2-*N*-Acylaminoalkylindoles: Design and Quantitative Structure−Activity Relationship Studies Leading to MT_2-Selective Melatonin Antagonists

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Several indole analogues of melatonin (MLT) were obtained by moving the MLT side chain from C_3 to C_2 of the indole ring. Binding and in vitro functional assays were performed on cloned human MT_1 and MT_2 receptors, stably transfected in NIH3T3 cells. Quantitative structure−activity relationship studies showed that 4-methoxy-2-(*N*-acylaminomethyl)indoles, with a benzyl group in position 1, were selective MT_2 antagonists and, in particular, *N*-[(1-*p*-chlorobenzyl-4-methoxy-1H-indol-2-yl)methyl]propanamide (**12**) behaved as a pure antagonist at MT_1 and MT_2 receptors, with a 148-fold selectivity for MT_2. We present a topographical model that suggests a lipophilic group, located out of the plane of the indole ring of MLT, as the key feature of the MT_2 selective antagonists.

Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine, MLT) is a neurohormone with a well-defined circadian nocturnal secretion by the pineal gland, being also producted by the photoreceptor cells of the retina.[1] Considerable attention is being devoted to the pharmacology of MLT in view of its potential applications in several therapeutic areas, but a clear depiction of the therapeutic applications still has to be established.[2] The circadian rhythms of many species, including human, is synchronized by MLT. This raises many possibilities for the use of MLT as a supplement to combat jet lag[3] or as a treatment for certain types of circadian-based sleeping disorders.[4] The pharmacological effects of MLT in humans have also been investigated in psychiatric disorders[5] and in the cardiovascular system.[6] The molecular mechanisms underlying the reported antioxidant,[7] neuroprotective,[8] anticarcinogenic,[9] and immunostimulatory[10] properties of melatonin are not yet fully understood.

Many of the MLT effects are mediated through high-affinity G-protein-coupled receptors expressed primarily in the brain, retina, pituitary, and blood vessels.[11] Recent cloning of several G-protein-coupled melatonin receptor genes revealed at least three melatonin receptor subtypes, two of which (MT_1 and MT_2) have been found in mammals.[12] These receptor subtypes are coupled to $G_{i/o}$ proteins associated with inhibition of adenylyl cyclase;[13] MT_1 receptors were also shown to activate other signal transduction pathways.[14] Another putative melatonin binding site called MT_3 was hypothesized from the observation of a binding site in hamster brain membranes[15] and in some peripheral hamster tissues including intestine, liver, kidney, lung muscle, and heart; this binding site has recently been characterized as the hamster homologue of the human enzyme quinone reductase 2.[16]

Despite these advances and the recent studies with chimeric receptors[17] and with MT_1-receptor-deficient mice,[18] there are still important issues, such as the exact interaction of agonists and antagonists with the amino acids of the binding site and the physiological role of the two receptor subtypes, that remain to be settled. These questions could be better answered if specific, potent, and subtype-selective agonists and antagonists were available.

The search for novel high-affinity MLT ligands led to the synthesis of several indole[19] and non-indole MLT analogues[20] and the establishment of a structure−activity relationship (SAR) for MLT binding affinity; the field was recently reviewed.[21]

On the basis of the MLT ligand affinity data on native receptors and on the amino acid sequence of the cloned MLT receptors, some three-dimensional receptor−ligand interaction models were proposed.[22] However, the molecular basis for the subtype selectivity is still unknown. Comparison of the potencies and binding affinities of MLT agonists, both to native MLT receptors[23] and to MT_1 and MT_2 receptors expressed in COS-7, COS-1, CHO, and NIH3T3 cellular lines, showed that most of these ligands exhibit little or no subtype selectivity.[15,24] Lack of subtype-selective agonists for MLT receptors has made it impossible to critically evaluate the contribution

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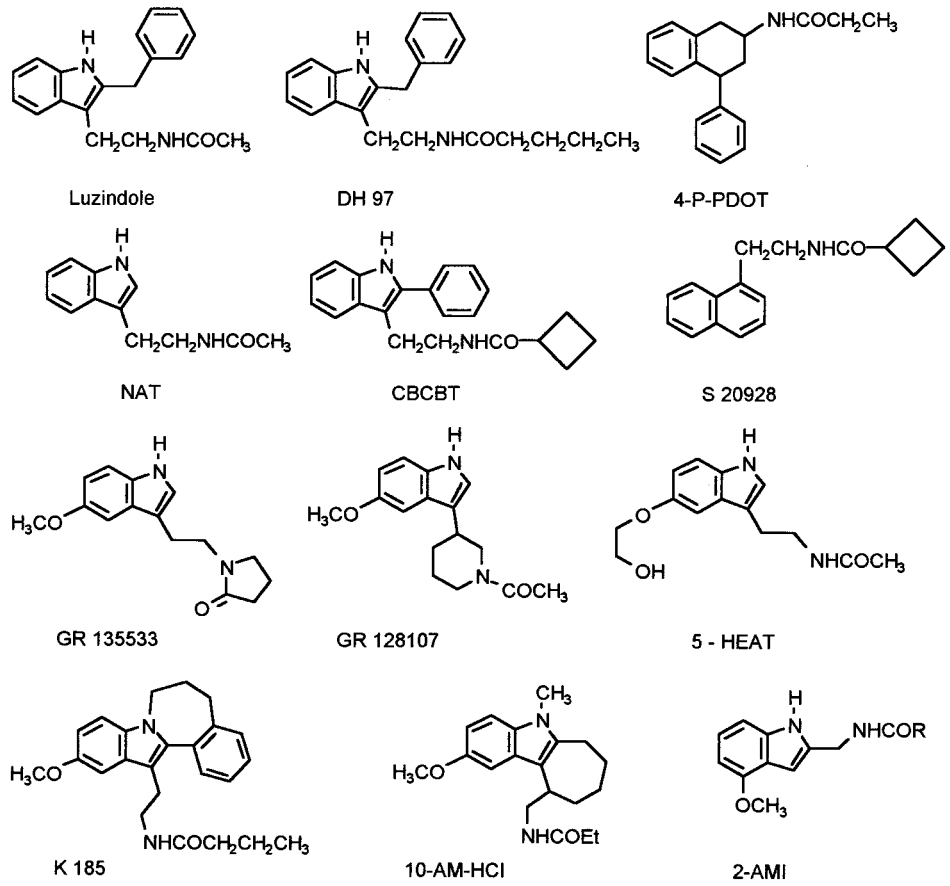


Figure 1. Chemical structures of melatonin antagonists or partial agonists.

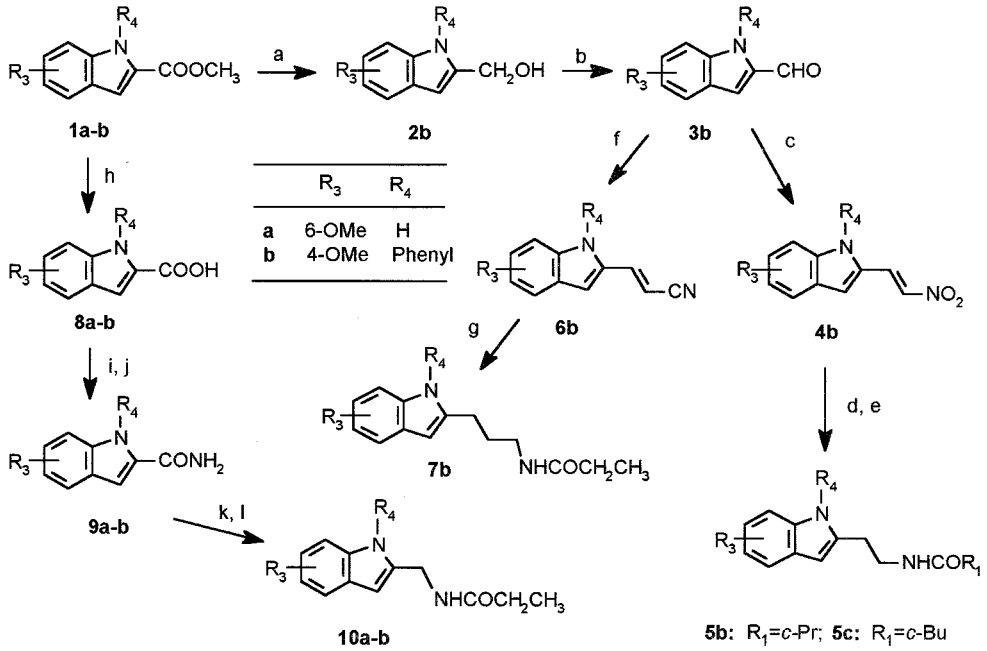
of MT_1 and MT_2 to the various MLT effects in the body. Moreover, no selective MLT antagonists were reported for the h-MT_1 receptor. Recently, partial agonists/antagonists with some degree of selectivity for MT_2 subtype appeared in the literature (Figure 1). Some of these compounds were successfully used to dissect MT_1 and MT_2-mediated melatonin effects in tissues coexpressing both subtypes.[25]

The first approach to design MLT antagonists involved variation of the 5-methoxy substituent. Some of these compounds, including *N*-acetyltryptamine,[26] 4-phenyl-2-acylaminotetralins,[13] luzindole,[27] and its congener DH97,[24g] are structurally characterized by the absence of the methoxy group. Substitution of the 5-OMe group with methyl or halogens did not yield antagonist activity.[24i] The 5-hydroxyethoxy derivative (5-HEAT)[28] was a full agonist at the h-MT_1 and an antagonist/weak partial agonist at the h-MT_2 receptor subtype. Other approaches lead to modification of the amide function such as in GR 135533 and GR 128107,[24b] where a tertiary amide replaces the secondary amide of the 3-ethylamido side chain. Other modifications of the amide side chain seem to depress intrinsic activity, such as the *N*-cyclobutylcarbonyl group of *N*-cyclobutylcarbonyl-2-phenyltryptamine (CBCPT)[29] or of the naphthalene derivative S 20928.[30]

Another approach called for a cycloheptane ring fused to the C_2-C_3 [*N*-ethylcarbonyl-10-(aminomethyl)-2-methoxy-5-methylhexahydrocyclohept[*b*]indole; 10-AM-HCl][19e] or to the N_1-C_2 (K 185)[24h] indole positions, and the compounds were claimed to possess an antagonist profile. Some of the putative antagonists, however, were reclassified as partial agonists when tested in different tissues or recombinant systems[31] or were partially characterized, with the notable exception of K 185.[24h]

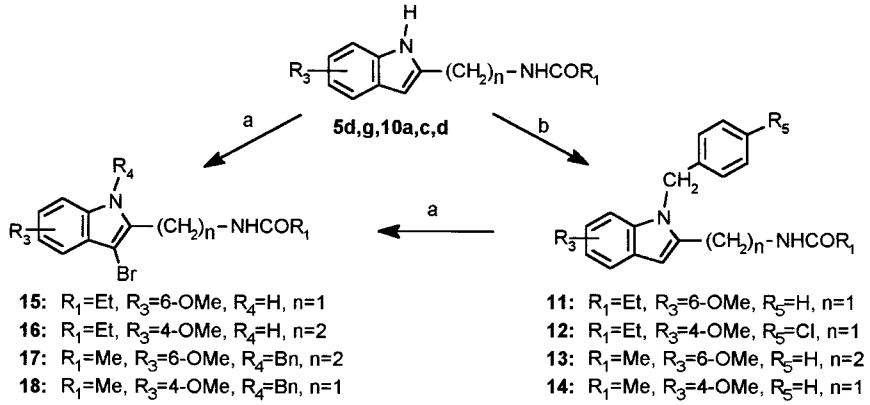
Recently we discovered a new series of h-MT_1 partial agonists and antagonists by transposing the MLT side chain from C_3 to C_2 of the indole nucleus (i.e., 2-acylaminomethyl-4-methoxyindole, 2-AMI), and we reported a preliminary Free−Wilson analysis of group contributions to MT_1 affinity and intrinsic activity.[32] The study showed that, whereas the N_1-benzyl group had a negative effect on MT_1 affinity, the phenyl group in the same position had a positive, statistically significant effect. This observation and the strong reduction of intrinsic activity caused by the shortening of the 2-acylaminoalkyl chain led us to predict for 1-phenyl-2-propionylaminomethyl-4-methoxyindole (**10b**) a pK_i value of 6.8 and an intrinsic activity of 0.3 in the test based on GTPγS binding. These predictions were considered encouraging because in the old series a pK_i around 7 could only be obtained at the expense of an increase of intrinsic activity to 0.5 or higher. This prompted us to pursue further this strategy to obtain relationships explaining selective receptor binding and activation, looking for new melatonin antagonists. Thus, to investigate in detail the influence of structural variation (length of the side chain, position of the methoxy group, N_1 and C_3 indole substitution) on affinity and intrinsic activity, the synthesis of additional compounds (**5b−c**, **7b**, **10a**,**b**, **11**, **12**, and **15−18**) has been performed. Furthermore, we evaluated the affinity and efficacy of these and of the previously synthesized 2-*N*-acylaminoalkyl derivatives (**5d−n**, **7c**, **10c−f**)[32] on human MT_2

Scheme 1[a]



[a] Reagents: (a) $LiAlH_4$, THF, room temperature, 45 min; (b) MnO_2, CH_2Cl_2, room temperature, 7 h; (c) CH_3NO_2, NH_4OAc, reflux, 1.5 h; (d) $LiAlH_4$, THF, room temperature, 5 h; (e) cyclopropanecarbonyl or cyclobutanecarbonyl chloride, TEA, THF, room temperature, 2 h; (f) $(Ph)_3P^+CH_2CNCl^-$, DBU, toluene, reflux, 30 min; (g) Raney nickel, H_2, 4 atm, $(EtCO)_2O$, THF, 50 °C, 6 h; (h) 3 N KOH, MeOH, THF, room temperature, 16 h; (i) $SOCl_2$, THF, 50 °C, 4 h; (j) NH_3, THF, room temperature, 16 h; (k) $LiAlH_4$, THF, reflux, 3 h; (l) $(EtCO)_2O$, TEA, THF, room temperature, 8 h.

Scheme 2[a]



[a] Reagents: (a) NBS, AcOH, room temperature, 2 h; (b) NaH, DMF, *p*-chlorobenzyl or benzyl chloride, room temperature, 16 h.

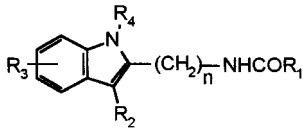
receptors, stably transfected in NIH3T3 cells, to delineate the structural features for h-MT_1/h-MT_2 subtype selectivity.

Chemistry

Melatonergic derivatives **5d**−**n**, **7c**, and **10c**−**f** were prepared as previously described.[32] The synthesis of the novel melatonin analogues **5b**,**c**, **7b**, **10a**,**b**, **11**, **12**, and **15**−**18** was achieved following the routes described in Schemes 1 and 2 and according to our previous methods for the synthesis of similar compounds.[32] Briefly, the methyl ester **1b**[32] was reduced with lithium aluminum hydride ($LiAlH_4$), followed by oxidation with MnO_2 to give the aldehyde **3b**.[32] Condensation of the aldehyde **3b** with nitromethane in the presence of NH_4OAc gave the nitroethenyl derivative **4b,**[32] which was then reduced with $LiAlH_4$ to give (4-methoxy-1-phenyl-1H-indol-2-yl)ethanamine. This crude amine was subsequently acylated with cyclopropanoyl or cyclobutanoyl chloride in the presence of triethylamine (TEA) to afford the corresponding target amides **5b**,**c**. The 2-acylaminopropyl derivative **7b** was prepared by hydrogenation over Raney nickel of the nitrile **6b** and concomitant N-acylation with propionic anhydride. The propenenitrile **6b** was obtained by subjecting the aldehyde **3b**[32] to Wittig reaction conditions[33] using cyanomethyltriphenylphosphonium chloride[34] in the presence of 1,7-diazabicyclo[4.5.0]undec-6-ene (DBU). The 2-acylaminomethyl derivatives **10a**,**b** were prepared by $LiAlH_4$ reduction of the corresponding amides **9a**,**b** followed by N-acylation with propionic anhydride. These amides were obtained by alkaline ester hydrolysis of indoles **1a**,**b**, followed by acylation of the acids **8a**,**b** with thionyl chloride and treatment of the crude acid chloride with a saturated solution of ammonia in dichloromethane.

N-alkylation of the 2-(*N*-acylaminoalkyl)indoles **10a**, **10c**, **5g**, and **10d** was carried out with sodium hydride and benzyl bromide or *p*-chlorobenzyl chloride to give

Table 1. Binding Affinity[a] and Intrinsic Activity of 2-(Acylaminoalkyl)indole Derivatives **5b**−**n**, **7b**,**c**, **10b**−**f**, **11**, **12**, and **15**−**18** for the Human MT_1 and MT_2 Melatonin Receptors Stably Expressed in NIH3T3 Cells



						human MT_1		human MT_2		
compd	R_1	R_2	R_3	R_4	n	$pK_i \pm$ SEM	IA_r[b]	$pK_i \pm$ SEM	IA_r[b]	$pK_{i1} - pK_{i2}$ [c]
MLT						9.54 ± 0.02	1	9.55 ± 0.02	1	−0.01
5b	*c*-Pr	H	4-OCH_3	Ph	2	6.48 ± 0.03	0.19	7.34 ± 0.12	0.26	−0.86
5c	*c*-Bu	H	4-OCH_3	Ph	2	6.16 ± 0.12	0.08	6.94 ± 0.11	0.31	−0.78
5d	CH_2CH_3	H	4-OCH_3	H	2	6.87 ± 0.07	0.49	6.65 ± 0.02	0.71	0.22
5e	CH_3	H	H	H	2	5.61 ± 0.03	0.09	5.79 ± 0.04	0.48	−0.18
5f	CH_3	H	7-OCH_3	H	2	5.42 ± 0.05	−0.06	5.37 ± 0.01	0.02	0.05
5g	CH_3	H	6-OCH_3	H	2	5.07 ± 0.05	−0.04	5.84 ± 0.02	0.02	−0.77
5h	CH_3	H	5-OCH_3	H	2	4.79 ± 0.11	−0.01	5.03 ± 0.04	0.18	−0.24
5i	CH_2CH_3	H	4-OCH_3	Ph	2	7.20 ± 0.03	0.47	8.78 ± 0.16	0.58	−1.58
5j	CH_3	H	4-OCH_3	Ph	2	7.06 ± 0.05	0.61	8.11 ± 0.08	0.59	−1.05
5k	CH_3	H	4-OCH_3	H	2	6.20 ± 0.02	0.60	6.03 ± 0.09	0.62	0.17
5l	CH_3	H	6-OCH_3	CH_3	2	5.74 ± 0.01	0.31	5.91 ± 0.05	0.50	−0.17
5m	CH_3	Br	6-OCH_3	H	2	5.40 ± 0.07	−0.07	6.45 ± 0.04	−0.13	−1.05
5n	CH_2CH_3	H	4-OCH_3	Bn[d]	2	6.08 ± 0.05	0.47	7.75 ± 0.08	0.56	−1.67
7b	CH_2CH_3	H	4-OCH_3	Ph	3	6.51 ± 0.02	0.10	7.89 ± 0.12	0.40	−1.38
7c	CH_3	H	5-OCH_3	H	3	5.53 ± 0.03	0.44	3.99 ± 0.04	0.16	1.54
10b	CH_2CH_3	H	4-OCH_3	Ph	1	6.65 ± 0.01	0.22	7.99 ± 0.05	0.49	−1.34
10c	CH_2CH_3	H	4-OCH_3	H	1	6.39 ± 0.06	0.05	6.54 ± 0.02	0.30	−0.15
10d	CH_3	H	4-OCH_3	H	1	5.91 ± 0.09	0.08	6.09 ± 0.01	0.42	−0.18
10e	*c*-Bu	H	4-OCH_3	H	1	6.28 ± 0.02	−0.01	6.19 ± 0.05	0.26	0.09
10f	CH_2CH_3	H	4-OCH_3	Bn	1	6.20 ± 0.04	0.03	8.12 ± 0.08	0.18	−1.92
11	CH_2CH_3	H	6-OCH_3	Bn	1	5.89 ± 0.04	0.00	6.56 ± 0.01	0.16	−0.67
12	CH_2CH_3	H	4-OCH_3	4-Cl-Bn	1	5.89 ± 0.09	0.04	8.06 ± 0.11	0.01	−2.17
15	CH_2CH_3	Br	6-OCH_3	H	1	6.56 ± 0.01	0.20	6.61 ± 0.02	0.01	−0.05
16	CH_2CH_3	Br	4-OCH_3	H	2	7.30 ± 0.05	0.61	6.84 ± 0.15	0.75	0.46
17	CH_3	Br	6-OCH_3	Bn	2	6.62 ± 0.05	0.16	6.70 ± 0.01	0.01	−0.08
18	CH_3	Br	4-OCH_3	Bn	1	5.50 ± 0.06	0.04	6.85 ± 0.16	0.04	−1.35

[a] pK_i values were calculated from IC_{50} values obtained from competition curves by the method of Cheng and Prusoff[40] and are the means of at least three independent determinations performed in duplicate. [b] The relative intrinsic activity values (IA_r) were obtained by dividing the maximal analogue-induced G-protein activation by that of MLT. [c] The difference ($pK_{i1} - pK_{i2}$) represents selectivity toward the MT_1 (positive values) or the MT_2 (negative values) subtype. [d] Bn = benzyl.

the desired compounds **11**−**14**. The 3-bromoderivatives **15**−**18** were synthesized by direct bromination of the 3-unsubstituted compounds **5d**, **10a**, **13**, and **14**.

Pharmacology

The biological evaluation of the compounds was performed by measuring the h-$MT_1$and h-MT_2 binding affinity and in vitro functional activity.

The binding affinity of compounds **5b**−**n**, **7b**,**c 10b**−**f**, **11**, **12**, and **15**−**18** was determined using 2-[^{125}I]-iodomelatonin (100 pM) as a labeled ligand in competition binding analyses on cloned human MT_1 and MT_2 receptor subtypes stably expressed in NIH3T3 rat fibroblast cells, and the results are summarized in Table 1. The characterization of NIH3T3-MT_1 and MT_2 cells was described in detail elsewhere.[24j,31c]

The relative intrinsic activity (IA_r) of the compounds was measured by the direct activation of the G protein after the binding of the compounds to MT_1 and MT_2 receptor subtypes. IA_r was estimated by means of the [^{35}S]-guanosine-5′-O-(3-thiotriphosphate) ([^{35}S]GTPγS) method in NIH3T3 cells expressing human cloned MT_1 or MT_2 melatonin receptors. The system provides a functional measure for the interaction between melatonin receptors and pertussis-toxin-sensitive G proteins. The detailed description and validation of this method was reported elsewhere.[24j,31c,35]

In cell lines expressing human MT_1 or MT_2 receptors, MLT 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, respectively. In the case of MLT analogues the amount of bound [^{35}S]GTPγS is proportional to the level of the analogue-induced G-protein activation, and it is related to the intrinsic activity of the compounds. Full agonists increased the basal [^{35}S]GTPγS binding in a concentration-dependent manner, like the natural ligand MLT, whereas partial agonists increased it to an extent much less than that of MLT, and antagonists were without effect. The IA_r values were obtained by dividing the maximum ligand-induced stimulation of [^{35}S]GTPγS binding by that of MLT as measured in the same experiment, and the results are summarized in Table 1.

The interaction between ligands and melatonin was investigated by competition experiments in which increasing concentrations of an antagonist inhibit the maximum melatonin-induced stimulation of [^{35}S]GTPγS binding.

Results

The affinity and intrinsic activity data for human MT_1 and MT_2 melatonin receptor subtypes of the new compounds **5b**,**c**, **7b**, **10b**, **11**, **12**, and **15**−**18** are reported in Table 1. Also included in Table 1 are the previously reported h-MT_1 pharmacological data[32] of **5d**−**n**, **7c**, and **10c**−**f** for comparison with h-MT_2 results of the same compounds obtained in the present study.

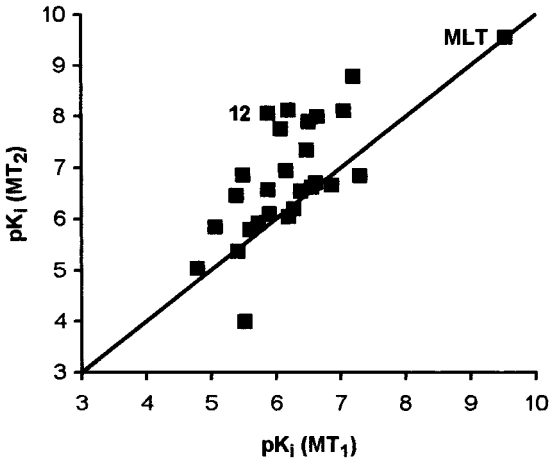


Figure 2. Plot of pK_i data on h-MT$_1$ and h-MT$_2$ receptors. Points on the straight line correspond to compounds having the same affinity for both receptors.

The affinities of all compounds are lower than those of MLT for both receptor subtypes. Nonetheless, it is apparent from past[32] and present studies that the shifting of the melatonin side chain from the C_3 to the C_2 indole position provides exclusively MT$_1$ and MT$_2$ receptor antagonists or partial agonists.

A plot of the MT$_2$ vs MT$_1$ affinity values reveals (Figure 2) that most of the compounds have some selectivity for the MT$_2$ subtype. Compound **12**, in particular, has the highest selectivity for the h-MT$_2$ receptor but not the highest affinity in the series.

With respect to relative intrinsic activity (IA$_r$; see Experimental Section), the effort to get antagonists with relatively high affinity gave different results for the two subtypes, as shown in Figure 3. At the bottom of the plot representing IA$_r$ vs pK_i for the MT$_1$ subtype, there are no points at pK_i values greater 6.5, and compounds with the highest affinity behave as partial agonists. In constrast, the analogous plot for the MT$_2$ is more scattered and some compounds (e.g., **10f** and **12**) have very low IA$_r$ but maintain good pK_i values. This could lead to the hypothesis that these compounds engage in additional interactions, leading to antagonist activity and high affinity at the MT$_2$ subtype but not at the MT$_1$ subtype. In the case of **5m** a small reduction of the baseline level of the [^{35}S]GTPγS binding is observed, suggesting a propensity to act as an inverse agonist.

The analysis of the pharmacological data, reported in Table 1, allows some preliminary considerations about the SAR for this series. Comparison of the simple acetylaminoethyl derivatives substituted on the benzene ring of indole (R$_3$), **5f**−**h** and **5k**, indicates that relative to **5e** the position of the methoxy group does not dramatically influence receptor affinity. Within the small variation observed, a 5- or a 7-methoxy group lowers receptor affinity on both MT$_1$ and MT$_2$, while a 6-methoxy one leads to lower affinity on MT$_1$ and no change on MT$_2$. Only a 4-methoxy group results in a small increase in affinity for both subtypes.

The pK_i and IA$_r$ values on MT$_1$ of compound **10b** are 6.65 and 0.22, respectively, confirming the prediction of our previous Free−Wilson analysis.[32] It is interesting to observe that its pK_i for the MT$_2$ receptor is 7.99, indicating that **10b** can discriminate between the two-receptor subtypes.

The data now available permit the study of the contribution to pK_i and IA$_r$ of the following fragments (italic entities are those of the reference compound): (a) the methoxy in two different positions of the indole ring, R$_3$ = *4-OCH$_3$* or 6-OCH$_3$; (b) the acylamino group from acetic, propionic, or cycloalkanecarboxylic acids (R$_1$ = *CH$_3$*, CH$_2$CH$_3$, or *c*-Bu); (c) lipophilic substituents on the indole nitrogen (R$_4$ = *H*, phenyl, or benzyl); (d) methylene or ethylene alkyl chains (*n* = 1, *2*); (e) bromine in C3 (R$_2$ = *H* or Br). Other minor structural modifications have also been occasionally tested, such as the elongation of the alkyl chain (compounds **7b** and **7c**), a methyl group on the indole nitrogen (**5l**), or a cyclopropanecarboxylic derivative (**5b**), but their effect does not deserve further exploration.

We have selected a set of 18 compounds among those of Table 1 (subset A, reference standard **5k**), characterized by features "a−e" appearing more than once. The data matrix for the Free−Wilson analysis is reported in Table 2, where each column corresponds to an *X* variable of the correlation equations. The multiple regression analysis (MRA) equations with less than the seven contemporary *X* variables give a poor fit in the case of the MT$_1$ receptor. This lack of correlation between the Free−Wilson variables, and the MT$_1$-affinity and MT$_1$-intrinsic activity data, may be due to nonadditivity of the group contributions or to a high ratio of noise to variation in the dependent variables. The inapplicability of the Free−Wilson analysis to this set might also be explained assuming different modes of interaction at the MT$_1$ receptor for the different compounds.

The affinity data for the MT$_2$ receptor correlate with the structure of the compounds better than those for the MT$_1$ receptor, and it is possible to obtain a statistically significant equation:

$$pK_i = 0.73(\pm0.25)\,(R_4 = \text{benzyl}) + 1.56(\pm0.26)\,(R_4 = \text{phenyl}) + 0.66(\pm0.21)\,(R_1 = CH_2CH_3) + 6.07 \quad (\pm0.17)$$

$$n = 18,\quad R^2 = 0.78,\quad s = 0.44,\quad F = 16.5,\quad Q^2 = 0.60,\quad \text{SDEP} = 0.52$$

According to this model, the contribution of R$_3$ = 6-OCH$_3$ is not significant despite the different MT$_2$ affinity of **10f** and **11**. This is likely due to the nonadditive properties of this contribution, as is argued from the similar affinities of **5k** and **5g**.

Assuming that 4- and 6-methoxy derivatives interact differently at the MT$_2$ receptor when R$_4$ = benzyl (see **10f** and **11**), we have considered the series of 4-methoxy compounds as a separate subset (subset B, 13 compounds, Table 2), and new quantitative structure−activity relationships (QSARs) were built. It was mandatory, in this case, to consider MRA equations with a small number of variables to avoid excessive risk of chance correlation.

When the Free−Wilson analysis was limited to the 4-methoxy derivatives, some interesting QSARs are observed (see Table 3). For MT$_1$ affinity, significant contributions are those of R$_1$ = CH$_2$CH$_3$ (positive) and of R$_4$ = benzyl and *n* = 1 (negative). The contribution of the phenyl group is positive but statistically not

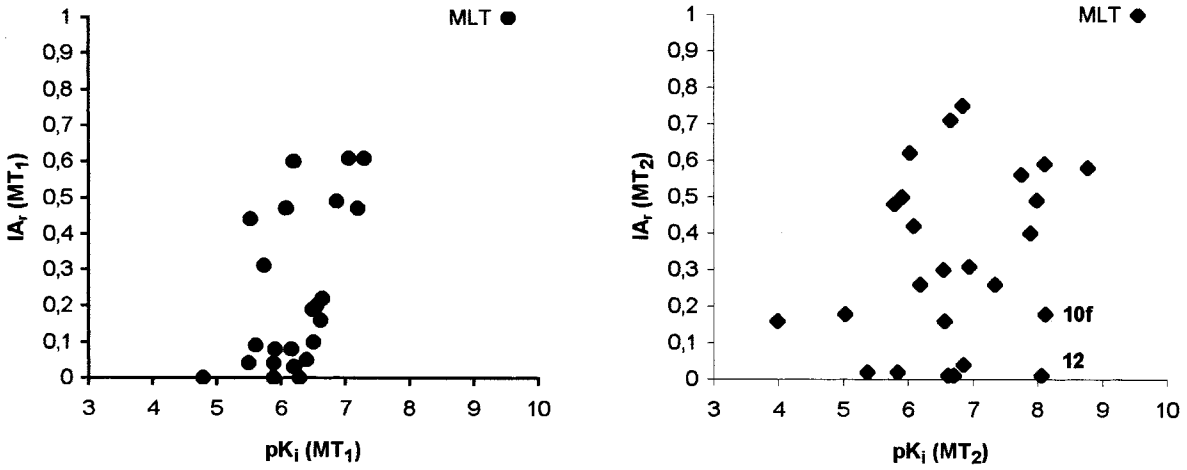


Figure 3. Affinity (pK_i) vs intrinsic activity (IA_r) on h-MT_1 (left) and h-MT_2 (right) receptors for the tested compounds.

Table 2. Data Matrix for the Free−Wilson Analysis

		R_1		R_2	R_3	R_4		n
compd	subset	CH_2CH_3	*c*-Bu	Br	6-OCH_3	phenyl	benzyl	1
5c	A, B	0	1	0	0	1	0	0
5d	A, B	1	0	0	0	0	0	0
5g	A	0	0	0	1	0	0	0
5i	A, B	1	0	0	0	1	0	0
5j	A, B	0	0	0	0	1	0	0
5k	A, B	0	0	0	0	0	0	0
5m	A	0	0	1	1	0	0	0
5n	A, B	1	0	0	0	0	1	0
10b	A, B	1	0	0	0	1	0	1
10c	A, B	1	0	0	0	0	0	1
10d	A, B	0	0	0	0	0	0	1
10e	A, B	0	1	0	0	0	0	1
10f	A, B	1	0	0	0	0	1	1
11	A	1	0	0	1	0	1	1
15	A	1	0	1	1	0	0	1
16	A, B	1	0	1	0	0	0	0
17	A	0	0	1	1	0	1	0
18	A, B	0	0	1	0	0	1	1

Table 3. Results of the Multiple Regression Analysis for the 13 Compounds in Subset B

	MT_1		MT_2	
	pK_i	IA_r	pK_i	IA_r
$n = 1$	-0.38 ± 0.18	-0.40 ± 0.06	*a*	-0.26 ± 0.06
R_4 = benzyl	-0.66 ± 0.22	*a*	1.05 ± 0.27	-0.22 ± 0.07
R_4 = phenyl	*a*	*a*	1.57 ± 0.24	*a*
R_2 = Br	*a*	*a*	*a*	*a*
R_1 = *c*-Bu	*a*	-0.28 ± 0.08	*a*	-0.24 ± 0.08
$R_1 = CH_2CH_3$	0.54 ± 0.18	*a*	0.77 ± 0.21	*a*
intercept	6.48 ± 0.16	0.53 ± 0.04	6.00 ± 0.19	0.65 ± 0.04
R^2	0.74	0.86	0.87	0.82
s	0.32	0.11	0.37	0.10
F	8.3	29.9	20.3	14.1
Q^2	0.46	0.52	0.71	0.47
SDEP	0.38	0.17	0.47	0.15

[a] Not significant.

significant (**5i** vs **5d**). The resulting equation (Table 3) gives a sufficient explanation of the pK_i values ($R^2 = 0.74$, $s = 0.32$), considering the uncertainty of the data and the simplified description of the structures inherent to the method. For what concerns MT_1 IA_r, chain shortening ($n = 1$) leads to a significant lowering of intrinsic activity, and the same is observed for the cyclobutyl group in the acylamino fragment. Other groups did not provide statistically significant contributions to IA_r on MT_1 receptor subtype.

A good MRA equation could be obtained for the pK_i data on MT_2 ($R^2 = 0.87$, $Q^2 = 0.71$), including the three variables corresponding to $R_1 = CH_2CH_3$, R_4 = benzyl, and R_4 = phenyl, all showing a positive contribution to receptor affinity (Table 3). The IA_r on the same receptor subtype generally decreased when $n = 1$, R_4 = benzyl, and R_1 = cyclobutyl, as indicated from the coefficients reported in Table 3.

Compound **10f**, according to these equations, has the best balance among receptor affinity ($pK_i = 8.12$), MT_2 selectivity (~100-fold), and functional antagonism, although the $IA_r = 0.18$ on MT_2 is that of a partial agonist. The introduction of a chlorine atom into the para position of the benzyl group gives compound **12**, a pure antagonist with increased MT_2 selectivity, thus confirming the previous findings.

Discussion

2-Acylaminoalkylindoles proved to be a suitable scaffold for the design of potent and selective MT_2 antagonists, whereas the same result could not be obtained for MT_1 antagonism because of the low affinity ($pK_i < 7.5$) or high relative intrinsic activity (approximately 0.5 for the most potent compounds). Compound **12** showed some interesting characteristics, such as 148-fold selectivity for h-MT_2 receptor and lack of significant effect on basal [^{35}S]GTPγS binding to NIH3T3 MT_1 or MT_2 receptors at any concentration tested (Figure 4). 4-Phenyl-2-propionylaminotetralin (4-P-PDOT)[24b] showed a very high selectivity but behaved as a partial agonist at the h-MT_2 receptor;[31b,c] other compounds, such as the pentanoyl derivative of luzindole (DH97)[24g] and the tetracyclic compound K185,[24h,k] (Figure 1) showed the same level of selectivity of **12**.

The role of the methoxy group remains undefined within the compounds of this work. The 4- and 6-methoxy derivatives, however, appear to be the most promising series because of the MT_1 affinity of compound **5k** and of the MT_2 selectivity of **5g**, with respect to **5e**. The set composed by the two series (subset A) leads to poor QSAR equations on the MT_1 receptor, even if it is possible to observe that the 4-methoxy derivatives are generally endowed with higher affinity. Restriction of the QSAR study to this last series (subset B) permits us to calculate the group contributions to receptor affinity and intrinsic activity. Thus, the change of the

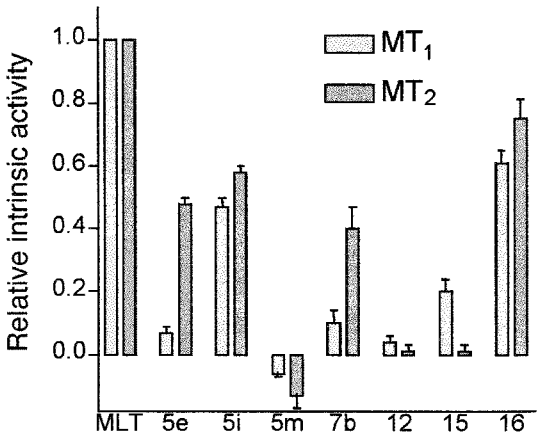


Figure 4. Effects of MLT and of selected analogues on the stimulation of the [^{35}S]GTPγS binding to NIH3T3-h-MT$_1$ and NIH3T3-h-MT$_2$ membranes.

ethylene into the methylene chain leads to a small but significant decrease of MT$_1$ affinity, whereas this is not observed for MT$_2$ affinity. On the other hand, a benzyl group on the indole nitrogen (R_4) has opposite effects on the two receptor subtypes, being favorable for MT$_2$ affinity and unfavorable for MT$_1$ affinity; in the same position, a phenyl group leads to an evident improvement of MT$_2$ affinity but not MT$_1$ affinity. The positive effect of the propionamido group (R_1 = CH_2CH_3) is shared by the two subtypes. Relative to the intrinsic activity, the only difference observed between the two subtypes relates to the contribution of the benzyl group, which is significant for the MT$_2$ only. It can be argued, from the coefficients reported in Table 3, that, in the case of the 4-methoxy-2-acylaminoalkylindole derivatives, a benzyl group discriminates the two receptor subtypes, favoring the interaction with the MT$_2$ one, although decreasing the intrinsic activity.

To provide an interpretation of these findings, we hypothesize that the benzyl group interacts with a lipophilic pocket of the MT$_2$ receptor whose occupation leads to antagonist activity. To test this hypothesis, the minimum-energy conformations of selective MT$_2$ antagonists and partial agonists were mutually compared, looking for the common arrangements of a lipophilic group (benzyl or phenyl), and added to the pharmacophore elements previously defined for nonselective melatonin receptor ligands.[22a,36] Initially, 4-P-PDOT and luzindole conformers were mutually superposed, searching for the best fit of the known pharmacophore elements (the condensed benzene ring and the amido group) and of the phenyl and benzyl groups in position 4 of the tetralin nucleus and in position 2 of the indole ring, respectively. The best superposition presents the phenyl group above or under the plane of the indole and above or under the plane of the tetralin nuclei, thus being on the same or on the opposite side relative to the amide chain. For this reason, we have named "syn" and "anti" the corresponding models. Subsequently, the selected conformations of luzindole have been superposed to the minimum-energy conformations of **10f**, thus permitting the selection of the best ones for the last molecule (see Experimental Section).

The chosen conformations of the three compounds are represented in Figure 5, which depicts the out-of-plane arrangement of the lipophilic groups. Table 4 quantifies some geometric features of the selected conformations and shows how, in the three selective MT$_2$ antagonists, the external phenyl ring is located out of the plane of the indole (or tetralin) nucleus, with an orthogonal orientation. It should be noted that the chirality of the models is not yet defined. Table 4 also indicates that both the trans and cis isomers of 4-P-PDOT fit these models (see Experimental Section).

Some results can be further discussed. While the different effect of the benzyl group on the two receptor subtypes could be attributed to the occupancy of a lipophilic pocket in the MT$_2$ receptor, the Free−Wilson analysis also pointed out a positive contribution of the N_1-phenyl group, statistically significant for the MT$_2$ receptor and not significant, although positive, for the MT$_1$ receptor. This contribution is expressed, for example, by the high MT$_2$ and moderate MT$_1$ affinity (pK_i = 8.78 and 7.20, respectively) shown by compound **5i**, which maintains a medium intrinsic activity. Comparison of the hypothesized hydrophobic cavity for MT$_2$ antagonists with the lipophilic pocket allocating the phenyl group of 2-phenyl-MLT previously evidenced by a 3D-QSAR investigation[36] helps us interpret these results. Figure 6 depicts the volumes occupied by the phenyl groups of the three MT$_2$ antagonists (cyan) and of 2-phenyl-MLT (yellow). Compound **5i** (magenta, right-hand side of the figure), once superposed on MLT structure, places the N_1-phenyl group at the boundary between the cyan and the yellow volumes, and this could explain its intermediate behavior, considering that 2-phenyl-MLT is a potent, nonselective agonist.

In conclusion, this work has identified new, readily available melatonin antagonists with subtype specificity for the h-MT$_2$ receptor. They are obtained by moving the MLT side chain from C_3 to C_2 indole position. The 2-(*N*-acylaminomethyl)indole derivative **12** appears as a promising new tool for investigating the role for the MT$_2$ receptor. We postulate that the N_1-benzyl group is the key feature to confer MT$_2$-selective antagonism on the basis of SAR and molecular modeling studies in this limited series of compounds.

Experimental Section

(**a**) **Chemical Materials and Methods.** 2-[^{125}I]iodomelatonin (specific activity = 2000 Ci mmol^{-1}) and [^{35}S]-GTPγS (specific activity = 1070 mmol^{-1}) were purchased from Amersham (Buckinghamshire, U.K.). 2-Iodomelatonin was obtained from RBI (Natick, MA). Compounds **1a**,[37] **1b**−**4b**,[32] and **8a**[37] were synthesized as described elsewhere. 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). EI-MS spectra (70 eV) were taken on a Fisons Trio 1000. Only molecular ions (M^+) and base peaks are given. Infrared spectra were obtained on a Bruker FT-48 spectrometer; absorbances are reported in ν (cm^{-1}). Elemental analyses for C, H, and N of the tested compounds were performed on a Carlo Erba analyzer.

2-(4-Methoxy-1-phenyl-1H-indol-2-yl)ethylamine. 4-Methoxy-2-(2-nitroethenyl)-1-phenyl-1H-indole **(4b)**[32] (0.294 g, 1 mmol) was added portionwise to a stirred ice-cooled suspension of $LiAlH_4$ (0.23 g, 6 mmol) in dry THF (15 mL) under nitrogen, and the mixture was stirred at room temperature for 5 h. The reaction mixture was cooled to 0 °C, then water was added dropwise to destroy the excess hydride. The resulting mixture was filtered on Celite, and the filtrate was concentrated in

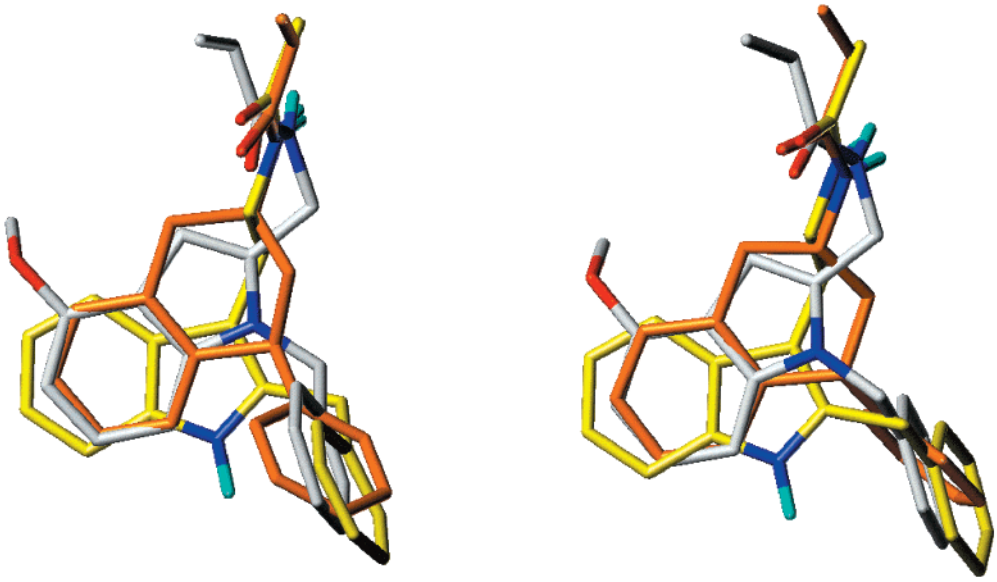


Figure 5. Superposition of 4-P-PDOT (orange carbons), luzindole (yellow carbons), and **10f** (white carbons), according to the models named syn (on the left) and anti (on the right), as described in the text.

Table 4. Superposition of MT_2 Selective Antagonists

compd	rms[a] (Å)	distance between centroids[b] (Å)	angle between planes[c] (deg)	height above the plane[d] (Å)
		syn Model[e]		
luzindole		6.26	82.0	2.21
(2S,4S)-*cis*-4-P-PDOT	0.57	4.82	69.9	2.00
(2R,4S)-*trans*-4-P-PDOT	0.67	4.86	82.5	2.64
10f	0.66	4.95	96.2	2.62
		anti Model[e]		
luzindole		6.30	98.5	2.59
(2R,4R)-*cis*-4-P-PDOT	0.63	4.82	69.9	2.00
(2S,4R)-*trans*-4-P-PDOT	0.88	4.86	81.3	2.63
10f	0.61	4.86	79.9	2.54

[a] Value obtained by superposition of the four atoms of the amido group and of the two aromatic centroids onto those of luzindole. [b] Distance between the centroid of the benzene fragment of the indole or tetralin ring and the centroid of the aromatic substituent. [c] Angle between the plane of the benzene fragment of the indole or tetralin ring and the plane of the phenyl ring of the aromatic substituent. [d] Distance between the plane of the benzene fragment of the indole or tetralin ring and the centroid of the aromatic substituent. [e] The names of the models refer to the apparent torsion angle defined by the following nonconsecutive atoms or pseudoatoms of luzindole: (1) C_α in the ethylamido chain, (2) C_β in the ethylamido chain, (3) methylene C of the benzyl group, and (4) the centroid of the phenyl group (see Figure 5).

vacuo and then acidified with 2 N HCl and partitioned between water and ethyl acetate. The aqueous phase was made alkaline with NaOH and then extracted with ethyl acetate. The combined organic layers were washed with brine, dried (Na_2SO_4), and then concentrated under reduced pressure to give a crude orange oily amine that was used without further purification. MS (EI): *m/z* 266 (M^+), 237 (100). ^{1}H NMR ($CDCl_3$): δ 1.90 (br s, 2H), 2.70−3.00 (m, 4H), 3.99 (s, 3H), 6.57 (d, 1H, J = 7.1), 6.59 (s, 1H), 6.71 (d, 1H, J = 8.1), 7.04 (t, 1H, J = 7.8), 7.29−7.33 (m, 5H).

***N*-[2-(4-Methoxy-1-phenyl-1H-indol-2-yl)ethyl)]cyclopropanamide (5b).** Cyclopropanecarbonyl chloride (0.091 mL, 1 mmol) was added to an ice-cooled solution of the crude amine in dry THF (5 mL) and TEA (0.13 mL, 1 mmol), and the mixture was stirred at room temperature for 2 h. The solvent was evaporated in vacuo, the residue was dissolved in ethyl acetate, and the solution was washed with a saturated $NaHCO_3$ aqueous solution and then with brine, dried (Na_2SO_4), and evaporated again. Purification by flash chromatography (silica gel, cyclohexane/EtOAc, 6:4, as eluent) gave 0.175 g (50%) of the desired product **5b** as a white solid: mp 125 °C (EtOAc/*n*-hexane). MS (EI): *m/z* 334 (M^+), 249 (100). ^{1}H NMR ($CDCl_3$): δ 0.64−0.73 (m, 2H), 0.90−0.96 (m, 2H), 1.17−1.32 (m, 1H), 2.85 (t, 2H, J = 6.7), 3.47 (m, 2H), 4.00 (s, 3H), 5.81 (br s, 1H), 6.58 (d, 1H, J = 8.1)), 6.60 (s, 1H), 6.72 (d, 1H, J = 8.3), 7.06 (t, 1H, J = 8.1), 7.31−7.51 (m, 5H). IR (cm^{-1}, Nujol): 3298, 1647. Anal. ($C_{21}H_{22}N_2O_2$) C, H, N.

***N*-[2-(4-Methoxy-1-phenyl-1H-indol-2-yl)ethyl]cyclobutanamide (5c). 5c** was obtained following the above procedure by using cyclobutanecarbonyl chloride (0.12 g, 1 mmol) instead of cyclopropanecarbonyl chloride; white solid (45% yield), mp 109 °C (EtOAc/*n*-hexane). MS (EI): *m/z* 348 (M^+), 249 (100). ^{1}H NMR ($CDCl_3$): δ 1.75−2.32 (m, 6H), 2.84 (t, 2H, J = 6.6), 2.86 (m, 1H), 3.44 (m, 2H), 3.99 (s, 3H), 5.48 (br s, 1H), 6.55 (s, 1H), 6.57 (d, 1H, J = 6.9), 6.71 (d,1H, J = 8.3), 7.05 (t, 1H, J = 8.0), 7.29−7.58 (m, 5H). IR (cm^{-1}, Nujol): 3303, 1650. Anal. ($C_{22}H_{24}N_2O_2$) C, H, N.

***(E)*-3-(4-Methoxy-1-phenyl-1H-indol-2-yl)propenenitrile (6b).** Cyanomethyltriphenylphosphonium chloride[34] (0.71 g, 2.1 mmol) was added to a stirred solution of 4-methoxy-1-phenyl-1H-indole-2-carboxaldehyde **(3b)**[32] (0.377 g, 1.5 mmol) and DBU (0.337 mL, 2.26 mmol) in toluene (25 mL), and the mixture was refluxed for 30 min. The mixture was cooled to room temperature, dichloromethane was added, and the organic phase was washed with water, dried (Na_2SO_4), and concentrated under reduced pressure. Purification by flash chromatography (silica gel; cyclohexane/EtOAc, 7:3, as eluent) and crystallization from dichloromethane/*n*-hexane gave 0.22 g (74% yield) of the desired compound **6b**; yellow solid (73% yield); mp 136 °C (dichloromethane/*n*-hexane). MS (EI): *m/z* 274 (M^+, 100). ^{1}H NMR ($CDCl_3$): δ 3.99 (s, 3H), 5.57 (d, 1H, J = 16.5), 6.56 (d, 1H, J = 7.8), 6.73 (d, 1H, J = 8.2), 7.14 (d, 1H, J = 16.5), 7.10−7.60 (m, 7H). IR (cm^{-1}, Nujol): 2215, 1622.

***N*-[3-(4-Methoxy-1-phenyl-1H-indol-2-yl)propyl]propanamide (7b).** A solution of **6b** (0.14 g, 0.52 mmol) in THF (10 mL) and propionic anhydride (1.1 mL) was hydrogenated over Raney nickel at 4 atm of H_2 for 6 h at 50 °C. The catalyst was filtered on Celite, the filtrate was concentrated in vacuo, and the residue was partitioned between ethyl acetate and 2 N NaOH. The organic layer was washed with brine, dried (Na_2SO_4), and evaporated under reduced pressure. Purification by flash chromatography (silica gel; EtOAc as eluent) and crystallization from ethyl acetate/*n*-hexane gave 0.245 g (79% yield) of the desired compound **7b** as a white solid; mp 83 °C. MS (EI): *m/z* 336 (M^+), 250 (100). ^{1}H NMR ($CDCl_3$): δ 1.07 (t, 3H, J = 7.6), 1.70−1.84 (m, 2H), 2.09 (q, 2H, J = 7.6), 2.67 (t, 2H,

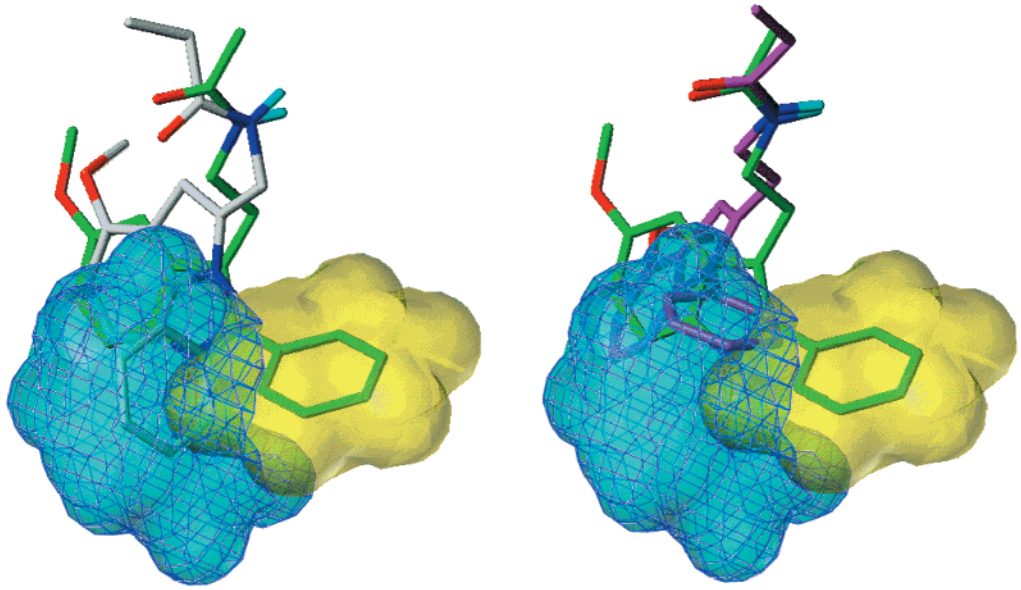


Figure 6. Representation of the volume occupied by the phenyl rings of 4-P-PDOT, luzindole, and **10f** (cyan), superposed according to the model syn, and of the volume occupied by the phenyl ring of 2-phenyl-MLT (yellow). 2-Phenyl-MLT is represented with green carbons and superposed onto **10f** (left, white carbons) or onto **5i** (right, magenta carbons).

J = 7.6), 3.23 (m, 2H), 3.98 (s, 3H), 5.27 (br s, 1H), 6.52 (s, 1H), 6.56 (d, 1H, J = 7.3), 6.70 (d, 1H, J = 8.2), 7.03 (t, 1H, J = 8.0), 7.30−7.55 (5H). IR (cm^{-1}, Nujol): 3318, 1644. Anal. ($C_{21}H_{24}N_2O_2$) C, H, N.

4-Methoxy-1-phenyl-1H-indol-2-carboxylic acid (8b). A solution of **1b**[32] (2.81 g, 10 mmol) in THF (20 mL), MeOH (20 mL), and 3 N KOH (10 mL) was stirred at room temperature for 16 h. The solvent was removed in vacuo, the residue was dissolved in water, and the solution was acidified with 6 N HCl and extracted with ethyl acetate. The organic phase was washed with brine, dried (Na_2SO_4), and evaporated in vacuo to give **8b** as white solid (92% yield); mp 218−219 °C (dichloromethane/*n*-hexane). MS (EI): m/z 267 (M^+, 100). ^{1}H NMR ($CDCl_3$): δ 3.99 (s, 3H), 6.55 (d, 1H, J = 7.8), 6.69 (d, 1H, J = 8.4), 7.21 (m, 1H J = 8.3 and J = 7.9), 7.25−7.51 (m, 5H), 7.67 (s, 1H).

6-Methoxy-1H-indol-2-carboxamide (9a). Thionyl chloride (1.29 mL) was added to a solution of the acid **8a**[32] (1.91 g, 10 mmol) in dry THF (30 mL), and the mixture was stirred at room temperature for 15 min under nitrogen, then heated at 50 °C for 4 h, and finally allowed to stand at room temperature for 2 h. The solvent and excess thionyl chloride were removed under reduced pressure, and the residue was dissolved in dry THF (40 mL). A saturated solution of ammonia in dichloromethane was poured into the ice-cooled solution of the acid chloride, and the mixture was stirred at room temperature for 16 h. Petroleum ether was added to the reaction mixture, and the brown solid that precipitated upon cooling to 0 °C was collected, washed with water, and dried (1.62 g; 85% yield). An analytical sample was prepared by recrystallization from ethyl acetate/petroleum ether; mp 194 °C. MS (EI): m/z 190 (M^+), 173 (100). ^{1}H NMR (DMSO-d_6): δ 3.49 (s, 3H), 6.16 (br s, 2H), 6.39 (d, 1H, J = 8.7), 6.57 (s, 1H), 6.72 (s, 1H), 7.13 (d, 1H, J = 8.5). IR (cm^{-1}, Nujol): 3369, 1650, 1628.

4-Methoxy-1-phenyl-1H-indol-2-carboxamide (9b). 9b was obtained following the above procedure by using the acid **8b** (2.67 g, 10 mmol) instead of the acid **8a**; beige solid (83% yield), mp 172 °C (ethyl acetate/petroleum ether). MS (EI): m/z 266 (M^+, 100). ^{1}H NMR ($CDCl_3$): δ 3.99 (s, 3H), 5.78 (br s, 2H), 6.57 (d, 1H, J = 7.8), 6.75 (d, 1H, J = 8.4), 7.19 (t, 1H, J = 7.9), 7.28−7.57 (m, 6H). IR (cm^{-1}, Nujol): 3375, 1642, 1629.

(4-Methoxy-1-phenyl-1H-indol-2-yl)methanamine. A solution of the amide **9b** (1.33 g, 5 mmol) in dry THF (25 mL) was added dropwise to a stirred ice-cooled suspension of $LiAlH_4$ (1.14 g, 30 mmol) in dry THF (25 mL) under nitrogen. Upon completion of the addition, the mixture was refluxed for 3 h. After the solution was cooled to 0 °C, water was added dropwise to destroy the excess hydride, the mixture was filtered on Celite, and the filtrate was concentrated *in a vacuum* and then acidified with 2 N HCl and partitioned between water and ethyl acetate. The aqueous phase was made alkaline with 6 N NaOH and then extracted (3×) with dichloromethane. The combined organic layers were washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure to give the crude amine, which was purified by trituration with diethyl ether; orange solid (yield 90%). MS (EI): m/z 252 (M^+, 100). ^{1}H NMR (acetone-d_6): δ 3.93 (s, 3H), 4.39 (s, 2H), 6.55−7.59 (m, 9H). IR (cm^{-1}, $CDCl_3$): 3465, 1587.

***N*-[(6-Methoxy-1H-indol-2-yl)methyl]propanamide (10a).** A solution of **9a** (0.95 g, 5 mmol) in dry THF (25 mL) was added dropwise to a stirred ice-cooled suspension of $LiAlH_4$ (1.14 g, 30 mmol) in dry THF (25 mL) under nitrogen. Upon completion of the addition, the mixture was refluxed for 3 h. After the mixture was cooled to 0 °C, water was added dropwise to destroy the excess hydride, the mixture was filtered on Celite, and the filtrate was concentrated in vacuo and partitioned between water and dichloromethane. The organic layer was washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure to give crude (6-methoxyindol-2-yl)methanamine, which was then used without any further purification. Propionic anhydride (0.64 mL, 5 mmol) was added to a solution of the crude amine in THF (15 mL) and TEA (0.65 mL, 5 mmol), and the mixture was stirred at room temperature for 6 h. The solvent was evaporated in vacuo, and the residue was dissolved in ethyl acetate. The solution was washed with a saturated $NaHCO_3$ aqueous solution and with brine, then it was dried (Na_2SO_4) and evaporated to give a solid residue that was purified by crystallization from ethyl acetate/hexane; white solid (80% yield), mp 138−139 °C. MS (EI): m/z 232 (M^+, 100). ^{1}H NMR ($CDCl_3$): δ 1.17 (t, 3H, J = 7.6), 2.24 (q, 2H, J = 7.6), 3.83 (s, 3H), 4.42 (d, 2H, J = 5.9), 6.06 (br s, 1H), 6.24 (s, 1H), 6.73−6.83 (m, 2H), 7.40 (d, 1H, J = 8.5), 8.92 (br s, 1H). IR (cm^{-1}, $CDCl_3$): 3400, 1650.

***N*-[(4-Methoxy-1-phenyl-1H-indol-2-yl)methyl]propanamide (10b).** Propionic anhydride (0.128 mL, 1 mmol) was added to a solution of (4-methoxy-1-phenyl-1H-indol-2-yl)-methanamine (0.27 g, 1 mmol) in THF (5 mL) and TEA (0.13 mL, 1 mmol), and the mixture was stirred at room temperature for 6 h. The solvent was evaporated in vacuo, and the residue was dissolved in ethyl acetate. The solution was washed with a saturated $NaHCO_3$ aqueous solution and with brine, then it

was dried (Na_2SO_4) and evaporated to give a solid residue that was purified by crystallization from dichloromethane/*n*-hexane; white solid (90% yield), mp 120 °C. MS (EI): *m*/*z* 308 (M^+, 100). ^{1}H NMR ($CDCl_3$): δ 1.08 (t, 3H, J = 7.6), 2.11 (q, 2H, J = 7.5), 3.98 (s, 3H), 4.50 (d, 2H, J = 5.0), 5.51 (br s, 1H), 6.57 (d, 1H, J = 7.7), 6.69 (s, 1H), 6.75 (d, 1H, J = 8.2), 7.08 (t, 1H, J = 7.7), 7.31−7.57 (m, 5H). IR (cm^{-1}, Nujol): 3390, 3323, 1658. Anal. ($C_{19}H_{20}N_2O_2$) C, H, N.

***N*-[(1-Benzyl-6-methoxy-1H-indol-2-yl)methyl]propanamide (11).** A solution of **10a** (0.232 g, 1 mmol) in dry DMF (4 mL) was added dropwise to a stirred suspension of sodium hydride (0.033 g of an 80% dispersion in mineral oil, 1.1 mmol) in dry DMF (2 mL) at 0 °C under a N_2 atmosphere. The mixture was stirred at 0 °C for 30 min, benzyl chloride (0.115 mL, 1 mmol) was then added, and the resulting mixture was stirred at room temperature for 16 h. After this time the reaction mixture was poured into ice/water (40 g) and extracted with ethyl acetate. The organic phase was washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure to give a residue that was purified by crystallization from CH_2Cl_2/*n*-hexane; white solid (51% yield), mp 160 °C. MS (EI): *m*/*z* 322 (M^+), 175 (100). ^{1}H NMR ($CDCl_3$): δ 0.92 (t, 3H, J = 7.6), 1.73 (q, 2H, J = 7.6), 3.79 (s, 3H), 4.58 (d, 2H, J = 5.4), 5.30 (br s, 1H), 5.32 (s, 2H), 6.45 (s, 1H), 6.71 (m, 1H), 6.81 (dd, 1H, J = 2.0 and J = 8.5), 6.92−7.25 (m, 5H), 7.49 (d, 1H, J = 8.3). IR (cm^{-1}, Nujol): 3305, 1642. Anal. ($C_{20}H_{22}N_2O_2$) C, H, N.

***N*-[(1-*p*-Chlorobenzyl-4-methoxy-1H-indol-2-yl)methyl]propanamide (12). 12** was prepared by N-alkylation of **10c**[32] (0.232 g, 1 mmol) with 4-chlorobenzyl chloride (0.16 g, 1 mmol) following the procedure described for compound **11**; white amorphous solid (73% yield), mp 218 °C (chloroform). MS (EI): *m*/*z* 356 (M^+), 175 (100). ^{1}H NMR ($CDCl_3$): δ 0.97 (t, 3H, J = 7.8), 1.91 (q, 2H, J = 7.8), 3.97 (s, 3H), 4.58 (d, 2H, J = 5.4), 5.33 (s, 2H), 5.40 (br s, 1H), 6.56 (d, 1H, J = 7.8), 6.62 (s, 1H), 6.83 (1H, J = 8.2), 6.85 (d, 2H, J = 8.5), 7.10 (m, 1H, J = 7.8 and J = 8.2), 7.21 (d, 2H, J = 8.5). IR (cm^{-1}, Nujol): 3307, 1639. Anal. ($C_{20}H_{21}ClN_2O_2$) C, H, N.

***N*-[2-(1-Benzyl-6-methoxy-1H-indol-2-yl)ethyl]acetamide (13). 13** was obtained following the above procedure for compound **11** by using the starting material **5g**[32] (0.232 g, 1 mmol) instead of **10a**; white solid (66% yield), mp 102 °C (ethyl acetate/*n*-hexane). MS (EI): *m*/*z* 322 (M^+), 263 (100). ^{1}H NMR ($CDCl_3$): δ 1.91 (s, 3H), 2.87 (t, 3H, J = 6.8), 3.52 (m, 2H), 3.79 (s, 3H), 5.30 (s, 2H), 5.63 (br s, 1H), 6.32 (s, 1H), 6.72 (d, 1H, J = 2.1), 6.80 (dd, 1H, J = 2.2 and, J = 8.5), 6.95−7.29 (m, 5H), 7.47 (d, 1H, J = 8.2). IR (cm^{-1}, Nujol): 3311, 1642.

***N*-[(1-Benzyl-4-methoxy-1H-indol-2-yl)methyl]acetamide (14). 14** was obtained following the above procedure for compound **11** by using **10d**[32] (0.218 g, 1 mmol) instead of **10a**; white solid (71% yield); mp 186−187 °C (dichloromethane/*n*-hexane). MS (EI): *m*/*z* 308 (M^+), 175 (100). ^{1}H NMR ($CDCl_3$): δ 1.60 (s, 3H), 3.98 (s, 3H), 4.58 (d, 2H), 5.33 (br s, 1H), 5.35 (s, 2H), 6.56 (d, 1H, J = 7.3), 6.62 (s, 1H), 6.87−7.26 (m, 7H). IR (cm^{-1}, Nujol): 3420, 1658. Anal. ($C_{19}H_{20}N_2O_2$) C, H, N.

***N*-[(3-Bromo-6-methoxy-1H-indol-2-yl)methyl]propanamide (15).** NBS (1.05 equiv) was added portionwise to an ice-cooled solution of **10a** (0.232 g, 1 mmol) in AcOH (4 mL), and the mixture was stirred at room temperature for 2 h. The mixture was then poured into an ice-cooled 30% NaOH solution and extracted (3×) with EtOAc. The combined extracts were washed with brine and dried (Na_2SO_4). Evaporation of the solvent gave a residue that was purified by chromatography (cyclohexane/EtOAc, 3:7, as eluent) to give **15** as a white solid (0.134 g, 45%); mp 131 °C dec (dichloromethane/*n*-hexane). MS (EI): *m*/*z* 312, 310 (M^+), 175 (100). ^{1}H NMR ($CDCl_3$): δ 1.17 (t, 3H, J = 7.5), 2.24 (q, 2H, J = 7.5), 3.83 (s, 3H), 4.50 (d, 2H, J = 5.8), 6.13 (br s, 1H), 6.80−6.84 (m, 2H), 7.36 (d, 1H, J = 8.8), 9.24 (br s, 1H). IR (cm^{-1}, Nujol): 3450, 1648. Anal. ($C_{13}H_{15}BrN_2O_2$) C, H, N.

***N*-[2-(3-Bromo-4-methoxy-1H-indol-2-yl)ethyl]propanamide (16). 16** was obtained following the above procedure for compound **15** by using **5d**[32] (0.246 g, 1 mmol) instead of **10a**; white amorphous solid (41% yield). MS (EI): *m*/*z* 324, 326 (M^+), 173 (100). ^{1}H NMR ($CDCl_3$): δ 1.14 (t, 3H, J = 7.5), 2.19 (q, 2H, J = 7.5), 3.02 (t, 2H, J = 6.5), 3.63 (m 2H), 3.95 (s, 3H), 5.77 (br s, 1H), 6.51−7.21 (m, 3H), 9.32 (br s, 1H). IR (cm^{-1}, Nujol): 3320, 1649. Anal. ($C_{14}H_{17}BrN_2O_2$) C, H, N.

***N*-[2-(1-Benzyl-3-bromo-6-methoxy-1H-indol-2-yl)ethyl)]acetamide (17). 17** was obtained following the above procedure for compound **15** by using **13** (0.322 g, 1 mmol) instead of **10a**; white solid (40% yield), mp 125 °C dec (dichloromethane/*n*-hexane). MS (EI): *m*/*z* 400, 402 (M^+), 91 (100). ^{1}H NMR ($CDCl_3$): δ 1.91 (s, 3H), 2.99 (t, 2H, J = 6.6), 3.40 (m, 2H), 3.79 (s, 3H), 5.38 (s, 2H), 5.65 (br s, 1H), 6.70 (s, 1H), 6.84−7.26 (m, 6H), 7.43 (d, 1H, J = 8.3). IR (cm^{-1}, Nujol): 3307, 1650. Anal. ($C_{20}H_{21}BrN_2O_2$) C, H, N.

***N*-[(1-Benzyl-3-bromo-4-methoxy-1H-indol-2-yl)methyl)]acetamide (18). 18** was obtained following the above procedure for compound **15** by using **14** (0.308 g, 1 mmol) instead of **10a**; white solid (47% yield), mp 150 °C (dichloromethane/*n*-hexane). MS (EI): *m*/*z* 386−388 (M^+), 91 (100). ^{1}H NMR ($CDCl_3$): δ 1.67 (s, 3H), 3.97 (s, 3H), 4.64 (d, 2H, J = 5.8), 5.45 (s, 2H), 5.56 (br s, 1H), 6.58 (d, 1H, J = 7.7), 6.87−7.23 (m, 7H). IR (cm^{-1}, Nujol): 3298, 1645. Anal. ($C_{19}H_{19}BrN_2O_2$) C, H, N.

(b) Pharmacology. Membrane Preparation. The preparation of membranes was described elsewhere.[24j, 31c] In short, NIH3T3 cells stably expressing the cloned human MT_1 or MT_2 receptor subtypes were grown to confluence. On the day of assay the cells were detached from the flasks with ethylenediaminetetraacetic acid (EDTA) (4 mM)/Tris-HCl (50 mM), pH 7.4, room temperature, and collected by centrifugation at 1000*g* for 10 min at 4 °C. The cells were suspended in EDTA (2 mM)/Tris-HCl (50 mM), homogenized in 10−15 volumes of ice-cold EDTA (2 mM)/Tris-HCl (50 mM) with an ultra-Turrax apparatus and centrifuged at 50000*g* at 4 °C for 25 min. The final pellet was then resuspended in ice-cold Tris-HCl (50 mM) assay buffer.

Membrane protein level was determined according to a previously reported method.[38]

2-[^{125}I]Iodomelatonin Binding Assays. Affinities of compounds were determined in competition binding assays by the displacement of 2-[^{125}I]iodomelatonin from MT_1 and MT_2 receptors expressed in NIH3T3 cells.

2-[^{125}I]iodomelatonin (100 pM) and the compound under test at 10 different concentrations were incubated with the receptor preparation for 90 min at 37 °C. The final membrane concentration was 5−10 μg protein per tube. The binding conditions were described in detail elsewhere.[39] IC_{50} values were determined by nonlinear fitting strategies, and pK_i values were calculated from the IC_{50} values using the Cheng−Prusoff equation.[40] In saturation studies 2-[^{125}I]iodomelatonin was added at concentrations ranging from 10 to 1000 pM.

Determination of the Intrinsic Activity: [^{35}S]GTPγS Binding Assays. [^{35}S]GTPγS binding studies in NIH3T3 cells expressing human cloned MT_1 or MT_2 receptors were performed as previously described.[24j,31c,32] The final pellet, obtained as described above (membrane preparation), was resuspended in ice-cold Tris-HCl assay buffer (50 mM) to give a final membrane concentration of 20−30 mg/mL. The membranes (15−25 μg of protein) were then incubated at 30 °C for 30 min in the presence and in the absence of melatonin analogues, 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). The final incubation volume was 100 μL. The incubation was terminated by the addition of ice-cold Tris-HCl buffer, pH 7.4 (1 mL), rapid vacuum filtration through Whatman GF/B glass-fiber filters, and three (3 mL) washes with ice-cold Tris-HCl buffer, pH 7.4. Bound radioactivity was determined by liquid scintillation spectrophotometry after overnight extraction in 4 mL of Filter-Count scintillation fluid. Basal binding was assessed in the absence of ligands, and nonspecific binding was defined using GTPγS (10 μM). Basal stimulation is the amount of [^{35}S]GTPγS specifically bound in the absence of compounds and was taken as 100%. The maximal G-protein activation was measured in each experiment by using MLT (100 nM). Compounds were added at three different concentrations (one concentration

equivalent to 100 nM of MLT, 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 to that of MLT. It was assumed that at the equivalent concentration the test compound occupies the same number of receptors as MLT, 100 nM. All measurements were performed in triplicate. The SEM values were below 15% of the mean.

(c) QSAR. The Fujita−Ban modification[41] of the Free−Wilson analysis[42] was applied to affinity and intrinsic activity data. In this approach, starting from a reference compound, the contribution of each chemical modification to biological activity (y) was calculated as

$$y = \sum_j b_j X_j + c$$

where b_j is the contribution of the chemical modification j calculated as the regression coefficient for the variable X_j, indicating the presence of that modification in each compound, and the intercept c is the calculated activity y for the reference compound. In this work the reference compound was defined by the following substituents: $R_1 = CH_3$, $R_2 = H$, $R_3 = 4\text{-}OCH_3$, $R_4 = H$, $n = 2$.

The affinity for each receptor subtype was expressed as pK_i, whereas the efficacy was expressed as relative intrinsic activity (IA_r), relating the maximal G-protein activation induced by each compound to that induced by MLT (see Table 1).

Multiple regression analysis (MRA) calculations were performed by an Excel (Microsoft Co., version 97) spreadsheet, employing the built-in statistical functions and automated macro procedures. For each subset of compounds, MRA models were calculated by including all the independent variables from Table 2, giving a nonsingular matrix, and all the combinations of smaller numbers of those variables. Typical statistical parameters[43] for some of these models are reported in Table 3. Standard deviation of the errors in prediction (SDEP) and the predictivity parameter Q^2 were calculated by cross-validation, omitting one compound at a time from the set according to the leave-one-out technique (LOO).[44] The best models, from a statistical point of view, were selected by looking for the best F (absence of nonsignificant X variables) value.

Statistical significance of the MRA models was considered to confirm the assumptions of the Free−Wilson analysis, i.e., that the change of biological activity resulting from a change of chemical structure is independent of all other modifications in the molecule and that these contributions are additive within congener series.[45]

(d) Molecular Modeling. Molecular modeling studies were performed by Sybyl 6.6[46] running on an SGI O2 workstation. Molecules were energy-minimized, using the standard Tripos force field[47] with the Powell method,[48] to an energy gradient of 0.01 kcal mol^{-1} $Å^{-1}$; the electrostatic contribution was ignored.

Compounds submitted to conformational analysis (luzindole, 4-P-PDOT, **5i**, and **10f**) were investigated by the systematic search routine of Sybyl. Rotatable bonds were rotated by steps of 30°, and the generated conformations were optimized to obtain minimum-energy geometry. The resulting conformers where then matched to reject duplicates.

For luzindole, the two single bonds of the benzyl group were the only bonds to be rotated because the acylaminoethyl chain was kept in the conformation proposed by a previously reported pharmacophore model for melatonin receptor ligands (model B in ref 22a). The conformational analysis of **5i** was limited to the three rotatable bonds corresponding to the ethylamino portion of the side chain in position 2 of the indole nucleus. In **10f**, four bonds were rotated: the two single bonds of the benzyl group and the two bonds around the methylene fragment of the side chain in position 2. The amido group was kept in trans conformation.

4-P-PDOT deserved particular attention because of its low conformational flexibility that can be very useful for the definition of a pharmacophore model for MT_2 receptor antagonists. 4-P-PDOT has a cis and a trans isomer, each isomer being a mixture of two enantiomers. Unfortunately, the literature does not report which isomer is the active one or if one of the enantiomers has selective affinity, and the authors do not specify the stereochemistry of the compound tested.[24b] For this reason all the isomers were studied.

For both the cis and the trans compounds, the conformers with the acylamino group in axial conformation were discarded on the basis of our published pharmacophore model for melatonin receptor ligands,[22a] where the acylamino group of 4-methoxytetralin derivatives was aligned in equatorial disposition. In the cis and trans isomers with an equatorial acylamino group, the 4-phenyl substituent adopts a pseudoequatorial or a pseudoaxial conformation, respectively. The minimum-energy conformations obtained by the Tripos force field were in good agreement with the crystal structures of 2-substituted 4-phenyltetralins in the Cambridge Structural Database.[49] The 2S,4R isomer of 4-P-PDOT was strictly superposable onto the 1R,3S-1-phenyl-3-amino-1,2,3,4-tetrahydronaphthalene (reference code: PEXLUZ)[50] for their common part, with a root mean square (rms) of 0.09 Å calculated for the carbon atoms of the tetralin and the phenyl rings.

For both the cis and trans isomers of 4-P-PDOT two enantiomers exist in which, maintaining a fixed orientation for the tetralin nucleus, the phenyl group is positioned above or beneath the plane of the nucleus. When the 2-acylamino chain is kept in equatorial conformation, it was possible to get a strict superposition of the 2S,4S cis isomer on the 2R,4S trans one, and the same was possible for the respective enantiomers. Only one out of each couple of diasteroisomers was therefore selected for the superposition with other compounds; in particular, the two cis enantiomers are represented in Figure 5.

Five conformers were found for luzindole, 27 for **10f**, and 23 for **5i**. In the following superposition, the four atoms of the amido group, the centroid of the benzene ring in the indole or tetralin nuclei, and the centroid of the aromatic substituent were taken as the points to be fitted. The lowest rms values were considered as the criterion for conformer selection.

The superposition of luzindole and 4-P-PDOT allowed the selection of three conformers of luzindole, one with the benzyl group above and two beneath the plane of the indole ring, considering the nitrogen atom on the bottom-right. The conformers of **10f** allowed the selection of two conformers of luzindole, one having the phenyl group above and the other beneath the plane.

Compounds **5i** and 2-phenyl-MLT were superposed to luzindole, fitting the four atoms of the amido group and the centroid of the benzene ring of the indole nucleus.

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