Communications to the Editor

Development of a High-Affinity Ligand That Binds Irreversibly to Mel_{1b} Melatonin Receptors

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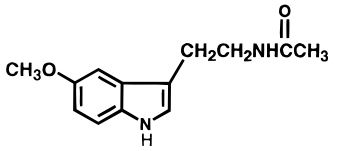
Melatonin is a hormone that is synthesized and secreted into the general circulation by the pineal gland.[1] The circadian production of melatonin is regulated by the suprachiasmatic nucleus of the hypothalamus and is synchronized by environmental light.[1,2] Melatonin entrains and synchronizes circadian and seasonal rhythms[2−4] and is involved in thermoregulation,[5,6] cardiovascular regulation,[7] and oncogenesis.[8]

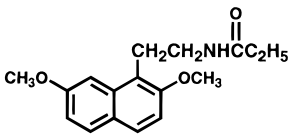
Melatonin may mediate its effects in vivo through melatonin receptors. Presently, two melatonin receptor subtypes have been defined as either the *ML-1* or *ML-2* type, based on pharmacological profiles.[2] However, only two human melatonin receptors have been cloned that belong to the G-protein-coupled receptor class of the *ML-1* type, defined as either the Mel_{1a}[9] or Mel_{1b}[10] subtype. Little is known about the role that the Mel_{1b} melatonin receptor plays in vivo as "tools" (i.e., selective Mel_{1b} antibodies or affinity labels selective for the Mel_{1b} receptor) have not yet been developed. Melatonin receptors of the Mel_{1b} melatonin receptor subtype may be involved in retinal physiology. This is supported by competitive receptor binding assays using the radioligand 2-[^{125}I]iodomelatonin and competitive melatonin receptor antagonists. It is shown that the pharmacological profile of these antagonists at the human Mel_{1b} melatonin receptors expressed in COS-7 cells is similar to that of the functional presynaptic melatonin heteroreceptor of the rabbit retina.[11] Additionally, the expression of the Mel_{1b} melatonin receptor mRNA is predominantly in the retina.[10]

In the past, attempts have been made to develop an affinity ligand (i.e., *N*-(bromoacetyl)-2-iodo-5-methoxytryptamine) for melatonin receptors.[12,13] Because melatonin binds to its receptors in the picomolar to low-nanomolar range, use of *N*-(bromoacetyl)-2-iodo-5-methoxytryptamine[12] has been minimal because of its low (∼50 nM) affinity for melatonin receptors.[3] In addition, whether this ligand displays any selectivity for either melatonin receptor subtype is unknown. Here, we report the development of an affinity ligand that binds specifically to and selectively alkylates Mel_{1b} melatonin receptors expressed in Chinese hamster ovary (CHO) cells.

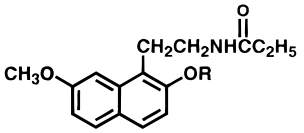
Recently, it was reported that *N*-[2-(2,7-dimethoxynaphthyl)ethyl]propionamide (**2**) is a ligand that has high affinity (K_i = 0.07 ± 0.004 nM) for melatonin receptors.[14] It was postulated that the 2-methoxy group binds to the accessory binding pocket at the receptor site. Thus, we designed and synthesized *N*-[2-[2-(chloroacetoxy)-7-methoxynaphthyl]ethyl]propionamide (**3a**) and *N*-[2-[2-(bromoacetoxy)-7-methoxynaphthyl]ethyl]-propionamide (**3b**, BMNEP) to alkylate the receptor at the pocket.



Melatonin 1



N-[2-(2,7-dimethoxynaphthyl)ethyl] propionamide 2



3a R = $COCH_2Cl$
3b R = $COCH_2Br$

The synthesis of target compounds **3a**,**b** is outlined in Scheme 1. 7-Methoxy-2-naphthol (**4**) was used as a starting material. Selective formylation of the 1-position of compound **4** was accomplished by reaction with chloroform in the presence of NaOH (Reimer−Tiemann reaction),[15] yielding the desired phenolic aldehyde **5**. The phenolic group in **5** was converted to benzyl ether by reacting **5** with benzyl bromide using K_2CO_3 as a base to yield compound **6** (100%). Condensation of aldehyde **6** with nitromethane in the presence of NH_4-OAc afforded nitroalkene **7** (96.5%). Reduction of **7** with $LiAlH_4$ followed by acylation with propionyl chloride furnished amide **8** (54.2% based on **7**). Cleavage of the benzyl ether in compound **8** by hydrogenation gave the phenol **9** (100%). Reaction of **9** with chloroacetyl chloride or bromoacetyl bromide using *N*,*N*-diisopropylethylamine as base yielded the target compounds **3a**,**b**, respectively.

Saturation analysis, which was previously performed on CHO cells expressing the human Mel_{1a} receptor (CHO-Mel_{1a}),[16] was performed on human Mel_{1b} melatonin receptors expressed in the same cells (CHO-Mel_{1b}) using 2-[^{125}I]iodomelatonin as already described.[16] Briefly, CHO-Mel_{1b} cells were resuspended in Tris (50 mM), pH 7.4, and added to tubes containing increasing concentrations of 2-[^{125}I]iodomelatonin (0−500 pM) in the absence (total binding) or presence of melatonin (nonspecific binding). All reactions were incubated for 1 h at room temperature, rapidly filtered, and counted in a gamma counter as already described.[16]

To determine whether BMNEP alkylated melatonin receptors, CHO-Mel_{1a} or CHO-Mel_{1b} cells were grown to confluence on 6-well dishes and were preincubated either with various concentrations of BMNEP (100 pM to 1 μM) in the absence or presence of melatonin (1 μM)

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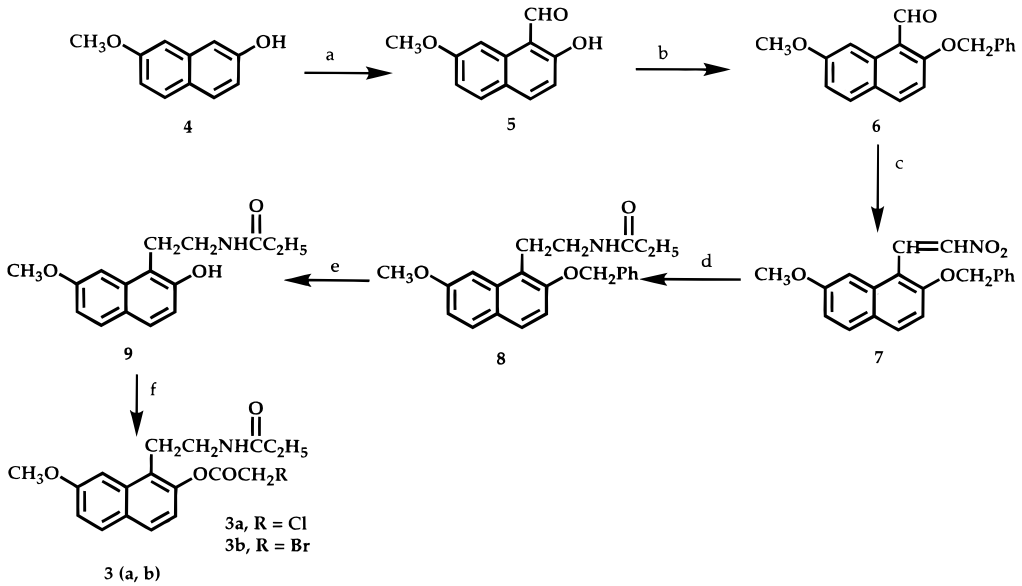
Scheme 1. Synthesis of Compounds **3a,b**[a]



[a] Reagents and conditions: (a) i. NaOH, $CHCl_3$, H_2O, ii. H_3O^+ (31.5%); (b) $PhCH_2Br$, K_2CO_3, acetone, reflux, 5 h, 100%; (c) CH_3NO_2, NH_4OAc, reflux, 2 h, 96.5%; (d) i. $LiAlH_4$, THF, 40 °C, 18 h, ii. C_2H_5COCl, Et_3N, CH_2Cl_2, rt, 1 h, 54.2% from **7**; (e) 5% Pd−C, MeOH, H_2, rt, 18 h, 100%; (f) XCH_2COX (X = Cl, Br), iPr_2NEt, CH_2Cl_2, 45 min, 72% for **3a**, 61% for **3b**.

for 1 h at 37 °C or with 1 nM BMNEP for various periods of time (0−120 min) at 37 °C. Following the incubation, cells were washed with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 0.62 mM KH_2PO_4), lifted in buffer (10 mM K_3PO_4, 1 mM EDTA, pH 7.4), and added to tubes containing 2-[^{125}I]iodomelatonin (500 pM) and Tris (50 mM, pH 7.4) buffer in the absence (total) or presence (nonspecific) of 1 μM melatonin.

Competition assays were performed as described previously.[16] Briefly, CHO-Mel_{1a} cells were grown to confluence in 10-cm diameter dishes and resuspended in Tris-HCl (50 mM), pH 7.4. Cell lysates were added to tubes containing Tris-HCl (50 mM) and 2-[^{125}I]-iodomelatonin (100 pM) in the absence (total) or presence of drugs (0.1 pM to 1 μM). All reactions were incubated for 1 h at room temperature, rapidly filtered, and counted in a gamma counter as already described.[16]

Bromoacetoxy and chloroacetoxy derivatives have been widely used as alkylating moieties.[17,18] This approach was used in the following study to develop alkylating agents against melatonin receptors. Because bromoacetoxy derivatives are more reactive than chloroacetoxy derivatives, the focus of this study was on the bromoacetoxy derivative BMNEP and its alkylating effects on melatonin receptors.

The expression of human Mel_{1a} melatonin receptors in CHO cells did not affect the pharmacology of this receptor as already described.[16] The expression of human Mel_{1b} melatonin receptor cDNA[10] in CHO cells (developed in collaboration with Dr. Margarita Dubocovich, Northwestern University Medical School) also did not affect the pharmacology of this receptor. Binding of 2-[^{125}I]iodomelatonin to CHO-Mel_{1b} cells was saturable, reversible, and of high affinity (K_D = 158 ± 1 pM; B_{max} = 424 ± 115 fmol/mg of protein, n = 3) which is similar to that reported for the Mel_{1b} receptors expressed in COS-1 cells.[10]

Preincubation of CHO-Mel_{1b} cells with BMNEP resulted in a significant time-dependent decrease in total 2-[^{125}I]iodomelatonin binding to Mel_{1b} melatonin receptors compared to untreated cells (n = 3−6 experiments)

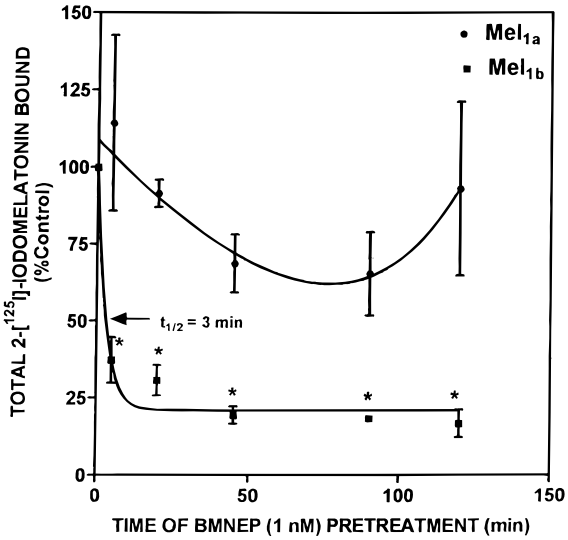


Figure 1. Time-dependent alkylation of Mel_{1b} melatonin receptors by BMNEP. Following a 2-h exposure to 1 nM BMNEP, no Mel_{1a} receptors and 83 ± 4% of Mel_{1b} melatonin receptors were alkylated by 5 min ($t_{1/2}$ = 3 min). Data were normalized against control (untreated) cells and are expressed as the mean ± SEM of 3−6 experiments. All data were analyzed by one-way ANOVA and Student's unpaired t-test where significance (*) is defined as $p < 0.05$; (*) comparing BMNEP-treated cells to control untreated cells.

where maximal inhibition (83 ± 4% Mel_{1b} receptors alkylated) occurred by 5 min (Figure 1). In addition, increasing concentrations of BMNEP significantly decreased 2-[^{125}I]iodomelatonin binding to CHO-Mel_{1b} cells compared to untreated control cells (p = 0.0044, n = 3−7 experiments). Melatonin (1 μM), added in combination with BMNEP, prevented Mel_{1b} receptors from alkylation at the 1 nM (p = 0.011, n = 4), 30 nM (p = 0.025, n = 3), and 1 μM (p = 0.001, n = 3) concentrations of BMNEP (Figure 2B). The decrease in 2-[^{125}I]iodomelatonin binding to CHO-Mel_{1b} cells following BMNEP/melatonin exposure was not due to a downregulation of Mel_{1b} melatonin receptors as melatonin-pretreated controls were run in parallel with untreated controls

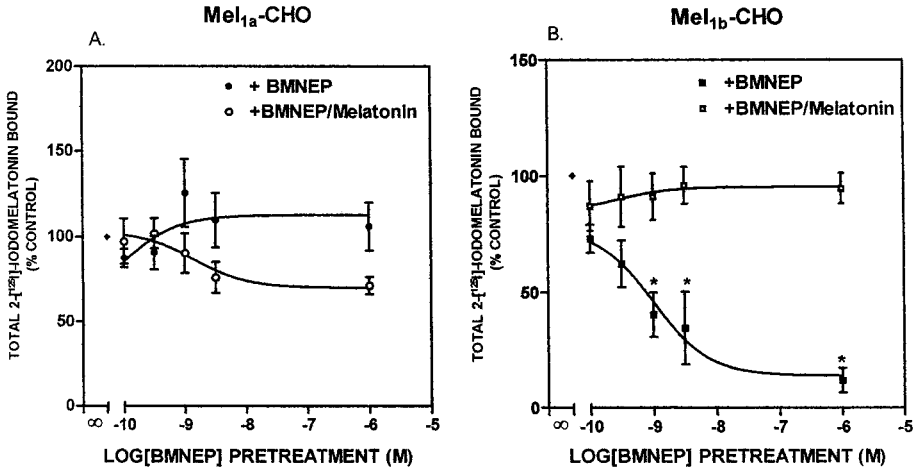
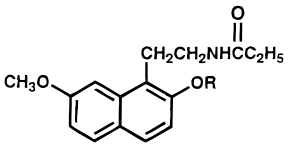


Figure 2. Concentration-dependent alkylation of Mel_{1b} melatonin receptors expressed in CHO cells. (A) Following a 1-h exposure to various concentrations of BMNEP (100 pM to 1 μM), no Mel_{1a} receptors were alkylated by BMNEP. (B) In contrast, 50% of the total Mel_{1b} melatonin receptors were alkylated by 1 nM BMNEP where maximal alkylation (88 ± 5% Mel_{1b} receptors alkylated) occurred at 1 μM BMNEP. Melatonin (1 μM), added in combination with BMNEP, prevented the effect of BMNEP (1 nM, 30 nM, and 1 μM)-mediated alkylation only at Mel_{1b} receptors. Data were normalized against control (untreated) cells and are expressed as the mean ± SEM of 3−7 experiments. All data were analyzed by one-way ANOVA and Student's unpaired *t*-test where significance (*) is defined as $p < 0.05$; (*) comparing BMNEP-treated cells to BMNEP/melatonin-treated cells.

Table 1. Competition of Melatonin and Related Analogues for 2-[^{125}I]Iodomelatonin Binding to $Me1_{1a}$ or $Me1_{1b}$ Melatonin Receptors Expressed in CHO Cells[a]



		K_i (nM) (range of SEM)	
compd	R	$Me1_{1a}$	$Me1_{1b}$
2	CH_3	0.099 (0.09−0.1)	0.38 (0.35−0.41)
3a	$COCH_2Cl$	11 (2.3−51)	1.6 (0.86−3.0)
3b	$COCH_2Br$	1.3 (0.84−2.0)	
melatonin		0.08 (0.06−0.11)	0.12 (0.07−0.19)

[a] All competition binding experiments were performed on CHO whole cell lysates using 80−100 pM 2-[^{125}I]iodomelatonin (NEN/DuPont, Boston, MA; 2200 Ci/mmol) at room temperature. The affinity of 2-[^{125}I]iodomelatonin for Mel_{1a} and Mel_{1b} melatonin receptors was 80 and 150 pM, respectively.

and no significant decrease in total 2-[^{125}I]iodomelatonin binding occurred (data not shown). Fifty percent of the total Mel_{1b} melatonin receptors alkylated by BMNEP occurred at ∼1 nM BMNEP (Figure 2B). In contrast, no time-dependent alkylation of Mel_{1a} melatonin receptors occurred under the same experimental conditions (Figure 1). In addition, increasing concentrations of BMNEP did not decrease 2-[^{125}I]iodomelatonin binding to CHO-Mel_{1a} cells (Figure 2A).

To determine whether the lack of alkylation of Mel_{1a} melatonin receptors by BMNEP was due to a lack of affinity of BMNEP for the receptor, competition binding studies were performed on CHO-Mel_{1a} cells as described previously.[16] As shown in Table 1, BMNEP displayed nanomolar affinity for Mel_{1a} melatonin receptors suggesting that the lack of alkylation of Mel_{1a} melatonin receptors by BMNEP was not due to a decreased affinity of BMNEP for the Mel_{1a} receptor. Thus, BMNEP selectively alkylated human Mel_{1b} and not human Mel_{1a} melatonin receptors expressed in CHO cells, and this is the first demonstration of such a phenomenon.

Alkylation of Mel_{1b} melatonin receptors by BMNEP could be used in various studies and greatly enhance our understanding of Mel_{1b} melatonin receptors in vivo. BMNEP could be used to characterize the Mel_{1b} melatonin receptor as affinity probes in the past have played important roles in molecular characterization of other receptors. Such receptors include the adenosine receptors,[19] steroid receptors,[20] and muscarinic receptors.[21−24] In addition, because BMNEP selectively alkylated the Mel_{1b} and not the Mel_{1a} receptors, this ligand may be used to determine the distribution of Mel_{1b} receptors throughout the body even in tissues that coexpress Mel_{1a} melatonin receptors. In conclusion, use of this affinity ligand will aid in a greater understanding of the molecular characteristics of Mel_{1b} melatonin receptors both in vitro and in vivo.

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