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Development of substituted *N*-[3-(3-methoxylphenyl)propyl] amides as MT_2-selective melatonin agonists: Improving metabolic stability

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ABSTRACT

A series of novel and selective *N*-[3-(6-benzyloxy-3-methoxyphenyl)propyl] amides has recently been shown to possess sub-nanomolar range binding affinity to the type 2 melatonin receptor (MT_2). Pharmacokinetics studies suggested that these compounds were subject to vigorous CYP450-mediated metabolism, resulting in a series of metabolites with significantly decreased or diminished binding affinities toward MT_2 receptor. The ether bonds were found to be the major positions susceptible to metabolism. In this study, the benzyl ether bond was either removed or replaced with a carbon–carbon bond in an attempt to improve metabolic stability and enhance their resistance towards phase I oxidation. The synthesis, receptor binding affinity, intrinsic potency and metabolic stability of modified structures are reported in this article. By removal or replacement of metabolic labile ether linkerage with carbon linkers, a novel compound was identified with good potency and MT_2 selectivity, and with increased metabolic stability.

1. Introduction

Melatonin is a multi-functional neurohormone primarily secreted by the pineal gland during the period of darkness. It regulates the circadian rhythm and has been used to treat diseases associated with the desynchronization of biological rhythms, such as jet-lag, disturbed sleep–wake cycles and seasonal disorders.[1,2] Besides its primary role of regulating circadian rhythm, melatonin is involved in a number of additional physiological effects and has a variety of therapeutic potentials for the treatment of depression, cancer and neurodegenerative pathologies.[3,4] Most of the biological functions of melatonin are mediated through activation of two subtypes of G protein-coupled receptors, MT_1 and MT_2, which are widely expressed in different tissues.[5] The exact role of MT_1 and MT_2 are not clearly defined due to the lack of receptor subtype-selective ligands in clinical uses. Developing potent and subtype-selective melatonin ligands have attracted huge interests from medicinal chemists.[6–9] We have previously characterized a series of novel *N*-[3-(6-benzyloxy-3-methoxyphenyl)propyl] amides exhibiting sub-nanomolar range binding affinity to MT_2 selectively.[10] Pharmacokinetic studies revealed that this series of compounds generally exhibit less desirable pharmacokinetic properties due to high metabolic clearance, which may jeopardize their in vivo pharmacological potency. Several sites susceptible to the attack of phase I enzymes were identified. The major metabolic pathways included carbon–oxygen ether bond cleavage (such as C6-debenzylation and C3-demethylation) and hydroxylation of the phenyl ring.[11]

In order to increase metabolic stability so as to ultimately improve pharmacokinetic properties of this series of compounds, additional *N*-phenylpropyl amides were designed and synthesized. As the *N*-[3-(3-methoxylphenyl)propyl] amide was identified as an optimal pharmacophore for binding towards melatonin receptor and the hydrophobic phenyl substituent on C6 position of the 3-methoxylphenyl scaffold was proven to be a determinant of subtype-selectivity, they were kept intact in the search for metabolically more stable compounds. As shown in Figure 1, the position for further structure modification was focused on the linker connecting the two phenyl rings. The oxygen–carbon ether bond of lead compound **1** was either removed or replaced with a carbon–carbon bond in an effort to block the degradation at this site.

2. Results and discussion

2.1. Chemistry

The syntheses toward the novel *N*-phenylpropyl amides (**7**, **8**, **9**, and **10**) are shown in Scheme 1. All of the compounds have a 3-methoxyphenyl group attached at C6 position on the scaffold 3-methoxylphenyl ring. Between the two phenyl rings, compound **7** has a carbon–carbon triple bond, compound **8** has an ethylene chain, compound **9** has one methylene carbon chain, and compound **10** has no chain. All of these compounds were prepared from a common triflite intermediate **6**, through palladium mediated carbon–carbon bond forming coupling reactions. The triflite

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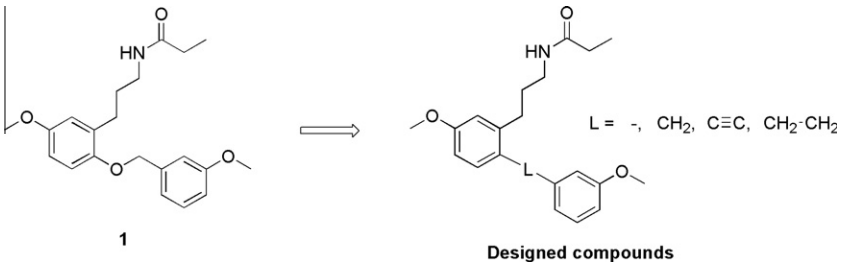
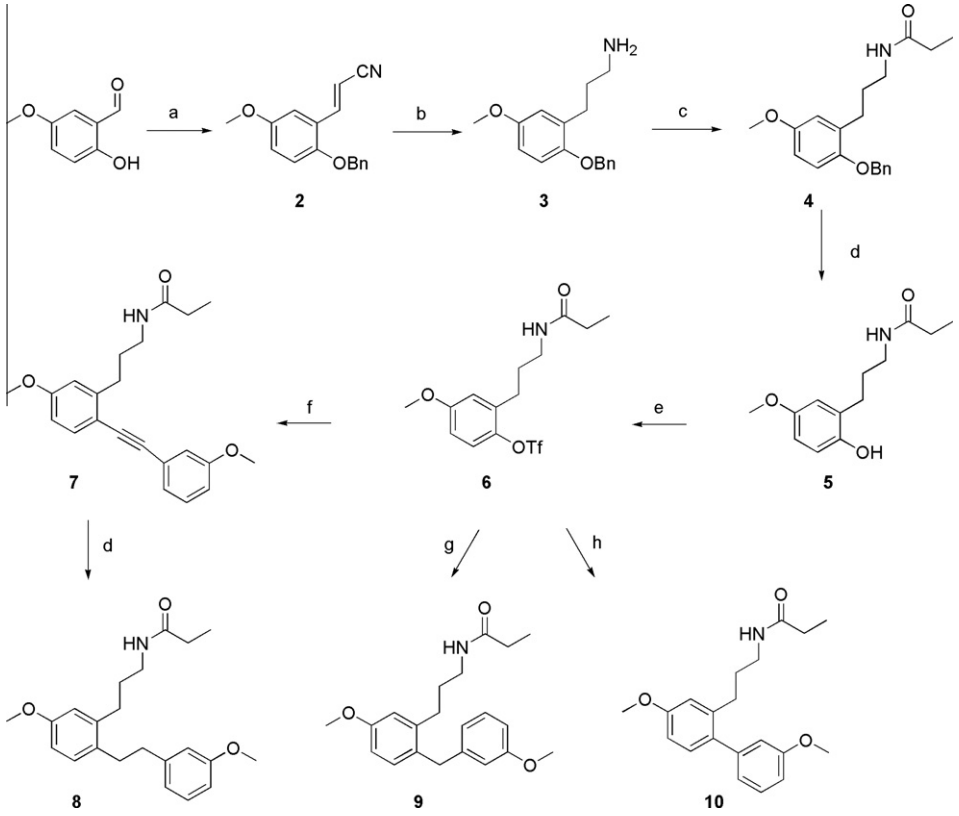


Figure 1. Design of metabolically more stable compounds.



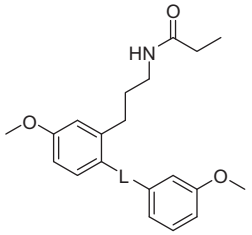
Scheme 1. Modification of the linker between the two phenyl rings. Reagents and conditions: (a) (1) BnBr, NaH, THF; (2) $(EtO)_2PO(CH_2CN)$, NaH, THF, (b) $LiAlH_4$, EtO_2, (c) EtCOCl, Et_3N, (d) Pd/C, H_2, MeOH, (e) Tf_2O, Et_3N, (f) m-MeOPhC≡CH, $Pd(PPh_3)_4$, CuI, (g) m-$MeOPhCH_2ZnBr$, $Pd(PPh_3)_4$, DMF, (h) m-$MeOPhB(OH)_2$, $Pd(PPh_3)_4$, Na_2CO_3, toluene.

6 was prepared from commercially available starting materials in six reaction steps. Starting with 2-hydroxy-5-methoxybenzaldehyde, alkylation with benzyl bromide under potassium carbonate in DMF, followed by Horner–Emmons olefination with diethylcyanomethylphosphate under sodium hydride in THF provided the α-β unsaturated nitriles **2** as a mixture of cis- and trans-isomers. Reduction of the unsaturated nitriles **2** with lithium aluminum hydride in refluxing diethyl ether generated the corresponding saturated amine **3**, which was reacted with propionyl chloride to give the amide **4**. Removing of the benzyl group of **4** by hydrogenation provided free phenol **5**, which was treated with trifluoroacetic anhydride to generate common intermediate triflite **6**. Coupling of triflite **6** with *m*-methoxyphenyl–acetylene provided alkyne **7**. Reduction of the alkyne triple bond of **7** by hydrogenation yielded compound **8** with an ethylene chain connecting the two phenyl rings. Coupling of triflite **6** with 3-methoxybenzyl zinc bromide in DMF gave compound **9** with a methylene chain connecting the two phenyl rings. Reaction of triflite **6** with 3-methoxyphenyl boronic acid bound the two phenyl rings directly to give biphenyl compound **10**.

2.2. Binding assay

Competitive binding characteristics of the compounds towards human MT_1 and MT_2 melatonin receptors stably expressed in Chinese hamster ovary (CHO) cells were determined by whole cell binding assays using 1 nM [^{3}H]melatonin as the probe. The K_d of melatonin for MT_1 and MT_2 receptors was 0.296 and 0.429 nM, respectively, as determined by saturation binding assays. The K_i values of modified compound **7**–**10** for MT_1 and MT_2 as well as their MT_1/MT_2 selectivity ratio are reported in Table 1. For comparison, the binding data of the original lead compound **1** with an oxygen–carbon linker was also listed. Compared with compound **1**, compound **7** with a carbon–carbon triple bond displayed even better affinity (K_i 0.073 nM) and selectivity (MT_1/MT_2 527-folds) toward MT_2. Replacement of the oxygen–carbon linker with an ethylene linker, compound **8**, displayed slightly reduced binding affinity toward MT_2 (K_i 0.55 nM) but increased binding affinity toward MT_1 (K_i 22.2 nM), thus decreasing selectivity (40-fold) toward MT_2. With a shorter methylene linker, compound **9** showed further reduction in binding affinity toward MT_2 (K_i 3.39 nM).

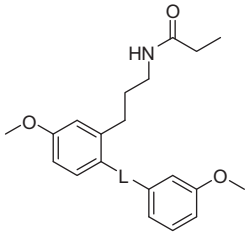
Table 1
Binding affinity of compound **1** and **7–10** towards human MT_1 and MT_2 receptors expressed in CHO cells



	–L–	K_i[a] (nM)		MT_1/MT_2
		MT_1	MT_2	
Melatonin		0.296	0.429	0.69
1	$-OCH_2-$	121	0.291	417
7	–C≡C–	38.2	0.073	527
8	$-CH_2CH_2-$	22.2	0.55	40
9	$-CH_2-$	39.5	3.39	12
10	—	32.7	0.339	96

[a] Test compound binding affinity was expressed as K_i (nM), while the ligand selectivity towards the two receptor subtypes was expressed as the MT_1/MT_2 K_i ratio. Data reported in the table were means of three trials done in duplicates. The corresponding K_i values were calculated using the mean pIC_{50} values.

Table 2
FLIPR dose–responses of compound **1** and **7–10** towards human MT_1 and MT_2 receptors expressed in CHO cells



Compound	–L–	EC_{50}[a] (nM)		MT_1/MT_2
		MT_1	MT_2	
1	$-OCH_2-$	9.03	0.05	181
7	–C≡C–	8.38	0.11	77
8	$-CH_2CH_2-$	16.3	0.024	679
9	$-CH_2-$	41.6	0.24	171
10	—	2.94	0.20	14

[a] Test compound potency was expressed as EC_{50} (nM), while the ligand selectivity towards the two receptor subtypes was expressed as the MT_1/MT_2 EC_{50} ratio. Data reported in the table were means of three or more experiments run at seven different concentrations in triplicates.

Interestingly, by removing the linker completely, compound **10** regained the binding affinity toward MT_2 (K_i 0.339 nM), providing higher MT_2 selectivity (96-fold). Overall, it is noteworthy that all of the novel compounds retained good potency toward MT_2 with single or sub-digit nanomolar binding affinities and compound **7** with a carbon–carbon triple bond linker displayed the highest potency and selectivity toward MT_2.

2.3. Functional assay

The intrinsic potency of these compounds was evaluated using Ca^{2+}-based FLIPR assays and the results are shown in Table 2. Two clonal cell lines expressing either hMT_1 (CHO–hMT_1) or hMT_2 (CHO–hMT_2) receptors with chimeric Gα protein 16z25 are efficiently coupled to the phospholipase Cβ/IP_3/Ca^{2+} pathway and triggered intracellular Ca^{2+} mobilization upon receptor activation.[12] The prototypic agonist, melatonin, activated both MT_1 and MT_2 in a non-selective manner with EC_{50} values of 0.16 ± 0.07 and 0.60 ± 0.10 nM, respectively. All compounds were tested with the assay system described above and appeared to be potent agonists toward MT_2 with MT_1/MT_2 selectivity ratio ranging from 14- to 679-folds. Comparing to compound **1** with an oxygen–carbon linker (EC_{50} 0.05 nM, selectivity 181), compound **7** with a carbon–carbon triple bond displayed similar potency toward MT_1 (EC_{50} 8.38 nM) and slightly reduced potency and selectivity toward MT_2 (EC_{50} 0.11 nM, selectivity 77-fold); compound **8** with an ethylene linker displayed better potency and selectivity toward MT_2 (EC_{50} 0.024 nM, selectivity 679-fold). Compound **9** with a methylene linker and compound **10** without a linker displayed similar MT_2 potency (EC_{50} ~0.2 nM), with compound **9** having higher MT_2 selectivity (171-fold). The most potent and selective compound toward MT_2 in this assay is compound **8** with an ethylene linker. Although compound **7** has a higher binding affinity than compound **8**, it was less efficacious in FLIPR assays. This discrepancy is probably due to differences in the compounds' intrinsic ability to induce conformational changes of the bound receptor. Despite their structural similarity, differences in the linker between the two phenyl rings in compounds **7** and **8** may result in distinct states of receptor conformation. The highly rigid alkyne-linkered diphenyl structure in compound **7** may limit the compound's molecular flexibility, leading to reduced efficacy. The lower intrinsic efficacy of compound **7** is thus manifested as a higher EC_{50} value in FLIPR assays.

2.4. Metabolic stability

The metabolic stability of compound **7–10** in rat and human liver microsomes was examined under the same experimental conditions as reported previously.[11] The in vitro half-life ($t_{1/2}$) was calculated according to the formula $t_{1/2} = -0.693/k$, where k is the slope of the linear regression of the natural logarithm of the parent remaining percentage versus incubation time. In vitro intrinsic clearance (Cl_{int}) was calculated from the $t_{1/2}$ value as reported previously and was used to predict the hepatic clearance. Compared with O-linkage structure **1**, compound **7** exhibited considerably improved stability with an in vitro half-life ($t_{1/2}$) of 21.7 and 18.3 min in rat and human microsomes, respectively. The in vitro intrinsic clearance (Cl_{int}) was 114.7 ml/min/kg in rat and 102.2 ml/min/kg in human for compound **7**. On the contrary, compounds **8, 9** and **10** showed no improvement in metabolic stability (Table 3). To investigate the reactions triggering the degradation of compounds **7–10**, attempts were made to identify the metabolites. Based on LC-UV/MS analysis, the principal biotransformation pathways of **7–10** were proposed to be demethylations of methoxyl moieties, hydroxylations on aromatic rings and combined reactions of demethylations and hydroxylations. No metabolite of carbon–carbon bond linker cleavage was found in metabolism medium, implying removal of carbon–oxygen ether linkage in original structure **1** successfully blocked the degradation in this site.

The major metabolites of compounds **7–10** were identified to be C3- or C3′-demethylated products and hydroxylated derivatives of aromatic rings. Previously SAR studies indicated that the key structural pharmacophores for both affinity and efficacy of melatonin receptor ligands are the presence and the relative spatial position of C3-methoxy group and *N*-alkylamido side chain.[7] Consequently, demethylation on C3 site would compromise the affinity of parent compound toward MT_2 receptor and generate metabolites with considerably reduced activity. In contrast, C3′-methoxy group plays a less important role for receptor binding. Compound without a substituent at C3′ also exhibited subnanomolar affinity to the MT_2 receptor.[10] Thus, metabolites derived from C3′-demethylation are expected to retain pharmacological activity. Hydroxylation on the aromatic ring is another principal metabolic

Table 3
Rat and human microsomal metabolic stability of compound **1** and **7**–**10**

Compound	RLM[a]		HLM[b]	
	$t_{1/2}$[c] (min)	Cl_{int}[d] (ml/min/kg)	$t_{1/2}$[c] (min)	Cl_{int}[d] (ml/min/kg)
Melatonin	75.3	33.1	73.2	25.5
1	9.5	262.3	5.5	339.8
7	21.7	114.7	18.3	102.2
8	8.3	300.6	5.1	366.7
9	8.6	290.1	6.0	311.7
10	7.5	332.6	5.3	352.8

[a] RLM, rat liver microsomes.
[b] HLM, human liver microsomes.
[c] $t_{1/2}$, calculated in vitro elimination half-life.
[d] Cl_{int}, in vitro intrinsic clearance.

pathway. The metabolites that retained structural characteristics necessary for binding to the MT_2 receptor might have a capacity to stimulate the MT_2 receptor.

3. Conclusion

In this study, we have described the successful synthesis and evaluations of novel *N*-[3-(3-methoxylphenyl)propyl] amides derivatives by modification of the metabolic liable benzyl ether linkage. Radioligand binding assay demonstrated that all of the new derivatives possessed high affinity toward MT_2 and moderate affinity toward MT_1. A carbon–carbon triple bond was found to be the best linkage in terms of binding affinity and selectivity toward MT_2. Calcium mobilization functional assay revealed that all of derivatives were potent agonists toward MT_2 with sub-digit nanomolar EC_{50} values. Compound **8** with an ethylene linker behaved as the most potent and selective agonist. Metabolic stability assays demonstrated that the initial benzyl ether cleavage was successfully blocked, and the major pathways involved combination of demethylations and hydroxylations of the phenyl rings for the new derivatives. Among them, compound **7** with a triple bond linker was found to have improved half-life compared to the original lead compound with an ether linker. These findings suggest that the modification of the ether linkage was successful in terms of retaining good potency and selectivity while improving metabolic stability. Although neither compound **7** or **8** is an ideal drug candidate yet, both compounds can serve as new lead compounds for further structure modifications. Strategies involving protection against demethylation and hydroxylation of the phenyl rings will be employed, and the results will be described in subsequent publications.

4. Experimentals

4.1. Chemistry

All reagents and starting materials were purchased from commercial sources and used without further purifications. The reaction condition and the yield are reported as it was and not optimized. ^{1}H NMR were recorded using a Varian 300 MHz spectrometer. Chemical shifts were measured in parts per million relative to tetramethylsilane as the internal standard. Coupling constants were measured in hertz.

4.1.1. 3-(2-Benzyloxy-5-methoxy-phenyl)-acrylonitrile (2)

To a flask charged with 2-hydroxy-5-methoxy-benzaldehye (3.0 g, 19.72 mmol) and benzyl bromide (2.8 mL, 23.66 mmol) in 80 mL THF under nitrogen, was added sodium hydride (60% in mineral oil, 1.26 g). The reaction was heated to 60 °C overnight. After aqueous workup and purification by column chromatography on silica gel, the desired product 2-benzyloxy-5-methoxy-benzaldehyde (4.79 g, 100%) was obtained as pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.5 (s, 1H), 7.35–7.42 (m, 5H), 7.33 (d, J = 3.2 Hz, 1H), 7.10 (dd, J = 8.8 Hz, 3.2 Hz, 1H), 6.99 (d, J = 8.8 Hz, 1H), 5.13 (s, 2H), 3.78 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 189.2, 155.6, 153.6, 136.1, 128.5 (2), 128.1, 127.2 (2), 125.3, 123.3, 114.9, 110.1, 71.2, 55.7.

To a dried flask charged with sodium hydride (60% in mineral oil, 594 mg) in 60 mL THF at 0 °C under nitrogen, was added diethyl cyanomethyl phosphonate (2.1 mL, 13.62 mmol) dropwise. After warmed up to room temperature for 30 min, 2-benzyloxy-5-methoxy-benzaldehyde (3.0 g, 12.38 mmol) in 5 mL THF was added via cannulation. The reaction was stirred at room temperature for 1 h and quenched with water. Aqueous workup and purification by column chromatography on silica gel provided the desired products (3.14 g, 95% yield) as mixtures of cis- and trans isomers. ESI-MS: 266.14 (M+1) cis-isomer: ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.74 (d, J = 2.4 Hz, 1H), 7.61 (d, J = 12.4 Hz, 1H), 7.33–7.39 (m, 5H), 6.90–6.96 (m, 2H), 5.39 (d, J = 12.0 Hz, 1H), 5.04 (s, 2H), 3.82 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 153.5, 151.0, 143.2, 136.4, 128.5 (2), 128.0, 127.3 (2), 123.5, 118.8, 117.6, 114.0, 111.9, 94.7, 71.3, 55.8. trans-Isomer: ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.60 (d, J = 16.8 Hz, 1H), 7.37 (m, 5H), 6.89 (m, 3H), 5.96 (d, J = 16.8 Hz, 1H), 5.03 (s,1H), 3.74 (s, 3H).

4.1.2. 3-(2-Benzyloxy-5-methoxy-phenyl)-propylamine (3)

To a solution of 3-(2-benzyloxy-5-methoxy-phenyl)-acrylonitrile (3.87 g, 15.57 mmol) in 140 mL diethyl ether under nitrogen, was added lithium borohydride (1.66 g, 43.73 mmol). The resulted mixture was heated to reflux overnight. The reaction was cooled to room temperature and quenched with 2.1 mL water dropwise. Another 3.2 mL aqueous sodium hydroxide (10%) and 5.5 mL water were added while stirring. After the gray solid turned white, the mixture was filtered through a plug of sodium sulfate and rinsed with a mixture of methanol/dichloromethane. The filtrate was concentrated and purified by column chromatography on silica gel to give the desired product (2.58 g, 65%) as pale yellow oil. ESI-MS: 272.08 (M+1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.31–7.43 (m, 5H), 6.82 (d, J = 8.8 Hz, 1H), 6.75 (d, J = 2.8 Hz, 1H), 6.67 (dd, J = 8.4, 2.8 Hz, 1H), 5.02 (s, 2H), 3.75 (s, 3H), 2.66–2.71 (m, 4H), 1.71–1.79 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 153.5, 150.6, 137.4, 132.1, 128.4 (2), 127.6, 127.0 (2), 116.1, 112.8, 110.8, 70.7, 55.6, 41.8, 34.0, 27.6.

4.1.3. *N*-[3-(2-Benzyloxy-5-methoxy-phenyl)-propyl]-propionamide (4)

To a solution of 3-(2-benzyloxy-5-methoxy-phenyl)-propylamine (0.70 g, 2.58 mmol) in 13 mL dichloromethane, was added triethylamine (0.65 mL, 4.64 mmol) followed by propionyl chloride (0.34 mL, 3.87 mmol). The reaction was stirred at room temperature for 1 h and quenched with water. After aqueous workup and purification by column chromatography on silica gel, eluting with 50% ethyl acetate in petroleum ether, the desired product (0.79 g, 94%) was obtained as a white solid.

Mp: 66–68 °C. HR-TOF-MS: Calcd for $C_{20}H_{26}NO_3$ (M+1) 328.1913. Found: 328.1915. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35–7.43 (m, 5H), 6.87 (d, J = 8.8 Hz, 1H), 6.70 (s, 1H), 6.71 (d, J = 8.4 Hz, 1H), 5.60 (s, 1H), 5.00 (s, 2H), 3.76 (s, 3H), 3.18 (m, 2H), 2.67 (t, J = 7.0 Hz, 2H), 1.75–1.86 (m, 4H), 1.00 (t, J = 7.6 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.5, 153.8, 150.6, 137.0, 131.4, 128.6(2), 128.0, 127.6(2), 116.2, 112.9, 111.3, 71.1, 55.6, 38.2, 30.1, 29.6, 27.2, 10.1.

4.1.4. *N*-[3-(2-Hydroxy-5-methoxy-phenyl)-propyl]-propionamide (5)

To a solution of *N*-[3-(2-benzyloxy-5-methoxy-phenyl)-propyl]-propionamide (0.47 g, 1.44 mmol) in 10 mL methanol, was added 5% palladium on carbon (0.12 g). The resulted mixture was stirred

under hydrogen atmosphere (balloon pressure) for 1 day. After filtered through a plug of celite, the filtrate was concentrated. Purification by column chromatography on silica gel provided the desired product (0.32 g, 92%) as a white solid. Mp: 83–84 °C. ESI-MS 224.07 (M+1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.15 (s, 1H), 6.77 (d, J = 8.8 Hz, 1H), 6.56–6.61 (m, 3H), 3.69 (m, 3H), 3.20 (q, J = 6.2Hz, 2H), 2.61 (t, J = 6.8 Hz, 2H), 2.16 (q, J = 7.6 Hz, 2H), 1.76 (m, 2H), 1.09 (t, J = 7.6 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.6, 152.8, 148.3, 128.6, 115.9, 115.6, 111.8, 55.6, 38.6, 29.8 (2), 27.0, 10.0.

4.1.5. *N*-[3-(2-Trifluoromethylsulfonyl-5-methoxy-phenyl)-propyl]-propionamide (6)

To a solution of *N*-[3-(2-hydroxy-5-methoxy-phenyl)-propyl]-propionamide (279 mg, 1.18 mmol) in 6 mLdry dichloromethane under N_2 at 0 °C, was added triethylamine (0.43 mL, 3.06 mmol), followed by trifluoroacetic anhydride (0.26 mL, 1.65 mmol) dropwise. The reaction was allowed to warm up to room temperature and stir for 3 h. Water was added and the mixture was extracted with dichloromethane (×3). The combined organic extract was dried, filtered and concentrated. Purification by column chromatography on silica gel recovered starting material 56 mg (20%) and provided the desired product (186 mg, 0.50 mmol, 43%). ESI-MS: 370.14 (M+1).

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.13 (d, J = 9.0 Hz, 1H), 6.83 (d, J = 2.8 Hz, 1H), 6.77 (dd, J = 8.9 Hz, 2.8 Hz, 1H), 5.70 (br s, 1H), 3.81 (s, 3H), 3.32 (q, J = 6.4 Hz, 2H), 2.70 (t, J = 7.6 Hz, 2H), 2.20 (q, J = 7.6 Hz, 2H), 1.85 (m, 2H), 1.15 (t, J = 7.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 174.1, 159.0, 141.3, 135.6, 122.2, 115.9, 112.8, 55.6, 38.7, 29.7, 29.6, 27,6, 9.7 and 124.8, 120.6, 116.4, 112.1 (quartet for CF3).

4.1.6. *N*-{3-[5-Methoxy-2-(3-methoxy-phenylethynyl)-phenyl]-propyl}-propionamide (7)

To a two-necked flask attached with a condenser, was added *N*-[3-(2-trifluoromethylsulfonyl-5-methoxy-phenyl)-propyl]-propionamide (47 mg, 0.127 mmol), $Pd(PPh_3)_4$ (14 mg, 0.012 mmol), CuI (7 mg, 0.037 mmol) and nBu$_4$NI (66 mg, 0.18 mmol). And the flask was degassed and filled with N_2 three times. A pre-degassed mixture of triethylamine and DMF (1:5, 1.5 mL) was added, followed by 1-ethynyl-3-methoxy-benzene (0.064 mL, 0.51 mmol). The mixture was heated to 70 °C for 24 h. The reaction was stopped and the solvent was evaporated under reduced pressure. Water was added and the mixture was extracted with dichloromethane (×3). The combined extract was dried, filtered and concentrated. Purification by column chromatography on silica gel, eluting with 20–25% acetone in petroleum ether, provided the desired product (24 mg, 0.068 mmol, 53%) together with starting material (22 mg, 46% recovery). ESI-MS: 352.29 (M+1). TOF-HRMS Calcd for $C_{22}H_{26}NO_3$ $(M+H)^+$ 352.1907. Found: 352.1907. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.45 (d, J = 8.2Hz, 1H), 7.26 (t, J = 8.4 Hz, 1H), 7.11 (d, J = 7.8 Hz, 1H), 7.04 (s, 1H), 6.89 (d, J = 8.4 Hz, 1H), 6.73–6.77 (m, 2H), 5.52 (br s, 1H), 3.83 (s, 3H), 3.82 (s, 3H), 3.30 (m, 2H), 2.88 (t, J = 7.3Hz, 2H), 2.03 (q, J = 7.6 Hz, 2H), 1.92 (m, 2H), 1.06 (t, J = 7.6 Hz, 3H).

^{13}C NMR (75 MHz, $CDCl_3$): δ 173.8, 159.9, 159.4, 145.4, 133.6, 129.5, 124.5, 123.9, 116.2, 114.6, 114.5 (2), 111.8, 91.5, 88.1, 55.3 (2), 38.8, 32.0, 30.4, 29.7, 9.9.

4.1.7. *N*-(3-{5-Methoxy-2-[2-(3-methoxy-phenyl)-ethyl]-phenyl}-propyl)-propionamide (8)

To a flask containing *N*-{3-[5-methoxy-2-(3-methoxy-phenylethynyl)-phenyl]-propyl}-propionamide (13 mg, 0.037 mmol), was added methanol (1 mL) and 10% palladium on carbon (5 mg). The mixture was degassed three times and stirred under hydrogen (1 atm) at room temperature overnight. The reaction was stopped and the mixture was filtered through a plug of celite. Purification by column chromatography on silica gel, eluting with 20% acetone in petroleum ether, provided the desired product (12 mg, 0.034 mmol, 91%). ESI-MS: 356.38 (M+1). TOF-HRMS Calcd for $C_{22}H_{30}NO_3$ $(M+H)^+$ 356.2220. Found: 356.2216. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.20 (t, J = 7.7 Hz, 1H), 7.08 (d, J = 9.3Hz, 1H), 6.70–6.78 (m, 5H), 5.46 (br s, 1H), 3.78 (s, 6H), 3.29 (q, J = 6.6 Hz, 2H), 2.82 (s, 4H), 2.58 (t, J = 7.8 Hz, 2H), 2.15 (q, J = 7.6 Hz, 2H), 1.74 (m, 2H), 1.12 (t, J = 7.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.7, 159.6, 157.9, 143.5, 140.5, 131.5, 130.2, 129.3, 120.8, 114.7, 114.2, 111.4, 111.1, 55.2, 55.1, 39.3, 37.8, 33.7, 30.8, 30.2, 29.7, 9.9.

4.1.8. *N*-{3-[5-Methoxy-2-(3-methoxy-benzyl)-phenyl]-propyl}-propionamide (9)

To a dry flask under N_2, was added zinc powder (131 mg, 2.0 mmol), DMF (0.4 mL) and 1,2-dibromoethane (0.014 mL, 0.08 mmol). The mixture was heated to 70 °C for 10 min, and cooled to room temperature. TMSCl (0.015 mL, 0.06 mmol) was added and the reaction was stirred for 30 min. After the mixture was cooled to 0 °C, a solution of 3-methoxybenzyl bromide (0.28 mL, 2.0 mmol) in 4 mL DMF was added dropwise in 2 h. After stirring for another 2 h, the zinc reagent was ready for use. To a flask, was added *N*-[3-(2-trifluoromethylsulfonyl-5-methoxy-phenyl) -propyl]-propionamide (36 mg, 0.097 mmol), $Pd(PPh_3)_4$ (8 mg, 0.0069 mmol). The flask was degassed three times and filled with N_2. A solution of the freshly prepared zinc reagent (1.5 mL) was added and the mixture was heated to 70 °C for 24 h. The reaction was stopped and the solvent was removed under reduced pressure. A solution of 10% HCl (~10 mL) was added and the mixture was extracted with dichloromethane (×3). The combined extract was dried, filtered and concentrated. The resulting residue was purified by column chromatography on silica gel, eluting with 10% acetone in petroleum ether to give the desired product (14 mg, 0.041 mmol, 42%). ESI-MS: 342.21 (M+1). TOF-HRMS Calcd for $C_{21}H_{28}NO_3$ $(M+H)^+$ 342.2064. Found: 342.2045. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.06 (t, J = 7.0 Hz, 1H), 6.71 (d, J = 8.8 Hz, 1H), 6.64–6.68 (m, 5H), 5.20 (br s, 1H), 3.92 (s, 2H), 3.79 (s, 3H), 3.75 (s, 3H), 3.22 (q, J = 6.5 Hz, 2H), 2.56 (t, J = 7.8 Hz, 2H), 2.12 (q, J = 7.5 Hz, 2H), 1.69 (m, 2H), 1.11 (t, J = 7.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.7, 159.7, 158.4, 143.0, 141.2, 131.7, 130.2, 129.3, 120.9, 115.0, 114.5, 111.2, 111.0, 55.2, 55.1, 39.2, 38.3, 31.9, 30.3, 29.4, 9.9.

4.1.9. *N*-[3-(4,3′-Dimethoxy-biphenyl-2-yl)-propyl]-propionamide (10)

To a flask, was charged with *N*-[3-(2-trifluoromethylsulfonyl-5-methoxy-phenyl)-propyl]-propionamide (30 mg, 0.081 mmol) and 3-methoxyphenylboronic acid (18.5 mg, 0.122 mmol). After the flask was degassed and filled with N_2 three times, toluene (0.6 mL) and aqueous sodium carbonate solution (2 M, 0.2 mL) were added, followed by $Pd(PPh_3)_4$ (9 mg, 0.0081 mmol). The mixture was degassed and filled with nitrogen again, and heated to 80 °C for overnight. After the reaction was stopped, the mixture was extracted with ethyl acetate (×3). The combined extract was dried, filtered and concentrated. Purification by column chromatography on silica gel, eluting with 20–25% acetone/petroleum ether, provided the desired product (10 mg, 0.03 mmol, 38%). ESI-MS: 328.31 (M+1). TOF-HRMS Calcd for $C_{20}H_{26}NO_3$ $(M+H)^+$ 328.1907. Found: 328.1900. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.32 (t, J = 7.9 Hz, 1H), 7.14 (d, J = 8.2Hz, 1H), 6.78–6.91 (m, 5H), 5.00 (br s, 1H), 3.84 (s, 3H), 3.93 (s, 3H), 3.09 (q, J = 6.3Hz, 2H), 2.64 (t, J = 7.5 Hz, 2H), 2.02 (q, J = 7.6 Hz, 2H), 1.61–1.70 (m, 2H), 1.06 (t, J = 7.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): □173.6, 159.3, 159.0, 143.0, 140.3, 134.3, 131.0, 129.2, 122.0, 115.3, 114.7, 112.1, 111.3, 55.3, 55.2, 38.6, 31.0, 30.2, 29.6, 9.8.

4.2. Radioligand binding assay

CHO cells stably expressing human MT_1 or MT_2 receptor have been described and characterized previously.[13] Competitive

binding assays were performed as described[14,15] with modifications for intact cells. Briefly, 1.5×10^5 cells were suspended in binding buffer (50 mM Tris, 2 mM $MgCl_2$, 1 mM EGTA, pH 7.4) containing 1 nM [^{3}H]melatonin and increasing concentrations of a test compound. Assays were carried out at 4 °C for 60 min with occasional agitation and then terminated by rapid filtration through GF/C filters pre-soaked in 10 mM Tris, pH 7.4. Bound radioactivity was counted in Wallac 1450 Microbeta Jet scintillation counter. Competitive curves were fitted using a one-site competition nonlinear regression (GraphPad Prism 3.03). Data were means of 2–3 independent experiments performed in duplicates. Standard errors were typically within 10% of the mean value. Melatonin was employed as standard reference in every assay with reproducible K_i. K_i values were calculated using the Cheng–Prusoff equation.

4.3. Functional assay

Fluorometric assay was performed using two clonal cell lines with stable expression of both melatonin receptor and chimeric Gα protein 16z25 to measure intracellular Ca^{2+} mobilization.[16] Cells in normal growth medium were seeded into clear-bottomed black-walled 96-well plates a day before assay. Cells in each well were labeled with 2 μM Fluo-4 (Invitrogen) in 200 μl of Hank's balanced salt solution (pH 7.5) containing 2.5 mM probenecid for 1 h at 37 °C prior to the addition of test compounds or melatonin. Changes in fluorescence upon the addition of test compounds or melatonin were detected in the fluorometric imaging plate reader FLIPR^{TETRA}™ (Molecular Devices/MDS Analytical Technologies) with an excitation wavelength of 488 nM. The real-time fluorescent signal was monitored for 3 min. Results were expressed as changes in relative fluorescence units (RFU). Concentration-response curves were generated by determining the maximal change in RFU of each data set. Numerical analysis of the statistics and EC_{50} (median effective concentration) values were performed on GraphPad Prism version 3.03.

4.4. Metabolic stability evaluation

In vitro metabolism was conducted in a system consisted of 50 μl of NADPH generating system (1 mM NADP, 1 mM NADH, 1 mM glucose-6-phosphate, 2 unit/ml glucose-6-phosphate dehydrogenase and 4 mM $MgCl_2$), 5 μl of 1 mM test compound and 425 μl Tris·Cl buffer (50 mM, pH 7.4). The mixture was pre-incubated at 37 °C for 30 min. Reactions were initiated by adding 12.5 μl rat/human liver microsomal suspensions (20 mg protein/ml) and shaken at 37 °C for 30 min with air exposure, and were subsequently terminated by adding 2 ml ice-cold dichloromethane containing internal standards. After extraction by shaking the sample tube and centrifugation at 3000 rpm for 5 min, the organic phase was transferred to a new tube and evaporated under N_2. The residue was reconstituted in 100 μl methanol, and analyzed by HPLC–DAD/MS system for evaluating stability and identifying metabolites.

Sample analysis was carried out using a HPLC system equipped with a Waters 600 controller quaternary pump, Waters 717 plus autosampler injector and Waters 2996 photodiode array detector (Waters Products, Milford, MA, USA). A reverse phase Water Sunfire C18 column (4.6 × 150 mm, 5 μM) was used as an analytical column. The UV detection wavelength was 290 nM for compound **7** and 220 nM for other compounds. The mobile phase consisted of 55% acetonitrile and 45% water. The flow-rate was 1 ml/min. The quantification methods were validated by determining linearity, accuracy and inter-/intra-day precise.

The metabolite identification was performed using a HPLC system coupled with an online Thermo-Finnigan LCQ Classic ion trap mass spectrometer equipped with electrospray ionization source. The separation of metabolites and parents were carried out by a Waters Sunfire C18 column (4.6 × 150 mm, 5 μM). Mobile phase consisted of acetonitrile and water and was delivered in a gradient program at the flow-rate 0.4 ml/min: 0 min, 40% ACN; 19 min, 40% ACN; 30 min, 60% ACN. For MS detection, positive mode was employed for the identification of metabolites. The major working parameters for mass spectrometer were as follows: sheath gas N_2 flow at 60 arbitrary units, auxiliary gas N_2 flow at 40 arbitrary units, ion-spray voltage 4.5 kV, capillary temperature at 200 °C. The metabolites were identified by MS full scan mode (m/z 100–1000) and CID-MS spectra were produced by collision-induced dissociation of each molecular ion of interest, using normalized collision energy of 20–50%.

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Supplementary data

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References and notes

1. Arendt, J. *J. Biol. Rhythm* **2005**, *20*, 291.
2. Samel, A. *Eur. J. Med. Res.* **1999**, *4*, 385.
3. Delagrange, P.; Boutin, J. *Chronobiol. Int.* **2006**, *23*, 413.
4. Olakowska, E.; Marcol, W.; Kotulska, K.; Lewin-Kowalik, J. *Bratisl. Lek. Listy* **2005**, *106*, 171.
5. Ekmekcioglu, C. *Biomed. Pharmacother.* **2006**, *60*, 97.
6. Mor, M.; Rivara, S.; Pala, D.; Bedini, A.; Spadoni, G.; Tarzia, G. *Expert Opin. Ther. Pat.* **2010**, *20*, 1059.
7. Spadoni, G.; Bedini, A. *Melatonin: From Molecules to Therapy*; Pandi-Perumal, S. R., Cardinali, D. P., Eds.; Nova Science: New York, 2007; p 33.
8. Mesangeau, C.; Fraise, M.; Delagrange, P.; Caignard, D. H.; Boutin, J. A.; Berthelot, P.; Yous, S. *Eur. J. Med. Chem.* **2011**, *46*, 1835.
9. Koike, T.; Hoashi, Y.; Takai, T.; Nakayama, M.; Yukuhiro, N.; Ishikawa, T.; Hirai, K.; Uchikawa, O. *J. Med. Chem.* **2011**, *54*, 3436.
10. Hu, Y. Q.; Ho, M. K. C.; Chan, K. H.; New, D. C.; Wong, Y. H. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 2582.
11. Zhu, J.; Hu, Y. Q.; Ho, M. K. C.; Wong, Y. H. *Xenobiotica* **2011**, *41*, 35.
12. Liu, A. M. F.; Ho, M. K. C.; Wong, C. S. S.; Chan, J. H. P.; Pau, A. H. M.; Wong, Y. H. *J. Biomol. Screen.* **2003**, *8*, 39.
13. New, D. C.; Wong, Y. H. *Assay Drug Dev. Technol.* **2004**, *2*, 269.
14. Ho, M. K. C.; New, D. C.; Wong, Y. H. *Neurosignals* **2002**, *11*, 115.
15. Tian, Y.; New, D. C.; Yung, L. Y.; Allen, R. A.; Slocombe, P. M.; Twomey, B. M.; Lee, M. M. K.; Wong, Y. H. *Eur. J. Immunol.* **2004**, *34*, 785.
16. Liu, A.; Ho, M.; Wong, C.; Chan, J.; Pau, A.; Wong, Y. *J. Biomol. Screen.* **2003**, *8*, 39.