors. We decided to connect the two anilinoethylamides by inserting the

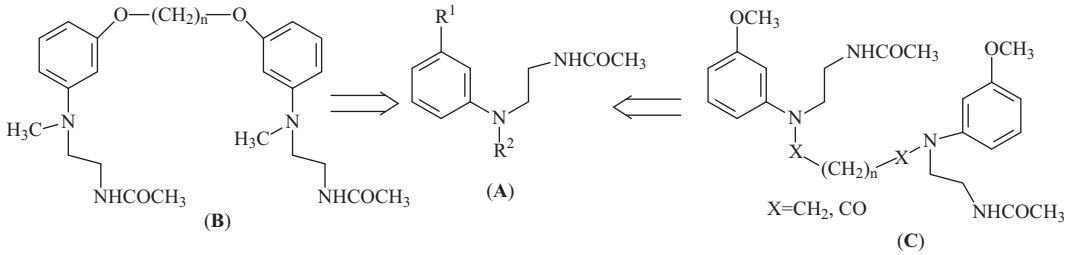
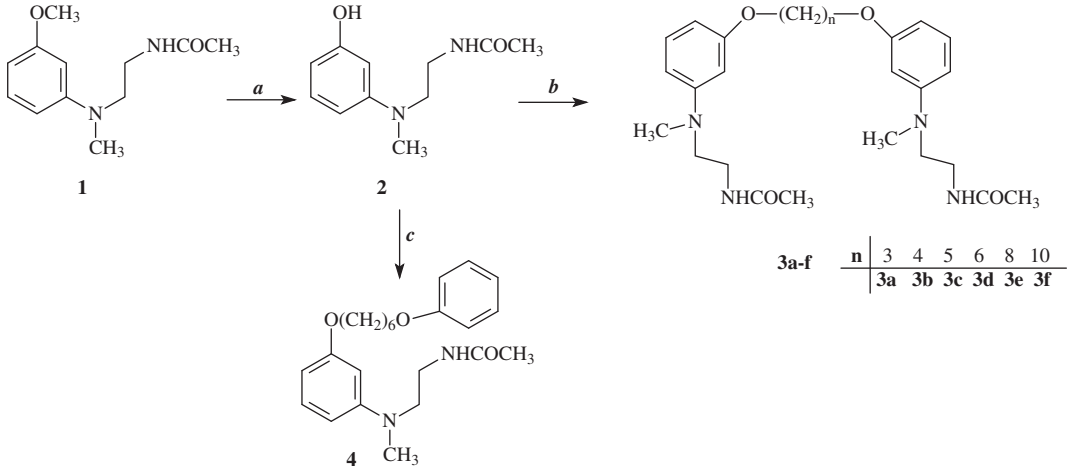
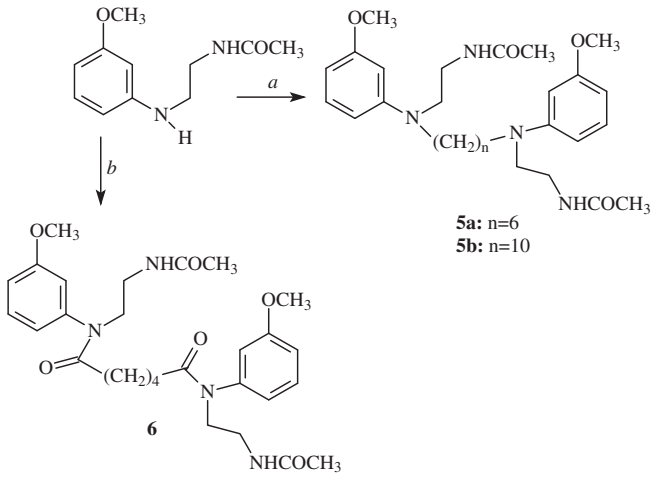


Figure 2. Approaches to new dimeric melatonin receptor ligands.



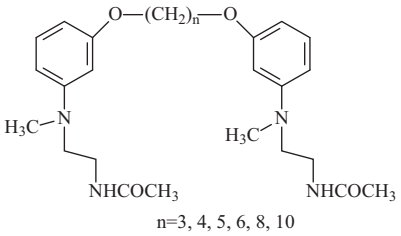
Scheme 1. Reagents and conditions: (a) BBr_3, CH_2Cl_2, rt; (b) $Br(CH_2)_nBr$, K_2CO_3, CH_3CN, reflux; (c) NaH, $PhO(CH_2)_6Br$, DMF.



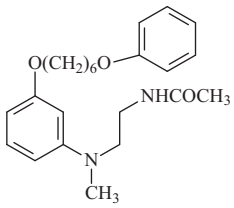
Scheme 2. Reagents and conditions: (a) $Br(CH_2)_nBr$, K_2CO_3, DMF, 100 °C; (b) adipoyl chloride, TEA, THF, rt.

linker between the methoxy groups (compounds **3a–f**) or between the aniline nitrogens (**5a–b** and **6**) because large substituents in these positions have been shown to be tolerated.[22] Several spacers have been generally used for dimer formation, including polymethylene, polyalkyloxy ether, polyesters, etc. We have initially selected a polymethylene spacer because of the ease of coupling of the pharmacophores to the spacer and the possibility to modulate the linker length by small increases. With the aim to find out the optimal length, we evaluated compounds **3a–f** bearing a spacer of 3, 4, 5, 6, 8 and 10 methylene units, respectively. Analysis of MT_1 and MT_2 binding data for compounds **3a–f** showed a gradual increase in MT_2 affinity with chain length, while MT_1 affinity varied in a more complex manner. All these dimers exhibited lower affinity than the monomer **1**, but, interestingly, they showed pronounced MT_1 subtype selectivity. The best MT_1 affinity values were obtained for the dimers with 6 and 10 methylene units (compounds **3d** and **3f**, pK_i = 8.47 and 8.40, respectively). The MT_1 affinity of compound **3d** was slightly lower than that of the monomer **1** (four-fold), but it displayed an 54-fold selectivity for the MT_1 receptor, while monomer **1** was a non-selective ligand. The SARs observed for this series of compounds resembled those for agomelatine analogs[21], even if agomelatine derivatives were reported to be generally more potent and more MT_1-selective than our compounds. In particular, also in the agomelatine series the most selective compounds had a trimethylene linker (K_{iMT2}/K_{iMT1} = 224), while longer chains gave significant increases in MT_1 affinity, with lower selectivity: the hexamethylene derivative of agomelatine, corresponding to **3d**, has pK_{iMT1} = 10.1 and K_{iMT2}/K_{iMT1} = 30. For the sake of comparison, it should be noted that binding data may also depend on the cell line employed. For the agomelatine series, tests were performed on HEK 293 cells, and in those conditions melatonin was more MT_1-selective than in our test (K_{iMT2}/K_{iMT1} = 4). In order to evaluate the influence of the second amido pharmacophore unit to the binding, the monovalent ligand **4**, characterized by a phenoxyhexane fragment, was synthesized. Compound **4**, having a hexamethylene spacer as the most interesting dimer **3d**, showed the same MT_2 affinity and a significantly lower MT_1 affinity, even if its pK_i (7.84) was among the highest values obtained on MT_1 receptors for compounds **3a–f**. While the irregular trend of pK_i values on MT_1 receptors does not allow a conclusive assessment of the role of a second MLT-like fragment, the relatively high MT_1 affinity of compound **4** suggests that a bivalent ligand is not necessary to generate MT_1 selectivity, as also shown in a recent series of agomelatine analogs.[24] Taken together, melatonin receptor affinity values of dimers 3a–f and of the monovalent ligand **4** indicate that the two pharma-

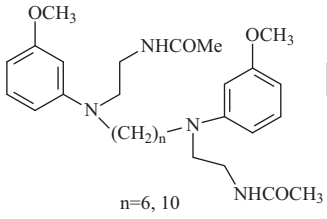
Table 1
Binding affinity (pK_i)[a] and intrinsic activity (IA_r)[b] of new compounds for human MT_1 and MT_2 receptors stably expressed in NIH3T3 cells



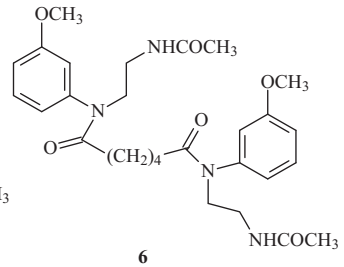
3a-f



4



5a-b



6

Compound	*n*	Human MT_1		Human MT_2		K_iMT_2/K_iMT_1
		pK_i	$IA_r \pm SEM$	pK_i	$IA_r \pm SEM$	
MLT		9.58 ± 0.18	1.00 ± 0.01	9.47 ± 0.13	1.00 ± 0.02	1.3
1[c]		9.09 ± 0.10	0.95 ± 0.08	9.19 ± 0.01	1.06 ± 0.05	0.8
3a	3	7.69 ± 0.14	0.26 ± 0.03	5.68 ± 0.10	0.33 ± 0.04	102
3b	4	7.37 ± 0.13	0.21 ± 0.03	6.00 ± 0.12	0.18 ± 0.04	23
3c	5	7.24 ± 0.09	0.46 ± 0.07	6.09 ± 0.04	0.64 ± 0.04	14
3d	6	8.47 ± 0.05	0.44 ± 0.01	6.74 ± 0.09	0.33 ± 0.06	54
3e	8	7.70 ± 0.31	0.47 ± 0.02	7.03 ± 0.12	0.38 ± 0.03	4.7
3f	10	8.40 ± 0.18	0.42 ± 0.02	7.39 ± 0.32	0.57 ± 0.02	10
4		7.84 ± 0.07	0.49 ± 0.02	6.80 ± 0.07	0.54 ± 0.04	11
5a	6	6.13 ± 0.19	−0.04 ± 0.02	7.44 ± 0.33	−0.09 ± 0.01	0.05
5b	10	6.42 ± 0.04	0.12 ± 0.05	6.51 ± 0.02	0.37 ± 0.12	0.8
6		6.54 ± 0.35	0.94 ± 0.08	6.61 ± 0.22	0.91 ± 0.05	0.85

[a] pK_i values were calculated from IC_{50} values, obtained from competition curves by the method of Cheng and Prusoff[32] and are the mean of at least three determinations performed in duplicate.
[b] The relative intrinsic activity values were obtained by dividing the maximum analogue-induced G-protein activation by that of MLT.
[c] Ref. 25.

cophores did not bind at two neighboring MLT binding sites because none of the dimers showed higher receptor affinity than that of the monomer **1**. The actual presence of receptor dimers has not been assessed in the transfected NIH3T3 cells employed for our in vitro tests. However, in these cells the MT_1 or MT_2 receptor was expressed at high density levels (>100 fmol/mg protein[27,28]), consistent with the formation of dimers, which had been observed in HEK293 cells at 20–100 fmol/mg protein.[15] Therefore, on the basis of the above data, it can be supposed that both the linker and one pharmacophore are bound to the receptor in a region of steric tolerance, that resulted more suitable in the MT_1 binding site. The compounds show moderate MT_2 binding affinity, slightly better for longer derivatives. This is different from what observed for agomelatine dimers, whose derivatives with longer linkers retained subnanomolar MT_2 affinity.[21]

The functional activity of the new compounds has been evaluated on both receptors (GTPγS assay) in comparison with MLT. None of the cited compounds (**3a–f**, **4**) showed full agonist activity, but they behave as partial agonists on both MT_1 and MT_2 receptors.

We also evaluated the possibility to link the two monomers **1** through their aniline nitrogens rather than their methoxy substituent, as we previously showed that N-substitution with a large hydrophobic substituent is favorable for binding to MT_2 receptor.[25] Compounds **5a** and **5b** (*n* = 6 and 10 respectively) displayed affinity values at MT_2 receptor in the same range as the corresponding dimers (**3d**, **3f**) connected through the methoxy substituent, but they exhibited considerably lower MT_1 binding affinity. This drop of MT_1 affinity is consistent with the reduced steric tolerance in the corresponding region of previously developed pharmacophore and 3D-QSAR models.[29] In fact, the shorter derivative, **5a**, is a moderately MT_2-selective full antagonist, even if the longer derivative, **5b**, also loses affinity for the MT_2 receptor. The steric arrangement of the linker chain with respect to the aniline scaffold also proved important for interaction with the melatonin receptors. Indeed, a linker connected through two planar amide groups led to a lack of subtype selectivity and to an increase in intrinsic activity. Compound **6**, despite its low binding affinity, behaves as a full agonist at both receptor subtypes.

4. Conclusions

We have synthesized the new MLT receptor ligands **3a–f**, **5a–b** and **6**, that were designed according to the 'bivalent ligand' approach by linking two moieties of the MT_1/MT_2 melatonin agonist *N*-{2-[(3-methoxyphenyl)methylamino]ethyl}acetamide (**1**), through their methoxy substituent or their aniline nitrogen, by polymethylene chains of variable length, with the aim to increase affinity and MT_1/MT_2 subtype selectivity. The dimers did not show MLT receptor affinities higher than that of the monomer **1**, and the similar binding profile shown by the asymmetric analog **4** suggests that they do not interact with two independent recognition sites; therefore, the bivalent ligand approach failed, in this case, to achieve more potent compounds. On the contrary, it is clear that dimerization of compound **1** through the methoxy substituent leads to an improvement in selectivity for MT_1 receptors. Considering that the development of MT_1 melatonin selective ligands can be still considered a difficult task to achieve, the most selective C_3-dimer **3a** (112-fold MT_1-selectivity) and dimer **3d** with a C_6 spacer (pK_{iMT1} = 8.47 and 54-fold MT_1 preference over MT_2 receptors) could be considered interesting lead molecules in the development of selective MT_1 ligands, the most selective C_3-dimer **3a** and dimer **3d** with a C_6 spacer (pK_{iMT1} = 8.47 and 54-fold MT_1 preference over MT_2 receptors) could be considered interesting lead molecules in the development of selective MT_1 ligands.

5. Experimental section

5.1. Chemistry

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). ESI-MS spectra were taken on a Waters Micromass ZQ instrument; only molecular ions (M+1) are given. EI-MS spectra (70 eV) were taken on a Fisons Trio 1000 spectrometer; only molecular ions (M^+) and base peaks are given. Infrared spectra were obtained on a Nicolet Avatar 360 FT-IR spectrometer; absorbances are reported in ν (cm^{-1}). Analyses indicated by the symbols of the elements (C, H, N) are within 0.4% of the 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 F_{254} plates.

5.1.1. *N*-{2-[(3-Hydroxyphenyl)methylamino]ethyl}acetamide (2)

A solution of BBr_3 (12.6 mmol) in dry CH_2Cl_2 (60 mL) was added dropwise to a solution of *N*-{2-[(3-methoxyphenyl)methylamino]ethyl}acetamide[25] (1.4 g, 6.3 mmol) in dry CH_2Cl_2 (47 mL) at 0 °C and the resulting mixture was stirred at room temperature for 18 h. The solvent was evaporated, the residue was neutralized with an aqueous saturated solution of $NaHCO_3$ and extracted with EtOAc. The organic phases were combined, washed once with brine, dried (Na_2SO_4) and concentrated under reduced pressure to give a crude residue, which was purified by flash chromatography (CH_2Cl_2–MeOH, 98:2 as eluent) and crystallization. Pink solid, 76% yield; mp 74–76 °C (Et_2O–light pet.). MS (EI): *m/z* 208 (M^+), 136 (100). ^1H NMR ($CDCl_3$) is in line with previous report.[30]

5.1.2. General procedure for the synthesis of dimeric derivatives 3a–f

K_2CO_3 (0.300 g, 2.17 mmol) was added to a solution of **2** (0.150 g, 0.72 mmol) in acetonitrile (2.5 mL). The resulting mixture was refluxed for 30 min and then the required dibromo-derivative (0.36 mmol) was added dropwise. The reaction mixture was refluxed 16 h, quenched with water and extracted with EtOAc. The organic phases were combined, washed once with brine, dried (Na_2SO_4) and concentrated under reduced pressure to yield a crude residue which was purified by flash chromatography (EtOAc–MeOH, 95:5 as eluent) and crystallization.

5.1.2.1. *N*-(2-{[3-(3-{3-[(2-Acetylaminoethyl)methylamino]phenoxy}propoxy)phenyl]methylamino}ethyl)acetamide (3a)

White solid, 61% yield; mp 120–121 °C (EtOAc–light pet.). ESI-MS (*m/z*): 457 (M+1). ^1H NMR (DMSO-d_6): δ 1.76 (s, 6H), 2.11 (m, 2H), 2.85 (s, 6H), 3.14 (m, 4H), 3.31 (m, 4H), 4.07 (t, 4H J = 6.0 Hz), 6.19–6.32 (m, 6H), 7.03 (t, 2H, J = 8.0 Hz), 7.93 (br t, 2H). IR (cm^{-1}, Nujol): 3314, 1635, 1614. Anal. Calcd for $C_{25}H_{36}N_4O_4$: C, 67.77; H, 7.95; N, 12.27. Found: C, 67.61; H, 7.80; N, 12.31.

5.1.2.2. *N*-(2-{[3-(4-{3-[(2-Acetylaminoethyl)methylamino]phenoxy}butoxy)phenyl]methylamino}ethyl)acetamide (3b)

White solid, 29% yield; mp 137–138 °C (EtOAc). ESI-MS (*m/z*): 471 (M+1). ^1H NMR (DMSO-d_6): δ 1.76 (s, 6H), 1.83 (m, 4H), 2.86 (s, 6H), 3.15 (m, 4H), 3.31 (m, 4H), 3.98 (m, 4H), 6.18–6.32 (m, 6H), 7.03 (t, 2H J = 8.0 Hz), 7.93 (br t, 2H). IR (cm^{-1}, Nujol): 3321, 1649, 1614. Anal. Calcd for $C_{26}H_{38}N_4O_4$: C, 66.36; H, 8.14; N, 11.91. Found: C, 66.29; H, 7.95; N, 12.05.

5.1.2.3. *N*-(2-{[3-(5-{3-[(2-Acetylaminoethyl)methylamino]phenoxy}pentyloxy)phenyl]methylamino}ethyl)acetamide (3c)

White solid, 49% yield; mp 109–111 °C (EtOAc). ESI-MS (*m/z*): 485 (M+1). ^1H NMR (DMSO-d_6): δ 1.55 (m, 2H), 1.76 (s, 6H), 1.80 (m, 4H), 2.85 (s, 6H), 3.14 (m, 4H), 3.29 (m, 4H), 3.93 (m, 4H), 6.16–6.30 (m, 6H), 7.01 (t, 2H J = 8.0 Hz), 7.95 (br t, 2H). IR (cm^{-1}, Nujol): 3319,1650, 1616. Anal. Calcd for $C_{27}H_{40}N_4O_4$: C, 66.92; H, 8.32; N, 11.56. Found: C, 66.67; H, 8.45; N, 11.84.

5.1.2.4. *N*-(2-{[3-(6-{3-[(2-Acetylaminoethyl)methylamino]phenoxy}hexyloxy)phenyl]methylamino}ethyl)acetamide (3d)

White solid, 48% yield; mp 118–120 °C (EtOAc). ESI-MS (*m/z*): 499 (M+1). ^1H NMR (DMSO-d_6): δ 1.46 (m, 4H), 1.71 (m, 4H), 1.76 (s, 6H), 2.85 (s, 6H), 3.14 (m, 4H), 3.33 (m, 4H), 3.92 (t, 4H J = 6.5 Hz), 6.16–6.32 (m, 6H), 7.02 (t, 2H J = 8.0 Hz), 7.93 (br t, 2H). IR (cm^{-1}, Nujol): 3322, 1650, 1615. Anal. Calcd for $C_{28}H_{42}N_4O_4$: C, 67.44; H, 8.49; N, 11.24. Found: C, 67.52; H, 8.57; N, 11.08.

5.1.2.5. *N*-(2-{[3-(8-{3-[(2-Acetylaminoethyl)methylamino]phenoxy}octyloxy)phenyl]methylamino}ethyl)acetamide (3e)

White solid, 60% yield; mp 108–110 °C (EtOAc). ESI-MS (*m/z*): 527 (M+1). ^1H NMR (DMSO-d_6): δ 1.35 (m, 8H), 1.67 (m, 4H), 1.76 (s, 6H), 2.85 (s, 6H), 3.14 (m, 4H), 3.30 (m, 4H), 3.89 (t, 4H J = 6.5 Hz), 6.15–6.30 (m, 6H), 7.01 (t, 2H J = 8.0 Hz), 7.94 (br t, 2H). IR (cm^{-1}, Nujol): 3321, 1650, 1615. Anal. Calcd for $C_{30}H_{46}N_4O_4$: C, 68.41; H, 8.80; N, 10.64. Found: C, 68.21; H, 8.59; N, 10.48.

5.1.2.6. *N*-(2-{[3-(10-{3-[(2-Acetylaminoethyl)methylamino]phenoxy}decyloxy)phenyl]methylamino}ethyl)acetamide (3f)

White solid, 63% yield; mp 105–107 °C (EtOAc). ESI-MS (*m/z*): 555 (M+1). ^1H NMR (DMSO-d_6): δ 1.28 (m, 12H), 1.66 (m, 4H), 1.76 (s, 6H), 2.84 (s, 6H), 3.14 (m, 4H), 3.28 (m, 4H), 3.88 (t, 4H J = 6.5 Hz), 6.15–6.30 (m, 6H), 7.01 (t, 2H J = 8.0 Hz), 7.94 (br t, 2H). IR (cm^{-1}, Nujol): 3320, 1649, 1615. Anal. Calcd for $C_{32}H_{50}N_4O_4$: C, 69.28; H, 9.08; N, 10.10. Found: C, 69.20; H, 9.01; N, 10.26.

5.1.3. *N*-(2-{Methyl[3-(6-phenoxyhexyloxy)phenyl]amino}ethyl)acetamide (4)

Sodium hydride (80% in mineral oil, 0.02 g, 0.66 mmol) and 6-bromo-1-phenoxyhexane[26] (0.265 g, 1.03 mmol) were added to a solution of **2** (0.13 g, 0.62 mmol) in dry DMF (2.6 mL) at −10 °C under nitrogen atmosphere. The reaction mixture was stirred at room temperature for 18 h, poured onto 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 (EtOAc as eluent) and crystallization. White solid, 79% yield; mp 67–68 °C (Et_2O–light pet.). MS (EI): *m/z* 384 (M^+), 94 (100). ^1H NMR ($CDCl_3$): δ 1.59 (m, 4H), 1.83 (m, 4H), 1.94 (s, 3H), 2.94 (s, 3H), 3.46 (m, 4H), 3.98 (m, 4H), 5.60 (br s, 1H), 6.29–6.40 (m, 3H), 6.88–6.97 (m, 3H), 7.14 (t, 1H J = 8.0 Hz), 7.25–7.32 (m, 2H). IR (cm^{-1}, Nujol): 3266, 1641, 1611. Anal. Calcd for $C_{23}H_{32}N_2O_3$: C, 71.84; H, 8.39; N, 7.29. Found: C, 72.05; H, 8.53; N, 7.51.

5.1.4. General procedure for the synthesis of dimeric ligands 5a,b

K_2CO_3 (0.36 g, 2.61 mmol) followed by the opportune dibromo-derivative (0.87 mmol) were added to a solution of *N*-[2-(3-methoxyphenylamino)ethyl]acetamide[25] (0.358 g, 1.74 mmol) in DMF (2 mL). The resulting mixture was heated at 100 °C for 24 h, quenched with water and extracted with EtOAc. The combined organic phases were washed once with brine, dried (Na_2SO_4) and concentrated under reduced pressure to yield a crude residue which was purified by flash chromatography (EtOAc as eluent) and crystallization. About 65% of non-reacted starting aniline was recovered after chromatography.

5.1.4.1. *N*-{2-[{6-[(2-Acetylaminoethyl)-(3-methoxyphenyl) amino]hexyl}-(3-methoxyphenyl)amino]ethyl}acetamide (5a) White solid, 15% yield; mp 123–124 °C (EtOAc–Et_2O). ESI-MS (*m/z*): 499 (M+1). ^{1}H NMR ($CDCl_3$): δ 1.33 (m, 4H), 1.58 (m, 4H), 1.95 (s, 6H), 3.25 (m, 4H), 3.42 (m, 8H), 3.79 (s, 6H), 5.74 (br s, 2H), 6.25–6.36 (m, 6H), 7.13 (t, 2H J = 8.5 Hz). IR (cm^{-1}, Nujol): 3256, 1638, 1609. Anal. Calcd for $C_{28}H_{42}N_4O_4$: C, 67.44; H, 8.49; N, 11.24. Found: C, 67.73; H, 8.72; N, 11.60.

5.1.4.2. *N*-{2-[{10-[(2-Acetylaminoethyl)-(3-methoxyphenyl) amino]decyl}-(3-methoxyphenyl)amino]ethyl}acetamide (5b) White solid, 13% yield; mp 101–102 °C (EtOAc–Et_2O). ESI-MS (*m/z*): 555 (M+1). ^{1}H NMR ($CDCl_3$): δ 1.28 (m, 12H), 1.55 (m, 4H), 1.95 (s, 6H), 3.25 (m, 4H), 3.42 (m, 8H), 3.79 (s, 6H), 5.64 (br s, 2H), 6.24–6.37 (m, 6H), 7.13 (t, 2H J = 8.5 Hz). IR (cm^{-1}, Nujol): 3256, 1638, 1608. Anal. Calcd for $C_{32}H_{50}N_4O_4$: C, 69.28; H, 9.08; N, 10.10. Found: C, 68.98; H, 8.95; N, 10.01.

5.1.5. Hexanedioic acid bis[(2-acetylaminoethyl)-(3-methoxyphenyl)amide] (6)

To a solution of *N*-[2-(3-methoxyphenylamino)ethyl]acetamide[25] (0.14 g, 0.67 mmol) and TEA (0.12 mL, 0.86 mmol) in dry THF (4 mL) was added adipoyl chloride (0.05 mL, 0.34 mmol). The resulting mixture was stirred at room temperature for 2 h, quenched with water and extracted three times with hot EtOAc. The organic phases were combined, dried (Na_2SO_4) and concentrated under reduced pressure to yield a crude solid which was triturated with EtOAc and filtered. White solid, 45% yield; mp 219–221 °C. ESI-MS (*m/z*): 527 (M+1). ^{1}H NMR (DMSO-d_6): δ 1.35 (m, 4H), 1.75 (s, 6H), 1.90 (m, 4H), 3.17 (m, 4H), 3.62 (m, 4H), 3.80 (s, 6H), 6.84–7.00 (m, 6H), 7.36 (t, 2H J = 8.0 Hz), 7.92 (br t, 7.92). IR (cm^{-1}, Nujol): 3307, 1647, 1604. Anal. Calcd for $C_{28}H_{38}N_4O_6$: C, 63.86; H, 7.27; N, 10.64. Found: C, 63.56; H, 7.35; N, 10.79.

5.2. Pharmacological evaluation

Binding affinities 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 was already described in detail.[27,28] 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. The membrane protein level was determined in accordance with a previously reported method.[31] 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 melatonin; IC_{50} values were determined by nonlinear fitting strategies with the program PRISM (GraphPad SoftWare Inc., San Diego, CA). The pK_i values were calculated from the IC_{50} values in accordance with the Cheng–Prusoff equation.[32] The pK_i values are the mean of at least three independent determinations performed in duplicate.

To define the functional activity of the new compounds at MT_1 and MT_2 receptor subtypes, [^{35}S]GTPγS binding assays in NIH3T3 cells expressing human-cloned MT_1 or MT_2 receptors were performed. The amount of bound [^{35}S]GTPγS is proportional to the level of the analog-induced G-protein activation and is related to the intrinsic activity of the compound under study. The detailed description and validation of this method were reported elsewhere.[27,28] Membranes (15–25 μg of protein, final incubation volume 100 μL) were incubated at 30 °C for 30 min in the presence and in the absence of melatonin analogs, in an assay buffer consisting of [^{35}S]GTPγS (0.3–0.5 nM), GDP (50 μM), NaCl (100 mM), and $MgCl_2$ (3 mM). Nonspecific binding was defined using [^{35}S]GTPγS (10 μM). In cell lines expressing human MT_1 or MT_2 receptors, melatonin 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 receptors, respectively. Basal stimulation is the amount of [^{35}S]GTPγS specifically bound in the absence of compounds and it was taken as 100%. The maximal G-protein activation was measured in each experiment by using melatonin (100 nM). Compounds were added at three different concentrations (one concentration was equivalent to 100 nM melatonin, a second one 10 times smaller, and a third one 10 times larger), and the percent stimulation above basal was determined. The equivalent concentration was estimated on the basis of the ratio of the affinity of the test compound over that of melatonin. It was assumed that at the equivalent concentration the test compound occupies the same number of receptors as 100 nM melatonin. All of the measurements were performed in triplicate. The relative intrinsic activity (IAr) values were obtained by dividing the maximum ligand-induced stimulation of [^{35}S]GTPγS binding by that of melatonin as measured in the same experiment.

The two radioligands 2-[^{125}I]iodomelatonin (specific activity, 2000 Ci/mmol) and [^{35}S]GTPγS ([^{35}S]guanosine-5′-*O*-(3-thio-triphosphate); specific activity, 1000 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Italy).

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References and notes

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