2-[*N*-Acylamino(C_1−C_3)alkyl]indoles as MT_1 Melatonin Receptor Partial Agonists, Antagonists, and Putative Inverse Agonists

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The synthesis of several novel indole melatonin analogues substituted at the 2-position with acylaminomethyl (**8**−**11**), acylaminoethyl (**5a**−**k**), or acylaminopropyl (**13**) side chains is reported. On the basis of a novel in vitro functional assay (specific binding of [^{35}S]GTPγS), which can discriminate agonist from partial agonist, antagonist, and inverse agonist ligands, **5a**,**g**,**h**,**j** and **13** were shown to be partial agonists, **5d**,**e** and **8**−**11** competitive antagonists, and **5b**,**c**,**k** putative inverse agonists. Binding and functional assays were performed on cloned human MT_1 receptor. Structure−activity relationship considerations indicate that *N*-[1-aryl-2-(4-methoxy-1*H*-indol-2-yl)(C_1−C_2)alkyl]alkanamides represent a lead structure for this type of ligands.

Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine, MLT) is the principal hormone of the vertebrate pineal gland. Its secretion, in humans and in diurnal and nocturnal animals alike, exhibits a circadian rhythm characterized by elevated peripheral blood levels at night. Recent studies on the pharmacology of MLT and on the distribution of its binding sites suggest that this neurohormone has a variety of biological effects.[1] MLT is involved in regulating circadian rhythms in birds, reptiles, and possibly mammals,[2] modulates various aspects of retinal physiology,[3] and has found practical use in inducing seasonally breeding animals to breed out of season.[4] The role exerted by MLT in humans is not yet completely understood; nevertheless, it has been suggested that MLT might be useful in the treatment of several disorders such as delayed sleep phase syndrome,[5] seasonal depression,[6] jet lag,[7] and shift work disturbances[8] and as a hypnotic agent.[9] These effects are mediated through high-affinity G-protein-coupled receptors[10] called MT_1 and MT_2;[11a,b] another putative low-affinity receptor for melatonin, termed MT_3,[11b,c] has also been described. Recent progress in this area has involved the cloning of melatonin receptors from *Xenopus* dermal melanophores[12] and from hamster, sheep, and human brains;[13] the physiological role of MT_1 and MT_2 receptor subtypes is beginning to be clarified.[14] MLT, in addition to its receptor-mediated effects, was also found to inhibit nitric oxide synthase[15] and to effectively scavenge free radicals of biological relevance.[16] Given this property, MLT probably has also a physiological neuroprotective role; further support of this hypothesis is lent by the results of studies using animal models in which MLT protected neurons from kainate-induced excitotoxicity,[17] thus suggesting that MLT might be considered for the treatment of neurodegenerative pathologies.[18] In other areas, MLT has been reported to modulate the immune system,[19] and it has been used as a coadjuvant in cancer therapy.[20]

Previous research focused on the synthesis of several potent indole and non-indole melatonin receptor agonists[21] in order to determine the structural parameters that regulate the binding of MLT to its receptor. A number of structure−activity relationships (SAR) have been identified, and recently we and other authors have proposed molecular models of the putative melatonin binding site.[22] The search for selective subtype agonists is, however, much less advanced[11c,23] and even less so for potent and possibly selective melatonin antagonists, because most of the currently available ligands, including luzindole,[24] 4-phenyl-2-(acylamino)tetralines,[11c,25] 2-phenyl-*N*-(cyclobutylcarbonyl)tryptamines,[26] S 20928,[27] GR 128107,[28] GR 135533,[28] and *N*-acetyltryptamine[29] (Chart 1), bind melatonin receptors with low to moderate affinity.[30] We therefore decided to concentrate on the discovery of melatonin receptor antagonists. For the evaluation of the intrinsic activity of our compounds, we relied on the method we had previously employed (GTPγS index[21c,22a]) and on the functional assay ([^{35}S]-GTPγS specific binding) (vide infra) which was recently developed to better define the profile of our compounds.[32]

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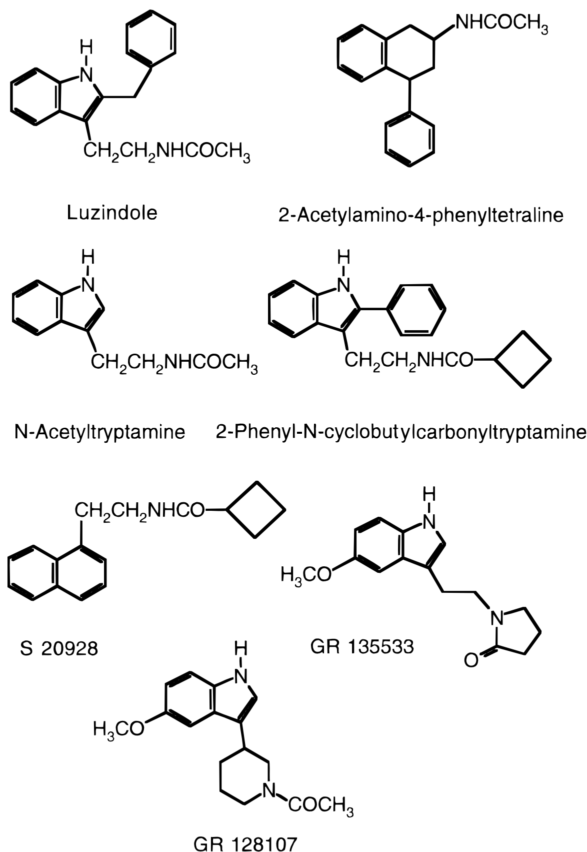
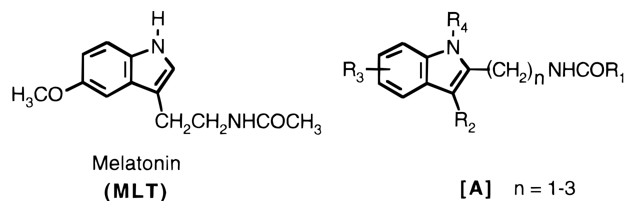
Chart 1. Chemical Structures of Melatonin Antagonists and Partial Agonists

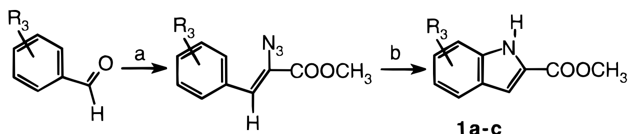


Little is known about the structural requirements for a competitive melatonin receptor antagonist; we started therefore from the assumption that such an antagonist could have a structure related to that of an agonist. The distance of the 5-methoxy and 3-alkylamido side chain from the center of the aromatic ring seems to be the essential feature for high-affinity agonist compounds. The presence or absence of either the methoxy[33] or the secondary amide of the 3-ethylamido side chain[28] seems to change the profile of the compounds from agonists to antagonists. Inspection of the structures of the antagonists and the partial agonists mentioned above (Chart 1) indicates that in some of these compounds the 5-methoxy group is absent (*N*-acetyltryptamine, luzindole, 4-phenyl-2-(acylamino)tetralines, 2-phenyl-*N*-(cyclobutylcarbonyl)tryptamine) and that in some other similar compounds a tertiary amide replaces the secondary amide of the 3-ethylamido side chain (GR 128107, GR 135533). In the hope of better modulating the fit to the MLT receptor (MT), we decided to retain both the secondary amide and the methoxy substituent but to modulate their relative distance by changing their position on the indole ring as well as the length of the ethylamido side chain. Furthermore, we substituted the indole nitrogen with a phenyl (**5f**,**h**), benzyl (**5j**,**11**), or methyl (**5i**) group and modified the nature of the acyl group (**5g**,**h**,**j**, **9**−**11**). In this paper, we report the synthesis of several indole melatonin analogues of general formula A (Chart 2), in which the methoxy group has been moved to the 4, 5, 6, and 7 positions of the indole ring, (C_1−C_3)alkyl-*N*-acylamino side chains stem from the 2-position of the indole ring, and the N_1 is variously substituted.

Chart 2. Chemical Structure of Melatonin and General Structure A of Compounds **5a**−**k** and **11**−**13**



Scheme 1[a]



[a] Reagents: (a) N_3CH_2COOMe, NaOMe, MeOH, −8 to 5 °C, 2 h; (b) xylene, reflux, 3 h.

Chemistry

Common starting materials for all target compounds **5a**−**k**, **8**−**11**, and **13** of general formula A (Chart 2) are the methyl indole-2-carboxylates **1a**−**f**, from which synthetic routes depart according to the length of the 2-alkylamido side chain (Schemes 2−4).

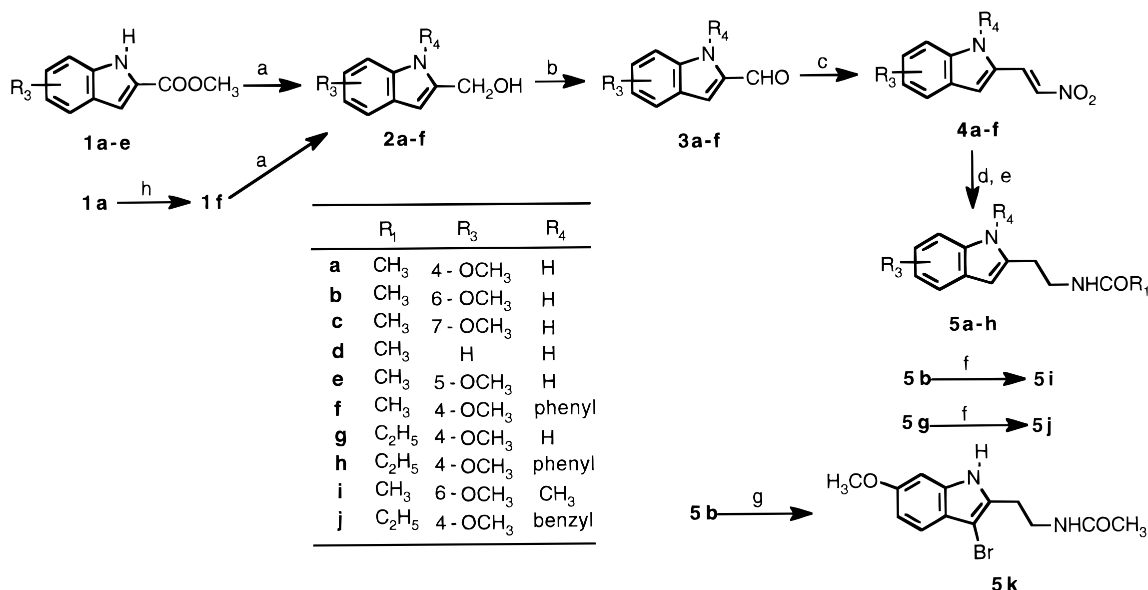
Indoles **1a**−**c** were synthesized by condensation of the 2-methoxy-, 4-methoxy-, and 3-methoxybenzaldehydes with methyl azidoacetate and pyrolysis of the resulting azidocinnamates according to a procedure previously described for related compounds[36] (Scheme 1). Indoles **1d**,**e** were obtained by esterification with diazomethane of the corresponding commercial acids. An Ullmann-type reaction[37] (iodobenzene, CuI, ZnO, K_2CO_3, 1-methyl-2-pyrrolidinone (NMP), 150 °C) was utilized for the synthesis of indole **1f** (Scheme 2).

The literature procedure[38] depicted in Scheme 2 was used for the synthesis of *N*-[2-(1*H*-indol-2-yl)ethyl]-acylamino derivatives **5a**−**h**. Methyl indole-2-carboxylates **1a**−**f** were reduced by $LiAlH_4$ to (2-indolyl)-methanol derivatives **2a**−**f**, which were oxidized by MnO_2 to indole-2-carboxaldehyde derivatives **3a**−**f**. Condensation of aldehydes **3a**−**f** with nitromethane in the presence of NH_4OAc afforded the corresponding nitroethenyl derivatives **4a**−**f**. Reduction of **4a**−**f** with $LiAlH_4$ followed by acylation with acetic (Ac_2O) or propionic anhydride in the presence of triethylamine (TEA) yielded the desired amides **5a**−**f** or **5g**,**h**, respectively (Scheme 2).

The *N*-indole-substituted compounds **5i**,**j** were prepared in dimethylformamide (DMF) by N-alkylation of **5b**,**g** with MeI or benzyl chloride, respectively, using sodium hydride as a base (Scheme 2). The 3-bromo derivative **5k** was synthesized by direct bromination of **5b** with *N*-bromosuccinimide (NBS) (Scheme 2).

The 2-acylaminomethyl target compounds **8**−**11** were prepared starting from methyl 4-methoxyindole-2-carboxylate, **1a**, as outlined in Scheme 3. 4-Methoxyindole-2-carboxylic acid (**6**), obtained by alkaline ester hydrolysis of **1a**, was reacted with thionyl chloride in dry tetrahydrofuran (THF), and the crude acid chloride was treated with a saturated solution of ammonia in dichloromethane to yield 4-methoxy-1*H*-indole-2-carboxamide, **7**. Reduction of **7** with $LiAlH_4$ (THF, reflux, 3 h) gave crude (4-methoxy-1*H*-indol-2-yl)methanamine which was subsequently acylated, with the suitable anhydride or

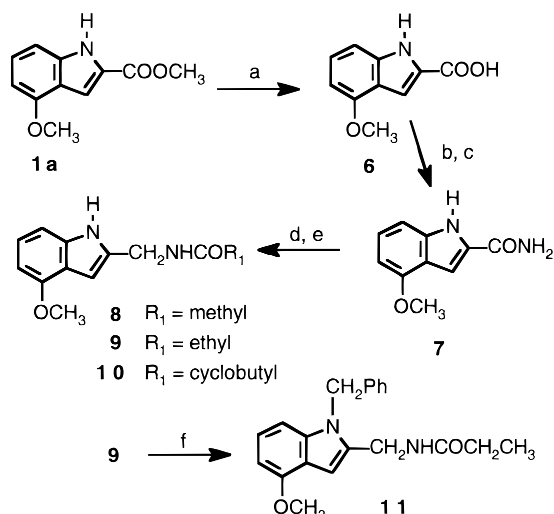
Scheme 2[a]



	R_1	R_3	R_4
a	CH_3	4-OCH_3	H
b	CH_3	6-OCH_3	H
c	CH_3	7-OCH_3	H
d	CH_3	H	H
e	CH_3	5-OCH_3	H
f	CH_3	4-OCH_3	phenyl
g	C_2H_5	4-OCH_3	H
h	C_2H_5	4-OCH_3	phenyl
i	CH_3	6-OCH_3	CH_3
j	C_2H_5	4-OCH_3	benzyl

[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) Ac_2O or $(EtCO)_2O$, TEA, THF, room temperature, 6 h; (f) NaH, DMF, MeI or benzyl chloride, room temperature, 16 h; (g) NBS, AcOH/dioxane, room temperature, 2 h; (h) iodobenzene, CuI, ZnO, K_2CO_3, NMP, 150 °C.

Scheme 3[a]

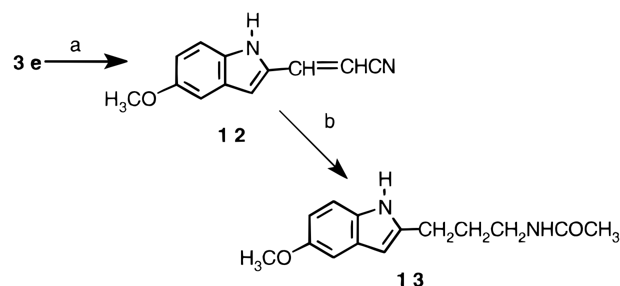


[a] Reagents: (a) 3 N KOH, MeOH, THF, room temperature, 16 h; (b) $SOCl_2$, THF, 50 °C, 4 h; (c) NH_3, THF, room temperature, 16 h; (d) $LiAlH_4$, THF, reflux, 3 h; (e) $(R_1CO)_2O$, TEA, THF, room temperature, 6 h (or cyclobutanoyl chloride, TEA, THF, room temperature, 2 h); (f) NaH, DMF, benzyl chloride, room temperature, 16 h.

acyl chloride in the presence of TEA, to afford carboxamides **8**−**10**. Compound **11** was obtained by N-alkylation of **9** with benzyl chloride in DMF, using sodium hydride as a base (Scheme 3).

Melatonin analogue **13**, *N*-[3-(5-methoxy-1*H*-indol-2-yl)propyl]acetamide, was obtained as described in Scheme 4. Aldehyde **3e**[38] was subjected to Wittig reaction conditions[39] using (cyanomethyl)triphenylphosphonium chloride[40] in the presence of 1,7-diazabicyclo[4.5.0]-undec-6-ene (DBU) to give an *E*/*Z* mixture of 5-methoxy-2-(cyanoethenyl)indole (**12**), which was converted to the final compound **13** by hydrogenation over Raney nickel and concomitant N-acylation with acetic anhydride (Scheme 4).

Scheme 4[a]



[a] Reagents: (a) $(Ph)_3P^+CH_2CN\ Cl^-$, DBU, toluene, reflux, 30 min; (b) Raney nickel, H_2, 4 atm, $(MeCO)_2O$, THF, 50 °C, 6 h.

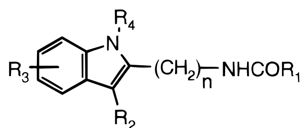
Pharmacology

Binding Studies. The affinity of the synthesized melatonin analogues (**5a**−**k**, **8**−**11**, **13**; Table 1) was determined using 2-[^{125}I]iodomelatonin (100 pM) as a labeled ligand in competitive binding analyses on cloned human MT_1 receptor subtype stably expressed in rat fibroblasts NIH3T3 cells (B_{max} = 620 fmol/mg of protein). The characterization of NIH3T3-MT_1 cells is described in detail elsewhere.[32] IC_{50} values were determined by nonlinear fitting strategies, and K_i values were calculated from the IC_{50} values using the Cheng−Prusoff equation.[41]

Intrinsic Activity Determination. The intrinsic activity of the novel compounds was measured by means of two methods (A and B).

Effect of Incubation with GTPγS and NaCl on the IC_{50} Values (Method A). This method is based on the measurement of the IC_{50} of the ligand for the G-protein-coupled[10,42] MT_1 receptor in the presence and absence of NaCl and guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS). According to the ternary complex model[43] the intrinsic activity of a ligand is related to the low-affinity/high-affinity state (K_{i-low}/K_{i-high}) ratio. Agonists possess higher affinity for the high-affinity conformation of the receptor (R_{high}), whereas the an-

Table 1. Binding Affinity[a] and Intrinsic Activity of *N*-[(1*H*-Indol-2-yl)alkyl]acylamino Derivatives **5a**–**k**, **8**–**11**, and **13** for the Human MT_1 Melatonin Receptor



compd	R_1	R_2	R_3	R_4	*n*	pK_i ± SEM	rel affin[b]	pRA[c]	GTPγS index[d] (method A)	G-protein activation[e] (method B)	rel intrin activity[f] (method B)	activity[g]
MLT						9.53 ± 0.04	1	0.00	1	325	1	A
NAS[h]						6.31			0.53		0.53	PA
N-CBCPT[h,i]						6.70			0.02		§	ANT
5a	CH_3	H	4-OCH_3	H	2	6.20 ± 0.02	2600	−3.41	0.34	235	0.6	PA
5b	CH_3	H	6-OCH_3	H	2	5.07 ± 0.05	32200	−4.51	0.025	90	−0.044	IA
5c	CH_3	H	7-OCH_3	H	2	5.42 ± 0.05	17000	−4.23	0.020	87	−0.057	IA
5d	CH_3	H	H	H	2	5.61 ± 0.03	9400	−3.97	0.15	120	0.09	ANT
5e	CH_3	H	5-OCH_3	H	2	4.79 ± 0.11	59700	−4.78	0.089	97	§	ANT
5f	CH_3	H	4-OCH_3	phenyl	2	7.06 ± 0.05	400	−2.60	0.23	238	0.61	PA
5g	CH_2CH_3	H	4-OCH_3	H	2	6.87 ± 0.07	690	−2.84	0.3	210	0.49	PA
5h	CH_2CH_3	H	4-OCH_3	phenyl	2	7.20 ± 0.03	260	−2.42	0.35	205	0.47	PA
5i	CH_3	H	6-OCH_3	CH_3	2	5.74 ± 0.01	6470	−3.81	0.19	170	0.31	PA
5j	CH_2CH_3	H	4-OCH_3	benzyl	2	6.08 ± 0.05	3300	−3.52	0.32	207	0.47	PA
5k	CH_3	Br	6-OCH_3	H	2	5.40 ± 0.07	15500	−4.19	0.036	85	−0.066	IA
8	CH_3	H	4-OCH_3	H	1	5.91 ± 0.09	6900	−3.84	0.021	118	0.08	ANT
9	CH_2CH_3	H	4-OCH_3	H	1	6.39 ± 0.06	2700	−3.44	0.024	113	0.05	ANT
10	cyclobutyl	H	4-OCH_3	H	1	6.28 ± 0.02	2700	−3.44	0.05	98	§	ANT
11	CH_2CH_3	H	4-OCH_3	benzyl	1	6.20 ± 0.04	3900	−3.59	0.09	106	0.03	ANT
13	CH_3	H	5-OCH_3	H	3	5.53 ± 0.03	13000	−4.12	0.13	200	0.44	PA

[a] pK_i values, calculated from the IC_{50} values using the Cheng–Prusoff equation,[41] are the means of at least three independent determinations. All the experiments were performed in duplicate. [b] Relative affinity = (IC_{50} compd)/(IC_{50} MLT) determined in parallel, in the same experiment. [c] pRA = −log of relative affinity. [d] GTPγS index = [(IC_{50} shift)compd]/[(IC_{50} shift)MLT]. [e] The data of [^{35}S]GTPγS binding experiments are given as percentage of basal binding, where the basal binding was fixed as 100%. [f] The relative intrinsic activity values were obtained by dividing the maximal net effect of a test compound by that of MLT. [g] A, agonist; PA, partial agonist; AN, antagonist. [h] See ref 30. [i] 2-Phenyl-*N*-(cyclobutylcarbonyl)tryptamine. § Compounds without effect on the basal activity.

tagonists cannot distinguish between R_{high} and the low-affinity conformation of the receptor (R_{low}) or have greater affinity for R_{low}. Guanine nucleotides and sodium ions are known to uncouple the G-protein from the receptor, promoting the R_{low} which has low affinity for agonists and high affinity for antagonists.[44] However, the previous characterization of the NIH3T3-MT_1 cells[32] showed that, even under extreme conditions [NaCl (700 mM) and GTPγS (100 μM)], it was impossible to shift the entire receptor population into the low-affinity state, and therefore we could not calculate the real K_{i-low} values through competition analysis. We took the IC_{50} shift, caused by the subtotal uncoupling of the G-protein-coupled receptor, as a measure of the K_{i-low}/K_{i-high} ratio, and we determined that shift by performing a series of experiments in the presence and absence of NaCl (700 mM) and GTPγS (100 μM) using 200 pM 2-[^{125}I]iodomelatonin as a labeled ligand. In the presence of GTPγS and NaCl the IC_{50} values of an agonist increase (the curve shifts to the right), whereas the IC_{50} values of an antagonist do not change or decrease (the curve shifts to the left).

A similar method had already been used to evaluate the intrinsic activity of melatonin analogues in quail optic tecta.[21c,22a]

Determination of the Activation of the G-Protein-Coupled MT_1 Receptor (Method B). This method is based on the measurement of the specific binding of [^{35}S]-GTPγS to the α subunit of the G-protein-coupled MT_1 receptor. The detailed description and validation of this method were published elsewhere;[32] we describe it here in its general outlines. One of the first events following agonist occupation of the binding domain of the G-protein-coupled receptors is the exchange reaction between GDP and GTP in the G_α subunit.[44] A dissociation of the G_α-GTP from the βγ subunit follows, with the subsequent downstream events stimulated by both G_α-GTP and βγ subunit. Therefore, the activation of the signal transduction cascade is directly dependent upon the rate of G_α-GTP formation. The activation of pertussis toxin-sensitive G-proteins after analogue occupation of membrane-bound receptors was previously determined in ligand-binding studies, using the increase of the [^{35}S]GTPγS binding to the membranes.[45]

The activation of the G-protein after the binding of an agonist to the MT_1 receptor was determined by measuring the specific binding of [^{35}S]GTPγS in the absence and presence of the test compounds in parallel with MLT as reference agonist. The amount of bound [^{35}S]GTPγS was directly proportional to the level of G-protein activation induced by the binding of the ligand to the receptor. In the case of method B, it is possible to observe not only the ability of the test compound to increase (agonist) or to leave either unaltered (antagonist) or almost completely unaltered (partial agonist) the binding of [^{35}S]GTPγS, but also its ability to decrease it (inverse agonist).

Results and Discussion

The affinity and the intrinsic activity of the compounds studied are shown in Table 1, and as predictable on the basis of the known structure–activity relationships of the MLT agonists, none of them behaved as a full agonist; rather, they were found to be antagonists, partial agonists, or putative inverse agonists depending on the assay performed (A or B). The two assays used

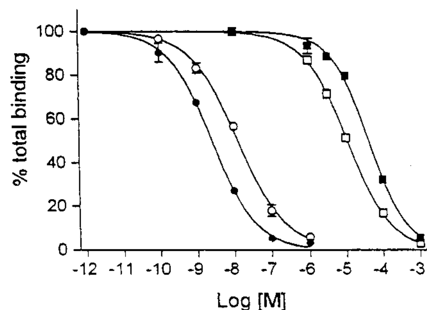


Figure 1. Example of the evaluation of the intrinsic activity by using the GTPγS index (method A). Representative competition binding curves to NIH3T3-MT_1 membranes of MLT (circles) and **5b** (squares) performed with a fixed concentration of the labeled ligand (200 pM 2-[^{125}I]iodomelatonin) in the absence (closed symbols) or presence (open symbols) of GTPγS (100 μM) and NaCl (700 mM). The experiment was carried out at 37 °C for 90 min. The MLT competition curve in the presence of GTPγS and NaCl is shifted to the right, whereas it is shifted to the left in the case of the antagonist **5b**.

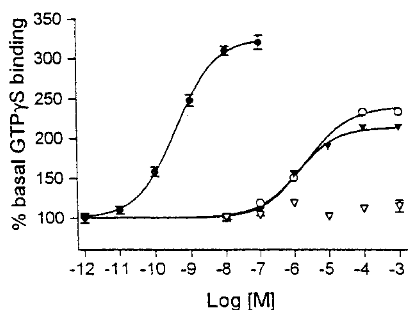


Figure 2. Examples of the evaluation of the intrinsic activity by measuring the stimulation of [^{35}S]GTPγS binding (method B) to NIH3T3-MT_1 membranes by MLT (closed circles), **5a** (open circles), **5j** (closed triangles), and **9** (open triangles). Values represent percentage of the basal binding, taken as 100%.

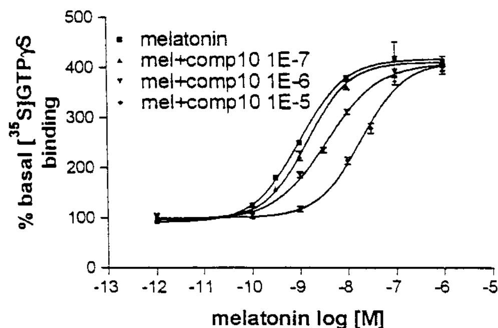


Figure 3. Representative experiment of concentration–effect relationship on the stimulation of [^{35}S]GTPγS binding to NIH3T3-MT_1 membranes by MLT in the absence and presence of three increasing concentrations of antagonist **10**.

to evaluate the intrinsic activity of the novel melatonin analogues gave similar results, although they are based on different approaches. The relative indices (GTPγS index and G-protein activation) are significantly correlated ($r = 0.879$, $p < 0.0001$), allowing the estimation of the intrinsic activity of a large number of MLT analogues by a rapid and reliable procedure. Method B was recently validated, in the same clone used here, with known well-described melatonin analogues, i.e., the full agonists 2-phenylmelatonin, 2-bromomelatonin, and 6-chloromelatonin, the partial agonist *N*-acetylserotonin (NAS), and the antagonist *N*-[(2-phenyl-1*H*-indol-3-yl)-ethyl]cyclobutanecarboxamide (*N*-CBCPT).[32] Furthermore **10**, taken as representative of the antagonist compounds reported here, antagonized melatonin-stimulated [^{35}S]GTPγS binding when investigated at three different concentrations (0.1, 1, and 10 μM) in a series of melatonin concentration–effect experiments. Incubation of **10** with MLT induced a dose-dependent rightward shift in the melatonin concentration–effect curve (Figure 3), thus exhibiting the behavior of a competitive antagonist. The K_b value of **10**, calculated by using the equation proposed by Lew and Angus,[46] was 350 nM, a value similar to the affinity constant (K_i = 520 nM) for MT_1 melatonin receptor subtype.

The compounds described here constitute an exploratory set, and the influence of structural variation on affinity and intrinsic activity was analyzed by using the Fujita–Ban modification[47] of the Free–Wilson approach. In our analysis, compounds **5c–e** and **13** were excluded because the calculation of their group contributions is trivial, whereas the three 6-methoxy derivatives (**5b**,**i**,**k**) were included, even though the CH_3 in R_4 (**5i**) and Br in R_2 (**5k**) led to single-point determinations (see Experimental Section for discussion). The contribution of the 6-OMe group was calculated as the average of the three cited compounds.

N-[(4-Methoxy-1*H*-indol-2-yl)ethyl]acetamide, **5a**, was taken as the reference compound, and the structural modifications considered were therefore R_4 = phenyl or benzyl or CH_3, R_1 = CH_2CH_3 or *c*-C_4H_7, R_3 = 6-CH_3O, $n = 1$. The relative affinity to MLT was given as its −log (pRA, see Table 1) and employed as a dependent variable, whereas to express the intrinsic activity, the GTPγS index (method A) and G-protein activation values (method B), respectively, were used. The data matrix was built as in Table 2; the coefficients of the independent variables, calculated by multiple regression analysis (MRA), indicate the gain (or loss, if negative) in the dependent variable, caused by the presence of the corresponding group with respect to the reference compound.

The results of MRA on the data matrix of Table 2 are reported in Table 3. Three equations were obtained (eqs 1–3) for affinity and intrinsic activity measured with the two methods (A and B, respectively); the affinity and intrinsic activity values were recalculated with the three models and are reported in Table 2. Most of the variance can be explained by the group contributions ($R^2 > 0.9$), with small standard errors for the three tests, confirming the additivity of these contributions. The relatively low values of F are due to the small number of residual degrees of freedom; method B gave a more significant model than the activity method A. The group contributions calculated from single-point contributions (R_4 = Me, R_1 = *c*-But, and R_3 = 6-OMe) have qualitative value, whereas the significance of the coefficient of the other variables can be evaluated on the basis of their standard errors. It can be observed that the affinity for the MLT receptor is significantly improved (around 0.7 log unit) when the hydrogen in R_4 is replaced by a phenyl group and to a lesser extent when the acetyl is replaced by the propionyl derivatives (R_1 = CH_2CH_3). With regard to intrinsic activity (method B), the only significant contribution (signifi-

Table 2. Data Matrix for the Free−Wilson Analysis and Calculated Values of Affinity, pRAc, and Intrinsic Activity, Ac and Bc, According to Eqs 1−3 (See Table 3)

	R_1		R_2	R_3	R_4						
compd	Et	*c*-But	Br	6-OMe	Bz[a]	Ph	Me	$n = 1$	pRAc[b]	Ac[c]	Bc[d]
5a	0	0	0	0	0	0	0	0	−3.39	0.295	232
5b	0	0	0	1	0	0	0	0	−4.51[e]	0.025[e]	90[e]
5f	0	0	0	0	0	1	0	0	−2.71	0.276	232
5g	1	0	0	0	0	0	0	0	−3.01	0.323	211
5h	1	0	0	0	0	1	0	0	−2.32	0.304	211
5i	0	0	0	1	0	0	1	0	−3.81[e]	0.190[e]	170[e]
5j	1	0	0	0	1	0	0	0	−3.37	0.343	209
5k	0	1	1	1	0	0	0	0	−4.19[e]	0.036[e]	85[e]
8	0	0	0	0	0	0	0	1	−3.76	0.020	127
9	1	0	0	0	0	0	0	1	−3.37	0.048	106
10	0	1	0	0	0	0	0	1	−3.44	0.050	98
11	1	0	0	0	1	0	0	1	−3.74	0.067	104

[a] Bz, benzyl. [b] Calculated value from eq 1. [c] Calculated value, method A, from eq 2. [d] Calculated value, method B, from eq 3. [e] Calculated from single-point determinations.

Table 3. Results of the MRA Analysis on the Independent Variable (**X** Matrix) Reported in Table 2

	eq 1 (pRA) coeff ± SE	eq 2 (method A) coeff ± SE	eq 3 (method B) coeff ± SE
$R_4 = CH_2Ph$	−0.367 ± 0.178	0.020 ± 0.051	−2.0 ± 8.2
R_4 = Ph	0.687 ± 0.178	−0.019 ± 0.051	0.0 ± 8.2
R_4 = Me[a]	0.697 ± 0.263	0.165 ± 0.075	80.0 ± 12.1
R_1 = Et	0.386 ± 0.152	0.028 ± 0.043	−21.0 ± 7.0
R_1 = *c*-But[a]	0.320 ± 0.234	0.030 ± 0.066	−29.0 ± 10.8
R_2 = Br[a]	0.310 ± 0.263	0.011 ± 0.075	−5.0 ± 12.1
R_3 = 6-OMe[a]	−1.115 ± 0.234	−0.270 ± 0.066	−142.0 ± 10.8
$n = 1$	−0.366 ± 0.152	−0.275 ± 0.043	−105.0 ± 7.0
intercept	−3.393 ± 0.142	0.295 ± 0.040	232.0 ± 6.5
R^2	0.975	0.960	0.994
s	0.186	0.053	8.6
n	12	12	12
F	14.5	8.92	67.2

[a] Calculated from single-point determinations.

cance level 95%, eq 3), not deriving by single-point determinations, is the shift toward antagonism caused by the shortening of the side chain ($n = 1$). The uncertainty of the contribution of the propionyl group was clarified by means of a model that excludes compounds and variables leading to single-point determinations and that selects, by a stepwise process,[48] the remaining significant variables; eq 4 was obtained:

$$B = 232.1(\pm 4.2) - [105.2(\pm 4.9) \times (n = 1)] - [21.8(\pm 4.9) \times (R_1 = \text{Et})] \quad (4)$$

$$N = 8 \quad R^2 = 0.990 \quad s = 6.7 \quad F = 247.2$$

This equation confirms the additivity and the statistical significance ($p < 0.05$) of the two contributions ($n = 1$, R_1 = Et), with a greater number of degrees of freedom.

Based on these results, it is possible to derive some information on the structural requirements of an antagonist. The 1-substitution (R_4) is quite important for the affinity and the activity of the compounds: the positive effect of the phenyl ring on affinity (see **5f**,**h** vs **5a**,**g**) is probably not simply related to the bulk or lipophilicity of the substituent, as the same effect is displayed by methyl (**5i**) and an opposite effect is produced by benzyl (**5j**) (see Table 3); on the contrary, the influence of 1-substitution on intrinsic activity seems negligible with the exception of the methyl derivative **5i** which has increased intrinsic activity with respect to **5b**. Another key role is played by the shortening of the side chain ($n = 1$, **8**−**11**), which leads to a decrease of the K_i values by a factor of ≈2 and to a remarkable, statistically significant loss of intrinsic activity. As for the acyl residue (R_1), the propionyl group is favorable for affinity and activity. The effect of the 3-bromo substitution (**5k** vs **5b**) is not very important. Moving the methoxy group from position 4 to position 5 (**5e** vs **5a**), 6 (**5b** vs **5a**), or 7 (**5c** vs **5a**), or abolishing it altogether (**5d** vs **5a**), decreases the affinity of the ligand by a factor of 23, 13, 6, and 4, respectively, but changes the profile from that of a partial agonist to that of a putative inverse agonist (**5b**,**c**,**k**) or antagonist (**5e**,**d**). It is important to note that **5h** (K_i = 63 nM), **5f** (K_i = 89 nM), and **5g** (K_i = 140 nM) have affinities for the MT_1 receptor at submicromolar level. Although it is difficult to compare the biological activity of compounds evaluated by different assays and by different groups, it is apparent that some of our antagonist/partial agonist compounds are endowed with affinities that, albeit low in absolute value, are among the highest reported for the MT_1 receptor subtype.[28,30]

In the case of **5b**,**c**,**k**, their inverse agonist profile, due to the small observed decrement of the [^{35}S]GTPγS binding and to the lack of a supporting functional assay, remains unproved. **5b**,**c**,**k** were tested six times each, and in each case there was a statistically significant reduction of the baseline level of the [^{35}S]GTPγS binding (**5b**, $P < 0.0001$; **5c**, $P < 0.0045$; **5b**, $P < 0.0166$), which in fact makes us classify these compounds as putative inverse agonists and not antagonists. A number of compounds have been reported to have a similar behavior, and they are referred to as inverse agonists.[49]

The close structural similarity of these ligands to each other and to MLT as well as their reversible binding to the receptor (competitive partial agonists/antagonists) makes it unlikely that they bind at sites different from those recognized by MLT. Although it is likely[28] that the affinities and the profile of the compounds are different for the MT_2 receptor, the problem of the selectivity of these compounds was not addressed at this time.

Conclusion

We have discovered, by using a novel in vitro functional test ([^{35}S]GTPγS), that chemical modifications of a series of 2-[*N*-acylamino(C_1−C_3)alkyl]indoles provide compounds acting as partial agonists, antagonists, or putative inverse agonists at the MT_1 receptor subtype.

The *N*-phenyl substitution of the indole ring, coupled with the 2-ethylamido side chain, is particularly important for enhancing the affinity of the compounds. This is shown by **5f**,**h**, which have submicromolar affinity for the receptor and can be classified as partial agonists. All the compounds with shortened chain length ($n = 1$, **8**−**11**) behaved as antagonists. These findings suggest that *N*-[1-aryl-2-(4-methoxy-1*H*-indol-2-yl)(C_{1-2})alkyl]-acylamines are lead structures for the discovery of MT_1-antagonist compounds.

Experimental Section

(a) Chemical Materials and Methods. Indoles **1d**,**e** were purchased from Aldrich Chemical Co. Compounds **3b**,**e**, **4c**,**e**,[38] and **5d**[50] were prepared according to the literature.

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 spectrometer. 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 were performed on a Carlo Erba analyzer. High-resolution mass spectra (HRMS) were performed by the peak-matching technique on a VG (Altrincham, UK) ZAB 2F instrument operating in EI conditions (70 eV, 200 μA) and with a resolving power of 5000 (10% valley definition).

General Procedure for the Synthesis of *N*-[2-(1*H*-Indol-2-yl)ethyl]alkanamides 5a−h. The following procedure for the synthesis of *N*-[2-(4-methoxy-1H-indol-2-yl)ethyl]-acetamide (**5a**) is representative of the synthesis of **5a**−**h**.

(4-Methoxy-1*H*-indol-2-yl)methanol (2a). A solution of methyl 4-methoxy--indole-2-carboxylate[36] (1.5 g, 7.31 mmol) in dry THF (25 mL) was added dropwise to a stirred suspension of $LiAlH_4$ (0.36 g) in dry THF (15 mL) at 0 °C under nitrogen. The mixture was stirred for an additional 45 min at room temperature, and the unreacted $LiAlH_4$ was destroyed by careful addition of water. The resulting mixture was filtered through a Celite pad, and the filtrate was dried over Na_2SO_4. The dried solution was concentrated under reduced pressure to give 1.23 g (95% yield) of a white solid. An analytical sample was obtained by crystallization from EtOAc/*n*-hexane: mp 151 °C; MS (EI) *m*/*z* 177 (M^+, 100); ^{1}H NMR ($CDCl_3$) δ 1.77 (t, 1H, $J = 6.1$), 3.95 (s, 3H), 4.81 (d, 2H, $J = 5.9$), 6.53 (d, 1H, $J = 7.7$), 6.54 (m, 1H), 6.99 (d, 1H, $J = 8.1$), 7.12 (dd, 1H, $J = 8.1$, 7.7), 8.35 (br s, 1H); IR (cm^{-1}, Nujol) 3506, 3253, 1591.

4-Methoxy-1*H*-indole-2-carboxaldehyde (3a). Activated manganese dioxide (4.3 g) was added to a solution of **2a** (1.23 g, 6.95 mmol) in dry dichloromethane (50 mL). The mixture was stirred for 7 h at room temperature and then filtered. The filter cake was washed with hot acetone (4 × 15 mL), and the combined filtrates were concentrated under reduced pressure to yield 0.62 g (51%) of **3a** as a brown solid which was used without any further purification: MS (EI) *m*/*z* 175 (M^+, 100); ^{1}H NMR ($CDCl_3$) δ 3.98 (s, 3H), 6.53 (d, 1H, $J = 7.7$), 7.02 (d, 1H, $J = 8.4$), 7.32 (dd, 1H, $J = 7.7$, 8.4), 7.39 (m, 1H), 8.95 (br s, 1H), 9.79 (s, 1H); IR (cm^{-1}, Nujol) 3278, 1652.

(*E*)-4-Methoxy-2-(2-nitroethenyl)-1*H*-indole (4a). A solution of **3a** (0.3 g, 1.71 mmol) and ammonium acetate (0.06 g) in nitromethane (2.4 mL) was heated at reflux for 1.5 h under nitrogen. After the mixture cooled to room temperature, ethyl acetate was added, and the organic phase was washed twice with water and dried (Na_2SO_4). The solvent was evaporated in vacuo to give a residue which was purified by column chromatography (silica gel, dichloromethane as eluent) followed by crystallization from acetone/*n*-hexane: yield 0.31 g (80%); mp 170−172 °C; MS (EI) *m*/*z* 218 (M^+), 157 (100); ^{1}H NMR ($CDCl_3$) δ 3.97 (s, 3H), 6.53 (d, 1H, $J = 7.7$), 6.99 (d, 1H, $J = 8.4$), 7.18 (m, 1H), 7.28 (dd, 1H, $J = 7.7$, 8.4), 7.48 (d, 1H, $J = 13.7$), 8.04 (d, 1H, $J = 13.7$), 8.36 (br s, 1H); IR (cm^{-1}, Nujol) 3418, 1608.

***N*-[2-(4-Methoxy-1*H*-indol-2-yl)ethyl]acetamide (5a).** Compound **4a** (0.22 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 vacuo and then partitioned between water and ethyl acetate. The organic layer was washed with brine, dried (Na_2SO_4), and then concentrated under reduced pressure to give a crude oily amine which was used without further purification. The crude amine was dissolved in THF (5 mL) and stirred at room temperature for 6 h with Ac_2O (0.093 mL, 1 mmol) in the presence of TEA (0.13 mL, 1 mmol). 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 followed by brine, dried (Na_2SO_4), and evaporated again. Purification by flash chromatography (silica gel, EtOAc as eluent) gave 0.098 g (42%) of the desired product **5a** as a white solid: mp 107 °C (EtOAc/*n*-hexane); MS (EI) *m*/*z* 232 (M^+), 173 (100); ^{1}H NMR ($CDCl_3$) δ 1.95 (s, 3H), 2.97 (t, 2H, $J = 6.6$), 3.60 (m, 2H), 3.95 (s, 3H), 5.70 (br s, 1H), 6.38 (m, 1H), 6.52 (d, 1H, $J = 7.7$), 6.98 (dd, 1H, $J = 8.0$, 0.7), 7.06 (dd, 1H, $J = 8.0$, 7.7), 8.47 (br s, 1H); IR (cm^{-1}, Nujol) 3411, 3172, 1662. Anal. ($C_{13}H_{16}N_2O_2$) C, H, N.

6-Methoxy-2-(2-nitroethenyl)-1*H*-indole (4b): yield 70%; mp 189−191 °C dec; MS (EI) *m*/*z* 218 (M^+, 100); ^{1}H NMR (acetone-d_6) δ 3.84 (s, 3H), 6.77 (dd, 1H, $J = 2.4$, 8.8), 6.90 (d, 1H, $J = 2.2$), 7.17 (m, 1H), 7.55 (d, 1H, $J = 8.8$), 7.82 (d, 1H, $J = 13.4$), 8.10 (d, 1H $J = 13.4$), 10.71 (br s, 1H); IR (cm^{-1}, Nujol) 3351, 3079, 1606.

***N*-[2-(6-Methoxy-1*H*-indol-2-yl)ethyl]acetamide (5b):** white solid (42% yield); mp 142−143 °C (EtOAc/*n*-hexane); MS (EI) *m*/*z* 232 (M^+), 173 (100); ^{1}H NMR (DMSO) δ 1.79 (s, 3H), 2.78 (t, 2H, $J = 7.3$), 3.29 (m, 2H), 3.72 (s, 3H), 6.06 (m, 1H), 6.57 (dd, 1H, $J = 2.0$, 8.3), 6.77 (d, 1H, $J = 2.0$), 7.26 (d, 1H, $J = 8.3$), 7.96 (br s, 1H), 10.75 (br s, 1H); IR (cm^{-1}, Nujol) 3297, 1641. Anal. ($C_{13}H_{16}N_2O_2$) C, H, N.

***N*-[2-(7-Methoxy-1*H*-indol-2-yl)ethyl]acetamide (5c):** beige solid (37% yield); mp 117−120 °C (diethyl ether); MS (EI) *m*/*z* 232 (M^+), 173 (100); ^{1}H NMR ($CDCl_3$) δ 1.96 (s, 3H), 2.99 (t, 2H, $J = 6.7$), 3.61 (m, 2H), 3.96 (s, 3H), 5.62 (br s, 1H), 6.27 (m, 1H), 6.62 (d, 1H, $J = 7.6$), 7.02 (dd, 1H, $J = 7.6$, 7.9), 7.17 (dd, 1H, $J = 7.9$, 0.6), 8.36 (br s, 1H); IR (cm^{-1}, Nujol) 3373, 3269, 1662. Anal. ($C_{13}H_{16}N_2O_2$) C, H, N.

***N*-[2-(1*H*-Indol-2-yl)ethyl]acetamide (5d):** corresponding to lit.[50]

***N*-[2-(5-Methoxy-1*H*-indol-2-yl)ethyl]acetamide (5e):** white solid (49% yield); mp 153 °C (EtOAc); MS (EI) *m*/*z* 232 (M^+), 173 (100); ^{1}H NMR ($CDCl_3$) δ 1.95 (s, 3H), 2.94 (t, 2H, $J = 6.3$), 3.58 (m, 2H), 3.85 (s, 3H), 5.74 (br t, 1H), 6.21 (m, 1H), 6.81 (dd, 1H, $J = 2.4$, 8.8), 7.02 (d, 1H, $J = 2.4$), 7.22 (d, 1H, $J = 8.8$), 8.48 (br s, 1H); IR (cm^{-1}, Nujol) 3400, 3179, 1655. Anal. ($C_{13}H_{16}N_2O_2$) C, H, N.

***N*-[2-(4-Methoxy-1-phenyl-1*H*-indol-2-yl)ethyl]acetamide (5f):** white solid (59% yield); mp 140 °C (EtOAc/*n*-hexane); MS (EI) *m*/*z* 308 (M^+), 249 (100); ^{1}H NMR ($CDCl_3$) δ 1.90 (s, 3H), 2.85 (t, 2H, $J = 6.4$), 3.46 (m, 2H), 3.99 (s, 3H), 5.56 (br s, 1H), 6.57 (m, 1H), 6.58 (d, 1H, $J = 7.6$), 6.72 (d, 1H, $J = 8.3$), 7.06 (dd, 1H, $J = 7.6$, 8.3), 7.30−7.58 (m, 5H); IR (cm^{-1}, Nujol) 3240, 1653. Anal. ($C_{19}H_{20}N_2O_2 \cdot {}^1/_4H_2O$) C, H, N.

Methyl 4-Methoxy-1-phenyl-1*H*-indole-2-carboxylate (1f). A mixture of **1a**[36] (2.05 g, 10 mmol), K_2CO_3 (1.75 g, 10.27 mmol), iodobenzene (3.5 g, 10.73 mmol), CuI (0.5 g), and ZnO (0.12 g) in NMP (20 mL) was heated at 150 °C for 6 h. After cooling to 0 °C, the insoluble materials were removed by filtration and the filtrate was partitioned between diethyl ether and 2 N NH_4OH. The organic layer was washed with brine, dried (Na_2SO_4), and evaporated under reduced pressure to give crude **1f** which was purified by flash chromatography (cyclo-

hexane/EtOAc, 9/1, as eluent) followed by crystallization from dichloromethane/*n*-hexane: white solid; 0.87 g, 31% yield; mp 91−92 °C; MS (EI) *m/z* 281 (M^+, 100); ^{1}H NMR ($CDCl_3$) δ 3.78 (s, 3H), 4.00 (s, 3H), 6.56 (d, 1H, *J* = 7.9), 6.69 (d, 1H, *J* = 8.3), 7.20 (dd, 1H, *J* = 7.9, 8.3), 7.32−7.55 (m, 6 H); IR (cm^{-1}, Nujol) 1718, 1616.

(4-Methoxy-1-phenyl-1*H*-indol-2-yl)methanol (2f) was prepared as described for **2a**, starting from **1f**: white solid, 94% yield; mp 97−98 °C (dichloromethane/*n*-hexane); MS (EI) *m/z* 253 (M^+, 100); ^{1}H NMR ($CDCl_3$) δ 3.99 (s, 3H), 4.66 (s, 2H), 6.57 (d, 1H, *J* = 7.7), 6.79 (m, 1H), 6.80 (d, 1H, *J* = 8.3), 7.10 (dd, 1H, *J* = 7.7, 8.3), 7.45−7.55 (m, 5H); IR (cm^{-1}, Nujol) 3395, 1597.

4-Methoxy-1-phenyl-1*H*-indole-2-carboxaldehyde (3f) was prepared as described for **3a**, starting from **2f**: white solid, 67% yield; mp 99−100 °C (Et_2O/*n*-hexane); MS (EI) *m/z* 251 (M^+, 100); ^{1}H NMR ($CDCl_3$) δ 4.01 (s, 3H), 6.57 (d, 1H, *J* = 7.7), 6.79 (d, 1H, *J* = 8.3), 7.28 (dd, 1H, *J* = 7.7, 8.3), 7.37−7.58 (m, 6H), 9.81 (s, 1H); IR (cm^{-1}, Nujol) 1674, 1612.

4-Methoxy-2-(2-nitroethenyl)-1-phenyl-1*H*-indole (4f) was prepared as described for **4a**, starting from **3f**: 83% yield; mp 184 °C (acetone/*n*-hexane); MS (EI) *m/z* 294 (M^+, 100); ^{1}H NMR ($CDCl_3$) δ 4.00 (s, 3H), 6.56 (d, 1H, *J* = 7.9), 6.72 (d, 1H, *J* = 8.3), 7.19 (d, 1H, *J* = 13.7), 7.21 (dd, 1H, *J* = 7.9, 8.3), 7.34−7.62 (m, 6H), 7.88 (d, 1H, *J* = 13.7); IR (cm^{-1}, Nujol) 1623, 1575.

***N*-[2-(4-Methoxy-1*H*-indol-2-yl)ethyl]propanamide (5g):** amorphous solid (58% yield); MS (EI) *m/z* 246 (M^+), 173 (100); ^{1}H NMR ($CDCl_3$) δ 1.12 (t, 3H, *J* = 7.6), 2.17 (q, 2H, *J* = 7.6), 2.99 (t, 2H, *J* = 6.4), 3.62 (m, 2H), 3.96 (s, 3H), 5.66 (br s, 1H), 6.38 (m, 1H), 6.53 (d, 1H, *J* = 7.5), 6.98 (d, 1H, *J* = 8.0), 7.08 (dd, 1H, *J* = 7.5, 8.0), 8.43 (br s, 1H); IR (cm^{-1}, Nujol) 3320, 3280, 1634. Anal. ($C_{14}H_{18}N_2O_2$) C, H, N.

***N*-[2-(4-Methoxy-1-phenyl-1*H*-indol-2-yl)ethyl]propanamide (5h):** amorphous solid (51% yield); MS (EI) *m/z* 322 (M^+), 173 (100); ^{1}H NMR ($CDCl_3$) δ 1.10 (t, 3H, *J* = 7.6), 2.12 (q, 2H, *J* = 7.6), 2.85 (t, 2H, *J* = 6.4), 3.45 (m, 2H), 3.99 (s, 3H), 5.55 (br s, 1H), 6.57 (m, 1H), 6.58 (d, 1H, *J* = 7.6), 6.71 (d, 1H, *J* = 8.3), 7.06 (dd, 1H, *J* = 7.6, 8.3), 7.31−7.55 (m, 5H); IR (cm^{-1}, Nujol) 3270, 1650; HRMS for $C_{20}H_{22}N_2O_2$ calcd 322.1676, found 322.1700 ± 0.003. Anal. ($C_{20}H_{22}N_2O_2$) C, H, N.

***N*-[(6-Methoxy-1-methyl-1*H*-indol-2-yl)ethyl]acetamide (5i).** A solution of **5b** (1 mmol) in dry DMF (3 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; after this time iodomethane (0.067 mL) was added, and the resulting mixture was stirred at room temperature for 16 h. The mixture was then 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 which was purified by crystallization from EtOAc/*n*-hexane: white solid (0.18 g, 72% yield); mp 108−109 °C; MS (EI) *m/z* 246 (M^+), 174 (100); ^{1}H NMR ($CDCl_3$) δ 1.96 (s, 3H), 2.96 (t, 2H, *J* = 6.7), 3.58 (m, 2H), 3.65 (s, 3H), 3.88 (s, 3H), 5.74 (br s, 1H), 6.22 (d, 1H, *J* = 0.6), 6.78 (m, 2H), 7.42 (d, 1H, *J* = 9.2); IR (cm^{-1}, $CHCl_3$) 3450, 1667. Anal. ($C_{14}H_{18}N_2O_2$) C, H, N.

***N*-[2-(1-Benzyl-4-methoxy-1*H*-indol-2-yl)ethyl]propanamide (5j).** A solution of **5g** (0.246 g, 1 mmol) in dry DMF (3 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) 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 which was purified by crystallization from CH_2Cl_2/*n*-hexane: white solid (77% yield); mp 137 °C; MS (EI) *m/z* 336 (M^+), 263 (100); ^{1}H NMR ($CDCl_3$) δ 1.10 (t, 3H, *J* = 7.6), 2.12 (q, 2H, *J* = 7.6), 2.89 (t, 2H, *J* = 6.4), 3.55 (m, 2H), 3.98 (s, 3H), 5.34 (s, 2H), 5.60 (br s, 1H), 6.49 (s, 1H), 6.56 (d, 1H, *J* = 7.63), 6.88 (d, 1H, *J* = 8.3), 7.08 (dd, 1H, *J* = 7.6, 8.3), 6.86−7.24 (m, 5H); IR (cm^{-1}, Nujol) 3309, 1635. Anal. ($C_{21}H_{24}N_2O_2$·$^1/_4H_2O$) C, H, N.

***N*-[2-(3-Bromo-6-methoxy-1*H*-indol-2-yl)ethyl]acetamide (5k).** NBS (1.05 equiv) was added portionwise to an ice-cooled solution of **5b** (0.232 g, 1 mmol) in AcOH (2.8 mL) and dioxane (1.4 mL), and the mixture was stirred at 0 °C 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 **5k** as a violet amorphous solid (0.265 g, 85%): MS (EI) *m/z* 312, 310 (M^+), 160 (100); ^{1}H NMR (DMSO-d_6) δ 1.78 (s, 3H), 2.81 (t, 2H, *J* = 7.3), 3.35 (m, 2H), 3.75 (s, 3H), 6.71 (dd, 1H, *J* = 1.9, 8.8), 6.83 (d, 1H, *J* = 1.9), 7.17 (d, 1H, *J* = 8.8), 7.98 (br s, 1H), 11.25 (br s, 1H); HMRS for $C_{13}H_{15}N_2O_2{}^{79}Br$ calcd 310.0313, found 310.0344 ± 0.003. Anal. ($C_{13}H_{15}BrN_2O_2$) H, N; C: calcd, 50.18; found, 49.38.

4-Methoxy-1*H*-indole-2-carboxylic Acid (6). A solution of **1a**[36] (2.05 g, 10 mmol) in THF (20 mL), MeOH (24 mL), and 3 N KOH (10 mL) was stirred at room temperature for 16 h. The solvents were 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 **6** (95% yield) as white solid, which was directly used without further purification: mp 240 °C.

4-Methoxy-1*H*-indole-2-carboxamide (7). Thionyl chloride (1.29 mL) was added to a solution of the crude acid **6** (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 (1.5 g, yield 80%). An analytical sample was prepared by recrystallization from methanol/diethyl ether/petroleum ether: mp 220 °C; MS (EI) *m/z* 190 (M^+), 173 (100); ^{1}H NMR (acetone-d_6) δ 3.92 (s, 3H), 6.53 (m, 1H), 6.69 (br s, 1H), 7.15 (m, 2H), 7.24 (m, 1H), 7.47 (br s, 1H), 10.79 (br s, 1H); IR (cm^{-1}, Nujol) 3461, 3368, 3283, 1647.

(4-Methoxy-1*H*-indol-2-yl)methanamine. A solution of **7** (1.9 g, 10 mmol) in dry THF (50 mL) was added dropwise to a stirred ice-cooled suspension of $LiAlH_4$ (2.28 g, 60 mmol) in dry THF (50 mL) under nitrogen. Upon completion of the addition, the mixture was refluxed for 3 h. After the mixture 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 the crude amine as a beige solid, mp 123−125 °C (yield 90%), which was then used without any further purification: MS (EI) *m/z* 176 (M^+), 72 (100); ^{1}H NMR ($CDCl_3$) δ 3.97 (s, 3H), 4.06 (s, 2H), 6.43 (m, 1H), 6.52 (dd, 1H, *J* = 7.6, 0.6), 6.98 (d, 1H, *J* = 7.9), 7.08 (dd, 1H, *J* = 7.6, 7.9), 8.52 (br s, 1H); IR (cm^{-1}, $CDCl_3$) 3465, 1587.

***N*-[(4-Methoxy-1*H*-indol-2-yl)methyl]acetamide (8).** The above crude amine (0.176 g, 1 mmol) was dissolved in THF (5 mL) and stirred at room temperature for 6 h with Ac_2O (0.093 mL, 1 mmol) in the presence of TEA (0.13 mL, 1 mmol). 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, and then it was dried (Na_2SO_4) and evaporated to give a solid residue which was purified by crystallization from ethyl acetate: white solid (85% yield); mp 174−5 °C; MS (EI) *m/z* 218 (M^+, 100); ^{1}H NMR

($CDCl_3$) δ 2.04 (s, 3H), 3.94 (s, 3H), 4.47 (d, 2H, *J* = 6.4), 6.04 (br s, 1H), 6.42 (m, 1H), 6.50 (d, 1H, *J* = 7.6), 6.96 (d, 1H, *J* = 8.3), 7.09 (dd, 1H, *J* = 7.6, 8.3), 8.98 (br s, 1H); IR (cm^{-1}, $CDCl_3$) 3453, 3052, 1664. Anal. ($C_{12}H_{14}N_2O_2$) C, H, N.

***N*-[(4-Methoxy-1*H*-indol-2-yl)methyl]propanamide (9)** was obtained following the above procedure by using propionic anhydride (0.128 mL, 1 mmol) instead of Ac_2O: white solid (85% yield); mp 117 °C (dichloromethane/*n*-hexane); MS (EI) *m*/*z* 232 (M^+, 100); ^1H NMR ($CDCl_3$) δ 1.17 (t, 3H, *J* = 7.6), 2.25 (q, 2H, *J* = 7.6), 3.94 (s, 3H), 4.47 (d, 2H, *J* = 6.0), 6.05 (br s, 1H), 6.41 (m, 1H), 6.51 (d, 1H, *J* = 7.6), 6.97 (d, 1H, *J* = 7.6), 7.09 (dd, 1H, *J* = 7.6, 7.9), 9.04 (br s, 1H); IR (cm^{-1}, $CDCl_3$) 3453, 3052, 1664. Anal. ($C_{13}H_{16}N_2O_2$) C, H, N.

***N*-[(4-Methoxy-1*H*-indol-2-yl)methyl]cyclobutanamide (10)** was obtained following the above procedure by using cyclobutanoyl chloride (0.12 g, 1 mmol) instead of Ac_2O: white solid (85% yield); mp 125 °C (dichloromethane/*n*-hexane); MS (EI) *m*/*z* 258 (M^+, 100); ^1H NMR ($CDCl_3$) δ 1.8−2.4 (m, 6H), 3.0 (m, 1H), 3.94 (s, 3H), 4.46 (d, 2H, *J* = 6.04), 5.95 (br s, 1H), 6.41 (m, 1H), 6.50 (dd, 1H, *J* = 7.6, 0.6), 6.98 (d, 1H, *J* = 7.9), 7.09 (dd, 1H, *J* = 7.6, 7.9), 9.07 (br s, 1H); IR (cm^{-1}, $CDCl_3$) 3449, 1663. Anal. ($C_{15}H_{18}N_2O_2$) C, H, N.

***N*-[(1-Benzyl-4-methoxy-1*H*-indol-2-yl)methyl]propanamide (11).** A solution of **9** (1 mmol) in dry DMF (2 mL) was added dropwise to a stirred ice-cooled suspension of sodium hydride (0.042 g of a 80% dispersion in mineral oil, 1.4 mmol) in dry DMF (3 mL) under a N_2 atmosphere. The suspension was stirred at 0 °C for 30 min, benzyl chloride (0.15 mL, 1.3 mmol) was added dropwise, the resulting mixture was stirred at room temperature for 16 h, and then it was poured into ice−water (25 g) and extracted with ethyl acetate (3 × 10 mL). The organic phase was washed with brine, dried (Na_2SO_4), and then concentrated under reduced pressure to give a residue which was purified by crystallization from ethyl acetate: white solid (80% yield); mp 199 °C; MS (EI) *m*/*z* 322 (M^+), 175 (100); ^1H NMR ($CDCl_3$) δ 0.93 (t, 3H, *J* = 7.6), 1.83 (q, 2H, *J* = 7.6), 3.98 (s, 3H), 4.6 (d, 2H, *J* = 5.4), 5.34 (br s, 1H), 5.36 (s, 2H), 6.57 (d, 1H, *J* = 7.6), 6.63 (s, 1H), 6.86−7.22 (m, 7H); IR (cm^{-1}, $CDCl_3$) 3534, 1673. Anal. ($C_{20}H_{22}N_2O_2\cdot{}^1/_4H_2O$) C, H, N.

(*E*)-3-(5-Methoxy-1*H*-indol-2-yl)propenenitrile (12). (Cyanomethyl)triphenylphosphonium chloride[40] (0.71 g, 2.1 mmol) was added to a stirred solution of **3e**[38] (0.26 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, 8/2, as eluent) and crystallization from dichloromethane/*n*-hexane gave 0.22 g (74% yield) of a 9:1 mixture (^1H NMR) of the *E*/*Z* geometrical isomers of **12**. An analytical sample of **12** was prepared by recrystallization from dichloromethane/*n*-hexane: MS (EI) *m*/*z* 198 (M^+, 100); ^1H NMR ($CDCl_3$) *E* isomer δ 3.90 (s, 3H), 5.63 (d, 1H, *J* = 16.6), 6.81 (br s, 1H), 7.01 (dd, 1H, *J* = 2.4, 8.8), 7.08 (s, 1H), 7.31 (d, 1H, *J* = 8.8), 7.39 (d, 1H, *J* = 16.6), 8.19 (br s, 1H); IR (cm^{-1}, Nujol) 3350, 2213, 1609.

***N*-[3-(5-Methoxy-1*H*-indol-2-yl)propyl]acetamide (13).** A solution of **12** (*E*/*Z*, 9:1 mixture) (0.14 g, 0.7 mmol) in THF (10 mL) and acetic anhydride (1.4 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) gave 0.13 g (81% yield) of the desired compound **13** as an oil: MS (EI) *m*/*z* 246 (M^+), 174 (100%); ^1H NMR ($CDCl_3$) δ 1.78 (m, 2H), 2.1 (s, 3H), 2.76 (t, 2H, *J* = 6.6), 3.37 (m, 2H), 3.8 (s, 3H), 5.65 (br s, 1H), 6.17 (m, 1H), 6.78 (dd, 1H, *J* = 8.7, 2.5), 7.01 (d, 1H, *J* = 2.5), 7.26 (d, 1H, *J* = 8.7), 9.28 (br s, 1H); IR (cm^{-1}, Nujol) 3281, 1624; HRMS for $C_{14}H_{18}N_2O_2$ calcd 246.1364, found 246.1374 ± 0.003. Anal. ($C_{14}H_{18}N_2O_2\cdot{}^1/_4CH_3COOC_2H_5$) C, H, N.

(b) Pharmacology. Membrane Preparation. NIH3T3 cells stably expressing the cloned human MT_1 receptor 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 ultra-Turrax, 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.[51]

2-[^{125}I]Iodomelatonin Binding Assays. Membranes were incubated (90 min at 37 °C) with 2-[^{125}I]iodomelatonin (100 pM) in the presence of melatonin analogues (10^{-11}−10^{-4} M). The final membrane concentration was 5−10 μg of protein per tube. The binding conditions are described in detail elsewhere.[52] In saturation studies 2-[^{125}I]iodomelatonin was added at concentrations ranging from 10 to 1000 pM. In competition curves with GTPγS (100 μM) and NaCl (700 mM), the radioligand concentration was 200 pM.[22a] 2-[^{125}I]iodomelatonin nonspecific binding was measured in the presence of 0.1 μM cold 2-iodomelatonin.

Method A. The IC_{50} shifts of the test compounds, induced by incubation with GTPγS (100 μM) and NaCl (700 mM), were calculated in competition analysis (2-[^{125}I]iodomelatonin, 200 pM) as the values of IC_{50} in the presence of GTPγS and NaCl vs the IC_{50} values in the absence of GTPγS and NaCl. The relative indices (GTPγS index) were calculated as the ratio [(IC_{50} shift)compound]/[(IC_{50} shift)MLT]. All measurements were made in duplicate and are reported as mean ± SEM of at least three independent experiments. The SEM values were 15% below the mean. Further details have been previously reported.[21c,22a]

[^{35}S]GTPγS Binding Assays (Method B). Agonist-stimulated [^{35}S]GTPγS binding was studied by using a modification of previously published methods.[45] Briefly, the final pellet, obtained as described above (membrane preparation), was resuspended into 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 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.

Basal binding was assessed in the absence of ligands, and nonspecific binding was defined using GTPγS (10 μM). The incubation was terminated by the addition of ice-cold Tris-HCl buffer, pH 7.4 (1 mL), and rapid vacuum filtration through Whatman GF/B glass-fiber filters followed by 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. The maximum intrinsic activity was measured in each experiment by using MLT (100 nM). All other compounds were tested at a concentration equivalent to 100 nM MLT. The equivalence was estimated on the basis of the ratio of the affinity of the test compound over that of MLT. It was assumed that at the equivalent concentration the test compound occupies the same number of receptors as MLT 100 nM. Partial agonist and antagonist compounds were also tested, whenever possible, at concentrations 10 and 100 times the equivalent dose to rule out the possibility of not reaching the maximum of the concentration−response curve.

Nonspecific binding, due to the high concentration (1 mM) of ligand used in the case of some low-affinity compounds, was ruled out by running the experiments in parallel, and according to the same protocol, on membranes from native NIH3T3 cells. MLT (10 μM) and **5a**−**d**,**f**,**g**,**k** (1mM) were tested against transfected and native NIH3T3 cells. None of these compounds modified the [^{35}S]GTPγS binding to the native NIH3T3 membranes.

The data from [^{35}S]GTPγS binding experiments are given as percentage of basal binding (100%) and are reported as

mean ± SEM of at least three independent experiments. All measurements were performed in triplicate. The SEM values were 15% below the mean. The relative intrinsic efficacy values are expressed as a fraction of the maximal net stimulation by melatonin.

(c) QSAR. The Fujita–Ban modification[47] of the Free–Wilson analysis was applied to the affinity and intrinsic activity data. This de novo method calculates the contribution to the activity (y) of any given compound, exerted by any given chemical substituent at any one position of the compound, relative to a reference molecule of activity μ, according to the equation:

$$y = S_j b_j X_j + \mu$$

where b_j is the contribution of the chemical modification j, calculated as the regression coefficient for the variable X_j, X_j indicates the presence ($X_j = 1$) or absence ($X_j = 0$) of that modification in any one compound, μ is the activity of the reference compound, and y is the calculated activity of compound i. MRA calculations were performed with an Excel (Microsoft Co., version 97) spreadsheet, employing the built-in functions.

The inclusion of single-point determinations deserves comment: it is not possible to evaluate the error associated with the contribution of groups that are used only once; however, their inclusion in the data set does not affect the coefficients or the SE of the contributions of the other groups as calculated by MRA. The coefficients deriving from single-point determinations, and the corresponding compounds, are indicated in Tables 2 and 3; the exclusion of the corresponding variables and compounds causes no changes in s and only small changes in R^2 (due to different initial variance) and F (due to different initial variance and different number of variables).

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