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High-throughput screening assay for new ligands at human melatonin receptors[1]

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Key words

melatonin; receptor; selectivity; agonist; antagonist

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Abstract

Aim: Melatonin (MT) is a neurohormone produced and secreted primarily by the pineal gland in a circadian manner, and mainly acts through 2 receptor subtypes: MT_1 and MT_2 in humans. The diversity in their tissue distribution is in favor of different functions for each receptor subtype. Selective modulators are therefore required to determine the physiological roles of these melatonin receptor subtypes and their implications in pathological processes. **Methods:** A homogenous MT_1/MT_2 receptor binding assay was established for high-throughput screening of new ligands at the hMT_1 and/or hMT_2 receptors. The functional properties (agonists or antagonists) were assessed by a conventional guanosine-5'[γ-^{35}S] triphosphate (GTP-γS) assay. **Results:** Three hMT_1 receptor-selective small molecule antagonists and 1 hMT_2 receptor-selective small molecule antagonist with novel structural features were identified following a high-throughput screening campaign of 48 240 synthetic and natural compounds. **Conclusion:** The findings may assist in the expansion of chemical probes to these 2 receptor subtypes.

Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine, MT) is a neurohormone synthesized and secreted primarily by the pineal gland in a circadian manner, with peak levels in all species occurring during the period of darkness. The synthesis of melatonin within the pineal gland is mainly regulated by the daily and seasonal changes in the environmental light/dark cycle. Melatonin, which is released into the blood circulation, is thought to transduce photoperiodic information to all the tissues expressing its receptors or other binding sites[1]. After its discovery and chemical characterization in 1959[2], many different physiological and behavioral responses have been ascribed to melatonin. It not only regulates the sleep/wake cycle[3], but also affects cardiovascular[4], reproductive[5], cell growth[6], and retinal processes[7].

The discovery of different melatonin receptors was facilitated by the introduction of the agonist radioligand 2-[^{125}I]iodomelatonin[8]. Two human melatonin receptor subtypes, hMT_1 and hMT_2, have been cloned and shown to be G-protein-coupled receptors (GPCR)[9–11]. MT_3, a third melatonin binding site, has been subsequently described in hamsters as the human homolog of the cytoplasmic quinone reductase-2[12,13]. The homology between hMT_1 and hMT_2 receptors at the amino acid level is 55% with approximately 70% overall identity within the transmembrane domains. The activation of these 2 receptor subtypes leads to the inhibition of adenyl cyclase, probably through the activation of a G_i-protein[9,10]. Studies of their tissue distribution in mammals have shown that these receptors are localized in different areas of the brain (suprachiasmatic nucleus, pars tuberalis, hypothalamus, cerebellum, cortex, hippocampus, and cerebral vessels), and at the peripheral level in the kidney, small intestine, and caudal arteries[9,10,14–19]. The differences in tissue distribution are in favor of different functions for each receptor subtype. Pharmacological investigations with selective agonists or antagonists are therefore necessary to determine the physiological roles of these melatonin

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receptor subtypes and their implications in pathological processes. This information will probably open new therapeutic perspectives for selective ligands, different from the chronobiotic properties of melatonin clearly demonstrated in humans[20,21].

Scintillation proximity assay (SPA) technology is a homogeneous approach that does not involve post-reaction liquid handling steps and is well-suited to automation and high-throughput screening (HTS). In the SPA system, membranes that express a particular receptor are attached to a microbead coated with wheat germ agglutinin. A radioisotope atom (eg [^{125}I]) is brought very close to a scintillant-impregnated microbead by binding to the receptor. Because the emitted β particles can only travel short distances in the bulk solution, the microbeads preferentially capture electrons from the bound radiolabeled ligand. Thus, the amount of light emitted from the scintillant in the microbeads is directly proportional to the amount of bound radiolabeled ligand[22].

In this study, we screened a library of 48 240 synthetic (91.2%) and natural compounds (8.8%) made in house, collected from various research institutions across China or purchased from commercial sources, with the goal of finding novel selective and potent hMT_1 and/or hMT_2 receptor agonists and antagonists. Using SPA technology, binding affinities were determined with 2-[^{125}I] iodomelatonin and their functional activities by guanosine-5'[γ-^{35}S]triphosphate ([^{35}S]-GTP-γS) binding assay[23]. As a result, we discovered a series of selective antagonists for hMT_1 and hMT_2 receptors.

Materials and methods

Reagents The radioligand 2-[^{125}I]iodomelatonin (specific activity: 81.4 TBq/mmol) and FlashBlue GPCR beads were purchased from PerkinElmer (Boston, MA, USA). [^{35}S]GTP-γS (specific activity: 37 TBq/mmol) was obtained from Amersham Biosciences (Buckinghamshire, England). Guanosine diphosphate (GDP), GTP-γS, saponin, bovine serum albumin (BSA), HEPES, and melatonin were from Sigma–Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS) was bought from Hyclone (Logan, UT, USA), and HamF12 medium was purchased from GIBCO BRL (Rockville, ML, USA).

Membrane preparation Chinese hamster ovary (CHO) cells stably expressing hMT_1 or hMT_2 receptors were provided by Servier (Neuilly-sur-Seine, France)[24]. The cells were cultured in HamF12 medium supplemented with 10% FBS, 2 mmol/L glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. They were grown at confluence, harvested in phosphate-buffered saline, and centrifuged at 1000×*g* for 5 min (4 °C). The resulting pellet was suspended in isotonic buffer (5 mmol/L Tris/HCl, 0.2 mmol/L $MgCl_2$, and 0.25 mol/L sucrose, pH 7.4), and homogenized using the BioNeb Cell Disruption System (Terre Haute, IN, USA). The homogenate was then centrifuged (20 000×*g* for 30 min at 4 °C), and the resulting pellet was suspended in binding buffer 1 (50 mmol/L Tris/HCl, pH 7.4, 5 mmol/L $MgCl_2$, 1 mmol/L EDTA). The protein content was determined using the Bradford assay[25]. Aliquots of membrane preparations were stored at –80 °C until use.

Homogenous binding assay The membranes were incubated overnight in binding buffer 1 containing 18 pmol/L 2-[^{125}I]iodomelatonin, 0.75 mg/mL FlashBlue GPCR beads, various titrations of melatonin from a stock solution of 40 μmol/L, and library compounds with an average concentration of 6.7 μmol/L (final volume: 100 μL) Non-specific binding was defined with 1 μmol/L melatonin. Data were analyzed with GraphPad PRISM (GraphPad Software, San Diego, CA, USA). The competitive inhibition constant (K_i) was calculated according to the Cheng–Prusoff equation: $K_i=IC_{50}/(1+L/K_d)$, where IC_{50} is the concentration that produced 50% inhibition, K_d is the dissociation constant of inhibitor-binder reaction, and L is the concentration of the radiolabeled ligand used[26].

[^{35}S]GTP-γS binding assay The membranes and test compounds were diluted in binding buffer 2 (20 mmol/L HEPES, pH 7.4, 100 mmol/L NaCl, 3 mmol/L $MgCl_2$, 12 μmol/L GDP, and 0.1% BSA) in the presence of 20 μg/mL saponin in order to enhance the agonist-induced stimulation level[27], and pre-mixed with 1 mg/mL FlashBlue GPCR beads. For the agonist test, incubation was started by the addition of 0.2 nmol/L [^{35}S]GTP-γS to the membranes and test compounds, and continued for 4 h at room temperature in a final volume of 100 μL. To study the antagonist activity, the membranes were pre-incubated for 2 h with melatonin (30 nmol/L) in conjunction with a given concentration of a test compound. Reaction begun with the addition of 0.2 nmol/L [^{35}S]GTP-γS followed by 4 h incubation at room temperature. Non-specific binding was assessed using non-radiolabeled GTP-γS (10 μmol/L). Data were analyzed with PRISM (GraphPad, USA) to calculate the 50% effective concentration (EC_{50}) and the maximal effect expressed as a percentage of that observed with 1 μmol/L melatonin, 100% (E_{max}) for agonists. K_B was used to describe antagonist potency: $K_B=IC_{50}/(1+[agonist]/EC_{50})$, where IC_{50} is the antagonist concentration that gives

50% inhibition of [^{35}S]GTP-γS binding in the presence of a fixed concentration of agonist, and EC_{50} is the 50% effective concentration of an agonist when tested alone[26]. The maximal inhibitory effect (I_{max}) was expressed as a percentage of that observed with 30 nmol/L melatonin for hMT_1 or hMT_2 receptors.

Results

HTS campaign In the validation process, various assay parameters were studied. The optimal concentrations of radioligand and microbeads were 18 pmol/L and 0.75 mg/mL, respectively, for both receptor subtypes, while protein concentrations were optimized to 2.6 μg/mL for the hMT_1 receptor and 0.9 μg/mL for the hMT_2 receptor, respectively (data not shown). The control compound, melatonin, was tested at both receptors to evaluate the system. As shown in Figure 1, the K_i values of melatonin were 0.87 nmol/L for the hMT_1 receptor and 2.07 nmol/L for the hMT_2 receptor, respectively, compatible to that reported in the literature[24]. In order to apply the assay to a HTS format, the Z′ value and the signal/background (S/B; ie specific *vs* non-specific binding) were studied. The Z′ factor, a screening window coefficient, is reflective of both the assay signal dynamic range and the data variation associated with the signal measurements. Therefore, it is suitable for the assay quality assessment[28]. As shown in Figure 2, the Z′ value for the hMT_1 receptor was 0.81 with a S/B ratio of 43.2 and a coefficient of variation value of 5.7%. In the case of the hMT_2 receptor, the corresponding values were 0.76, 9.08, and 5.4%, respectively. These characteristics suggest that the 2 assay systems are of high quality and suitable for HTS[28]. The number of hits at different inhibition rates discovered from a parallel HTS campaign directed towards hMT_1 and hMT_2 receptors are shown in Figure 3. Of the 48 240 samples screened (which did not include 4 P-PDOT and other 17 ligands known to react with hMT_1 and hMT_2 receptors[24]), 205 and 199 initial hits were identified for hMT_1 and hMT_2 receptors (≥85% binding activities), respectively. Among them, 63 (hMT_1) and 54 (hMT_2) hits were subsequently confirmed

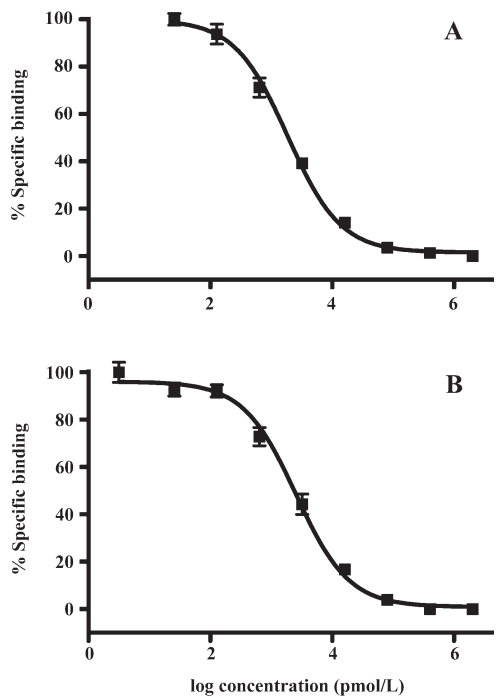

Figure 1. Displacement of 2-[^{125}I]iodomelatonin binding to hMT_1 receptor- (A) or hMT_2 receptor-expressing CHO cell membrane preparation (B) by melatonin. Data shown are mean±SD of triplicate measurements and representative of at least 3 independent experiments.

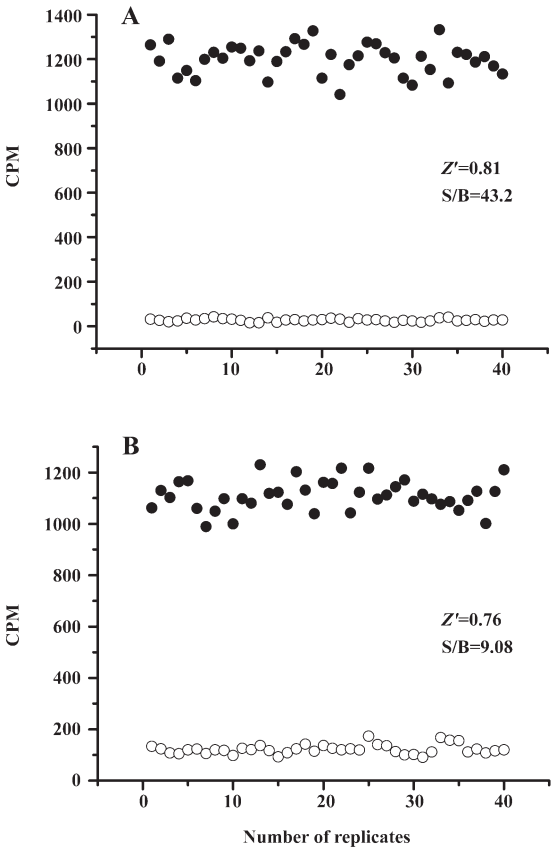

Figure 2. Z′ value and S/B ratio determination. Homogenous hMT_1 (A) or hMT_2 (B) receptor binding assay was performed at optimized conditions with respective receptor-expressing CHO cells, 2-[^{125}I] iodomelatonin (18 pmol/L) and melatonin (1 μmol/L). Background is the radioactivity counts in 2.5% DMSO solution where melatonin is dissolved. Forty replicates of S/B readouts were studied.

and subjected to IC_{50} determination, and the values of these confirmed hits were within the range of dozens of nmol/L to a few μmol/L for both hMT_1 and hMT_2 receptors (Table 1 and data not shown).

Functionality characterization In order to examine the pharmacological properties (agonist or antagonist) of these compounds at each melatonin receptor subtype, [^{35}S]GTP-γS binding assays were performed. For both subtypes, the optimal concentrations of saponin, $MgCl_2$,, and GDP were 20 μg/mL, 3 mmol/L and 12 μmol/L, respectively (data not shown), similar to that reported previously[24]. The agonist activities of melatonin at hMT_1 (EC_{50}=1.91 nmol/L) and hMT_2 receptors (EC_{50}=1.37 nmol/L) are shown in Figure 4. Based on the novelty in molecular structures and potency in binding activities, we selected 18 confirmed hits identified from the HTS campaign for simultaneous functionality studies with both receptor subtypes. As shown in Table 1, melatonin acted as a full agonist for both hMT_1 and hMT_2 receptors. Of the 18 compounds, NC00090702, NC00096199, and NC00099763 displayed selective hMT_1 receptor antagonist activities with K_B values of 0.26, 0.50, and 1.06 μmol/L, respectively; NC00080421 exhibited selective hMT_2 receptor antagonist activities with a relatively more potent K_B value of 0.15 μmol/L. The chemical structures of the 4 selective antagonists, all obtained from a commercial source (Specs, Delft, the Netherlands), are presented in Figure 5. Figure 6 depicts both the agonist and antagonist features of these compounds, as defined by E_{max} and K_B/I_{max}. Apart from NC00080421, which demonstrated some partial agonist activities (at the hMT_1 receptor in particular), these antagonists are highly selective for a particular receptor subtype.

Discussion

SPA is an innovative approach to assay development and biochemical screening that allows the rapid and sensitive measurement of a wide variety of molecular interactions in a homogeneous system. In the present study, we employed this technology in the HTS of 48 240 samples against 2 melatonin receptor subtypes: hMT_1 and hMT_2. Not only did it show strong reliability in terms of consistent EC_{50} values measured for the control ligand (melatonin) and assay parameters exemplified by the high Z' values (0.81 for the hMT_1 receptor and 0.76 for the

Table 1. Binding properties and functionality characteristics of 18 confirmed hits at two melatonin receptor subtypes (MT_1 and MT_2).

Compound	Receptor binding K_i (nmol/L)		GTPγS assay hMT_1				GTPγS assay hMT_2				Structural feature
	hMT_1	hMT_2	EC_{50} (nmol/L)	E_{max} (%)	K_B (nmol/L)	I_{max} (%)	EC_{50} (nmol/L)	E_{max} (%)	K_B (nmol/L)	I_{max} (%)	
Melatonin	0.87	2.07	1.91	100	NA	NA	1.37	100	NA	NA	Indole
NC00004068	43	61	101	85	NA	24	98	71	NA	<10	Indole
NC00076880	56	21	NA	<10	463	62	$>10^4$	47	NA	33	Thieno[2,3-d]pyrimidine
NC00080421	238	348	NA	22	6324	75	NA	11	153	71	Tetrahydroisoquinoline
NC00085323	1573	ND	NA	<10	15401	128	NA	<10	9028	119	Benzenesulfonamide
NC00085862	ND	34	NA	<10	$>10^4$	57	62	130	NA	<10	Naphthalene
NC00085864	79	ND	NA	12	514	83	442	78	NA	<10	Naphthalene
NC00085892	475	ND	727	58	NA	33	300	97	NA	<10	Thiophene
NC00086791	ND	86	620	77	NA	39	257	71	NA	<10	Thiophene
NC00087441	ND	208	NA	<10	6588	92	NA	62	$>10^4$	39	Thieno[2,3-d]pyrimidine
NC00089216	ND	2535	NA	<10	2591	103	NA	8.5	4750	88	Benzoimidazole
NC00090038	18	ND	NA	83	NA	41	NA	19	NA	42	Dibenzo[a,c]cycloheptene
NC00090702	512	ND	NA	<10	264	120	NA	<10	NA	35	Dibenzylamide
NC00094488	1402	1216	NA	<10	NA	55	NA	4.9	12605	75	2-Oxo-[1,3]dithiole
NC00096199	1370	ND	NA	<10	501	120	NA	<10	NA	50	Benzsulfamide
NC00096661	222	306	1290	126	NA	NA	97	120	NA	<10	Phenylethylamide
NC00098918	842	ND	NA	<10	NA	35	NA	16	NA	15	2-Hydroxy-naphthalene
NC00099763	1609	ND	NA	<10	1056	100	NA	<10	NA	<10	Propionamide
NC00100636	124	ND	$>10^4$	58	$>10^4$	47	NA	<10	NA	30	Dibenzo[a,c]cycloheptene

ND, not determined as the compound was not an HTS hit at this receptor subtype; NA, not available, unable to calculate or the value was more than 20 μmol/L.

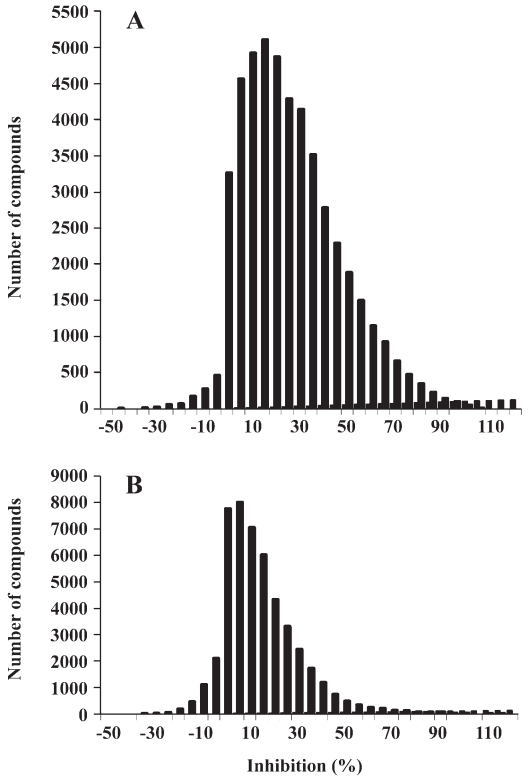

Figure 3. HTS of 48 240 compounds using hMT_1 (A) or hMT_2 (B) receptor binding assays. Results are expressed as percentage inhibition of radiolabeled 2-[^{125}I]iodomelatonin binding to the receptors by the number of hit compounds at each 5% interval.

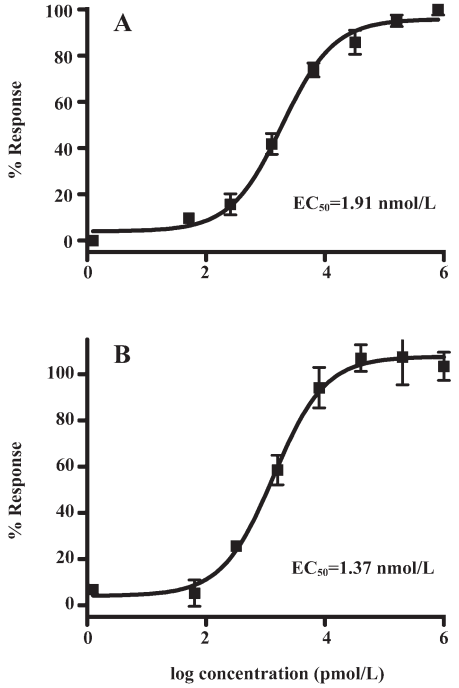

Figure 4. Dose-response curves of melatonin at hMT_1 (A) and hMT_2 (B) receptors measured by respective GTP-γS assay from which EC_{50} values were calculated. Data shown are mean±SD of triplicate measurements and representative of at least 3 independent experiments.

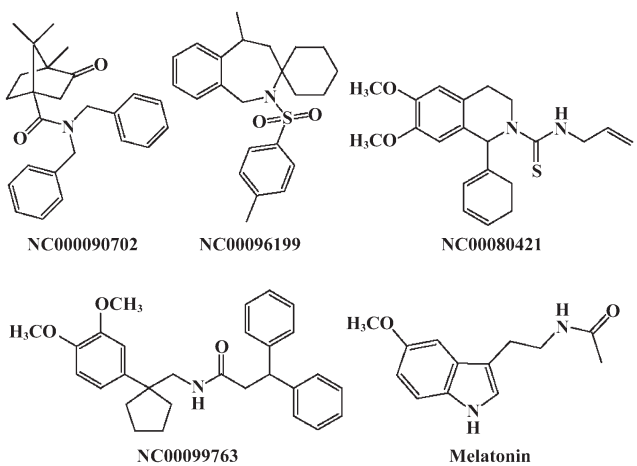

Figure 5. Structures of newly-discovered selective melatonin receptor antagonists and melatonin.

hMT_2 receptor), this affinity detection-based HTS method successfully identified a series of active compounds with a confirmed hit rate of 0.13% and 0.11% for the hMT_1 and hMT_2 receptors, respectively. Subsequent IC_{50} determination and functionality studies with GTP-γS assays verified the results obtained from the HTS campaign. It was noted that the S/B window of our GTP-γS assay was somehow reduced (data not shown), probably due to the lack of wash step, as compared to that reported elsewhere[24]. Nevertheless, the system was robust enough for the eventual identification of several novel melatonin receptor modulators.

Of the 4 functionally-selective compounds, 1 was characterized as a hMT_2 antagonist and 3 as hMT_1 receptor antagonists. NC00080421, with a similar structure to known selective hMT_2 receptor antagonist 4P-PDOT[29], showed approximately 40-fold selectivity at the hMT_2 receptor. However, NC00090702, NC00096199, and NC00099763, with selective hMT_1 receptor antagonist activities, exhibited little structural similarity to melatonin. Because hMT_1 receptor modulators are scarcely available, our findings certainly provide some new knowledge for continued pursuit in this direction using the dual melatonin receptor subtype assay system[30]. Unfortunately, we have not found selective hMT_1 or hMT_2 receptor agonists in this study, but further structure–activity relationship analyses on these leads and relevant structural modifications may

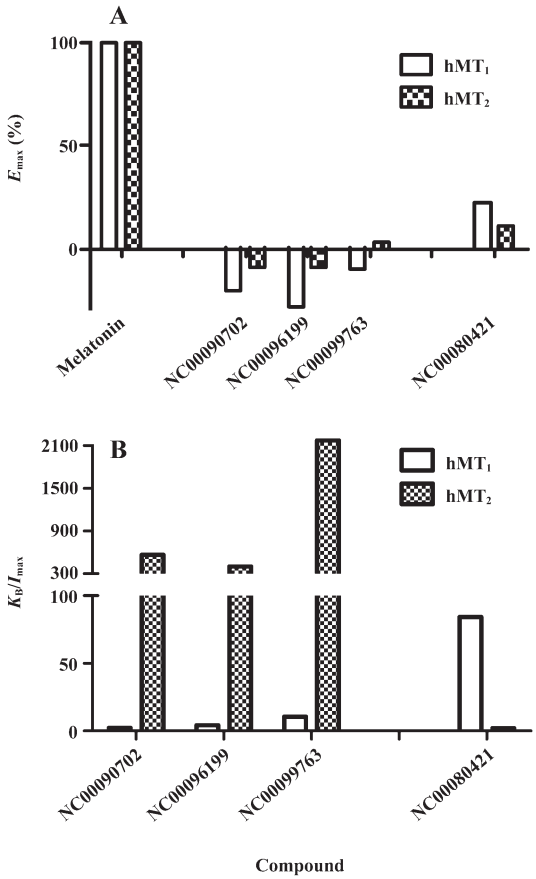

Figure 6. Subtype selectivity of 4 newly-discovered melatonin receptor (hMT_1 and hMT_2) modulators. All compounds show antagonist activities. K_B values at the hMT_1 receptor were compared with that of hMT_2 (hMT_1/hMT_2). (A) agonist feature (E_{max}); (B) antagonist feature (K_B/I_{max}).

dial in agonist activity.

In humans, it has been suggested that melatonin has a variety of clinical applications, such as the treatment of delayed sleep phase syndrome, jet lag, work shift disturbances[31], seasonal affective disorders[32], and aging[33]. Melatonin was also implicated to have immunomodulating properties[34]. Recent studies demonstrated that melatonin may play a role in Alzheimer's disease[35], anesthesia[36], depression[37], and obesity[38]. However, the very short biological half-life (20–30 min), extensive metabolism, and lack of selectivity of melatonin at its target sites prompt the search for metabolically-stable agonists and antagonists. Although studies involving animal models relevant to human pathologies and appropriate clinical trials have indicated that circadian sleep disorders and insomnia are probably the sole therapeutic indications today for selective hMT_1 or hMT_2 receptor ligands[39], the selective antagonists described in this study may provide important tools in further understanding the physiological functions of the different receptor subtypes (hMT_1 or hMT_2) *in vivo*.

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Author contribution

Ming-wei WANG and Jean A BOUTIN designed research; Jian-hua YAN and Hao-ran SU performed research; Jean A BOUTIN and M Pierre RENARD contributed new analytical tools and reagents; Jian-hua YAN and Ming-wei WANG analyzed data; Ming-wei WANG wrote the paper.

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