Identification of Bivalent Ligands with Melatonin Receptor Agonist and Fatty Acid Amide Hydrolase (FAAH) Inhibitory Activity That Exhibit Ocular Hypotensive Effect in the Rabbit

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Abstract

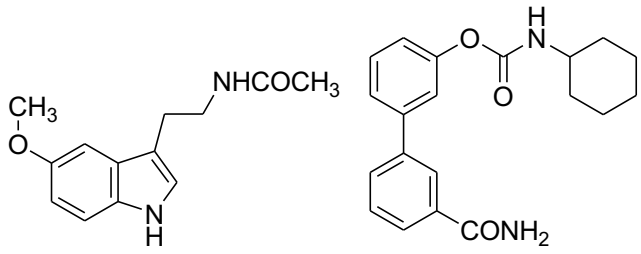
Activation of melatonin receptors and inhibition of fatty acid amide hydrolase (FAAH) have both shown potential benefits for the treatment of glaucoma. To exploit the combination of these biological activities in single therapeutic agents, we designed dual-acting compounds sharing the pharmacophore elements required for the two targets, in search for balanced potencies as MT_1/MT_2 agonists and FAAH inhibitors. In particular, the N-anilinoethylamide scaffold, previously developed for melatonergic ligands, was decorated at *meta* position with a polymethylene linker bound to an O-arylcarbamate group, substituted consistently with known structure-activity relationships for FAAH inhibition. For the most active series, the N-anilinoethylamide portion was also replaced with the indole scaffold of melatonin. O-biphenyl-3-ylcarbamate derivatives were characterized by remarkable and balanced activity at both targets, in the nanomolar range for compound **29**. Topical administration reduced elevated intraocular pressure in rabbits, with a longer action and improved efficacy compared to the reference compounds melatonin and URB597.

Introduction

Glaucoma is an invalidating eye disease characterized by high intraocular pressure (IOP) and progressive death of retinal ganglion cells (RGC). Chronically elevated IOP itself is a risk factor for the development of glaucoma and can lead to visual field impairment and loss of visual function as a consequence of optic nerve damage.[1] Current first-line treatment of this condition is generally based on daily topical administration of therapeutics aimed at lowering IOP. These drugs counteract the leading causes of high IOP, i.e., they reduce the production of aqueous humor or promote its impaired outflow through the trabecular meshwork of the uveoscleral pathway. Widely used compounds are β-adrenergic antagonists, α_2-agonists, carbonic anhydrase inhibitors, prostaglandin analogs and miotic parasympathetic agents.[2] Combinations of drugs are also available for those patients in which IOP is not properly controlled by a single agent, to achieve an enhanced activity and to reduce side effects. Despite the availability of several active principles, the search for new IOP lowering agents is highly active and aims at developing more potent compounds with a more persistent action and lower side effects compared to currently available drugs.

The neurohormone melatonin (MLT, **1**, Figure 1) has significant IOP-lowering properties. MLT is primarily produced by the pineal gland with highest concentrations reached at night. A number of biological activities have been ascribed to MLT, ranging from central nervous system (CNS)-related activities, such as coordination of circadian rhythms and neuroendocrine processes, to peripheral effects, related, for example, to regulation of the immune system, glucose handling and cardiovascular homeostasis. In mammals, the majority of MLT actions are driven by the activation of the two G-protein-coupled receptors named MT_1 and MT_2 expressed in the CNS and in the periphery, which signal photoperiodic information and regulate physiological functions.[3,4] Other binding sites have been described for MLT, such as the MT_3 binding site, which has been identified with the cytosolic enzyme quinone reductase 2, nuclear transcription factors of the retinoic acid superfamily, and other proteins such as calmodulin and calreticulin.[5] Moreover, MLT exerts direct antioxidant effects at high concentrations, which can be related to different biological activities

observed after its administration. MLT is produced locally in the eye and its receptors have been identified in several areas, such as in the retina, ciliary body, cornea, lens and sclera.[6] Topical and systemic administration of MLT has been shown to transiently reduce IOP in normotensive and hypertensive/glaucomatous animals as well as in humans.[7] MLT potentiates the IOP lowering effect exerted by the adrenergic system, as it increases expression of the α_2 and reduce expression of the β_2 adrenergic receptors that control the synthesis and drainage of aqueous humor in ciliary nonpigmented epithelium.[8] Besides the natural ligand, synthetic MLT receptor agents, such as agomelatine, IIK7 and 5-MCA-NAT, have shown IOP lowering effects.[8,9,10]



melatonin, **1** URB597, **2**

Figure 1. Melatonin and FAAH inhibitor URB597.

Reduction of IOP is also a well-known effect observed in cannabis users.[11] In fact, a functional endocannabinoid system, which includes endogenous cannabinoids, enzymes involved in their synthesis and deactivation and the cannabinoid receptors CB_1 and CB_2, has been identified in eye tissues and cells, comprising retina and ciliary body.[12] Reduction of IOP has been observed after administration of endogenous or exogenous cannabinoids, such as Δ^9-tetrahydrocannabinol, the main active principle of cannabis, the synthetic cannabinoid receptor ligand WIN55212-2 and the endocannabinoid arachidonoylethanolamide (anandamide, AEA), in studies performed in animals and humans.[13] The IOP-lowering effect exerted by topical AEA was counteracted by concomitant administration of the CB_1 receptor antagonist SR141716A (rimonabant), supporting a role for this receptor in the modulation of IOP.[14] However, cannabinoid actions on IOP are also mediated by other, CB_1-independent, mechanisms such as activation of the TRPV1 channel and the orphan

receptor GPR55 and by arachidonic acid-based COX products.[15] Experimental findings suggest that the endocannabinoid system participates to the control of IOP modulating both production and outflow of aqueous humor. The serine-hydrolase enzyme fatty acid amide hydrolase (FAAH) is the main AEA-degrading enzyme.[16,17] FAAH is also responsible for the hydrolysis of several other fatty acid ethanolamides (FAEs) which have been found in the eye and might contribute to the regulation of IOP through specific mechanisms. Interestingly, FAAH has been described as responsible for the regulation of diurnal variation of IOP. In fact, FAAH is expressed with a circadian rhythm and high FAAH levels drive the production of N-arachidonoyl glycine which reduces IOP by activating GPR18 receptor.[18] However, despite the relevance of FAAH on IOP modulation, the effect of FAAH inhibitors on IOP has never been described.

In this study, we report the design of novel dual-acting compounds that behave as MLT receptor agonists and FAAH inhibitors, and their evaluation as IOP lowering agents. We combined the pharmacophore elements required for activation of melatonergic receptors with those for FAAH inhibition, exploiting the relevant structural elements of two well-known classes of compounds. In particular, N-anilinoethylamides (Figure 2) are a class of versatile melatonergic ligands in which modulations of substituents at the aniline nitrogen and on benzene ring have allowed fine tuning of binding affinity, receptor subtype selectivity and intrinsic activity.[19,20] The benzene ring of the aniline structure tolerates bulky, lipophilic alkyloxy substituents, which can also provide some selectivity for the MT_1 receptor.[21] On the other hand, N-alkyl-O-arylcarbamates are widely used FAAH inhibitors which irreversibly carbamoylate the catalytic serine residue.[22] A representative compound from this class, URB597 (**2**, Figure 1), has become the reference FAAH inhibitor in pharmacological studies investigating the physio-pathological roles of this enzyme and its substrates.[23] We devised therefore two new series of N-anilinoethylamide derivatives carrying on the aniline ring an alkoxy linker bound to a carbamoyl portion, intended to exert FAAH inhibition (Figure 2). In one series, an N-cyclohexyl-O-phenylcarbamate portion was joined to the aniline O-alkyl side chain through a second bridging oxygen atom. In a second series, the cyclohexyl

substituent of URB597 was replaced by the alkyl chain stemming from the aniline portion of melatonergic ligands, as known structure-activity relationships for O-aryl-N-alkyl carbamates had evidenced that a linear alkyl chain is tolerated without significantly affecting FAAH inhibitory potency.[22] The length of the alkyl spacer and the substitution patterns were previously investigated by molecular modelling, docking the modelled compounds within their putative binding sites at the two protein targets. While for FAAH several crystal structures are available, for MLT receptors a homology-based model of the MT_1 subtype was employed. As the second series of compounds resulted the most promising one at preliminary biological tests, it was further explored replacing the N-anilinoethylamide portion with the original indole structure of melatonin (Figure 2).

The compounds were tested on human MT_1 and MT_2 receptors to evaluate their binding affinity and intrinsic activity, on rat FAAH for their inhibitory potency and in a rabbit model of ocular hypertension to investigate their IOP-lowering potential.

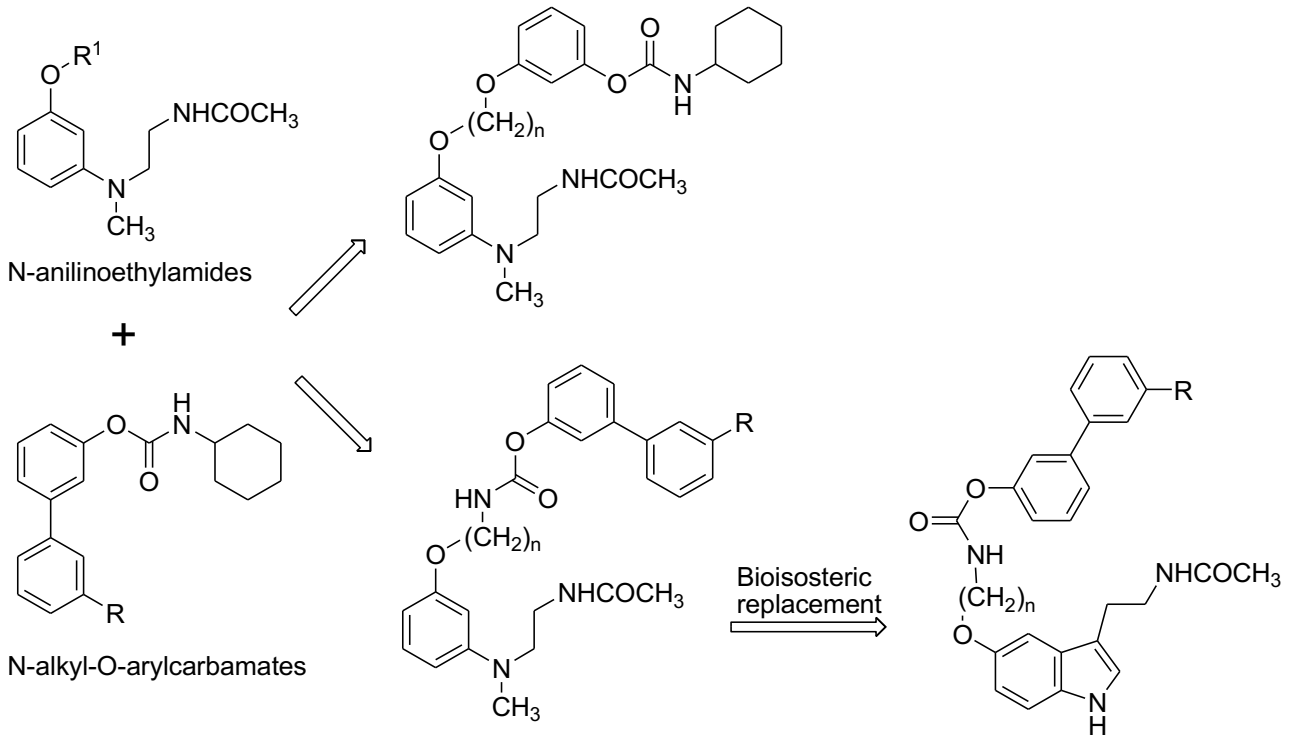


Figure 2. Design of dual-acting compounds by combination of relevant structural features of N-anilinoethylamide melatonergic ligands and N-alkyl-O-arylcarbamate FAAH inhibitors.

Chemistry

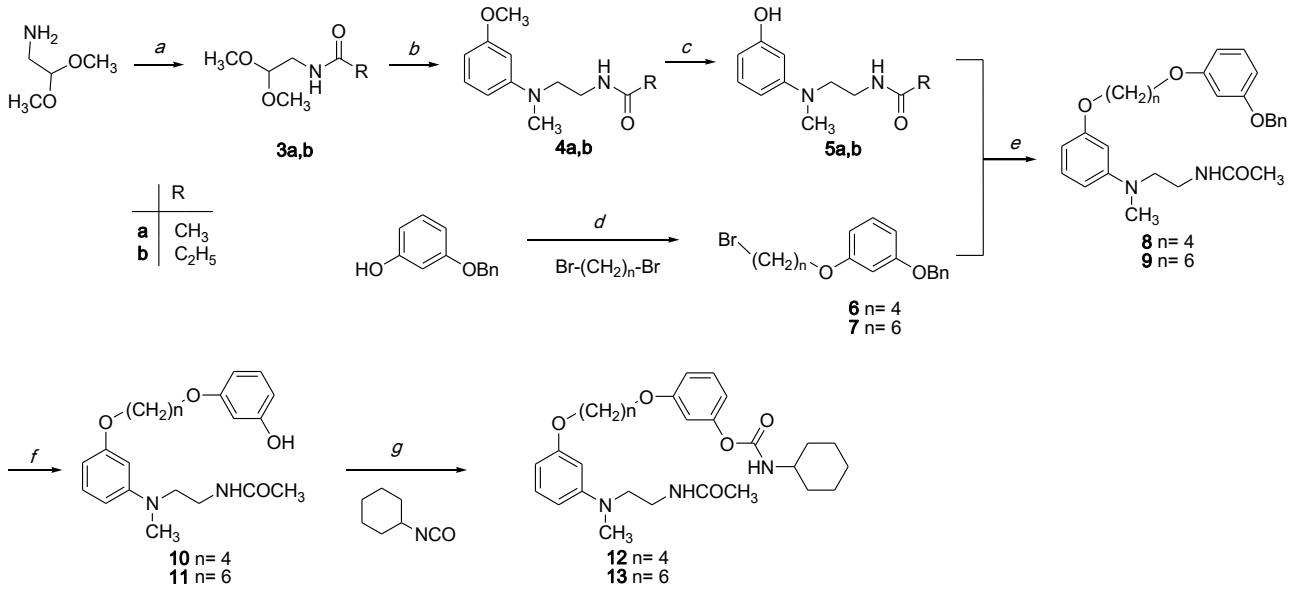
Compounds **10-13** (Scheme 1) were obtained by O-alkylation of (3-hydroxyanilinoethyl)amides (**5a-b**) with the bromoalkylphenoxy derivative **6** or **7** (which in turn were prepared by O-alkylation of 3-(benzyloxy)phenol with the proper dibromoalkane) in the presence of NaH, followed by O-debenzylation by hydrogenolysis (**10-11**) and subsequent O-carbamoylation with *c*-hexylisocyanate in the presence of Et_3N (**12-13**). The key intermediates **5a-b** were synthesized according to a well-validated route to similar compounds,[24] involving a reductive N-alkylation of 3-methoxy-*N*-methylaniline with the suitable *N*-acylaminoacetaldehyde dimethyl acetal (**3a**,**b**) in the presence of TFA/Et_3SiH, followed by methyl ether cleavage of intermediates **4a**,**b** using boron tribromide.

Compounds **20a-e** and **20g** were prepared in a convergent fashion following the steps outlined in Scheme 2. First, the aminoalkoxyphenyl intermediates **16a-d** were synthesized by O-alkylation of the previously cited phenol derivatives **5a,b** with the appropriate *tert*-butyl (ω-bromoalkyl)carbamate **14a-c** in the presence of K_2CO_3/NaI and subsequent *N*-Boc-deprotection (TFA). The activated carbonates **19d-f** were then prepared by reaction of 1,1-biphenyl-3-ol derivatives (**18d-f**) [commercially available (**18d**) or prepared by a Suzuki coupling reaction of 3-bromophenol with the suitable phenylboronic acid **17e-f**] with ***p***-nitrophenyl chloroformate in the presence of *N,N*-diisopropylethylamine (DIPEA). Finally, carbamates **20a-f** were obtained by the convergent coupling of the aminoalkoxyphenyl intermediates **16a-d** with the appropriate 4-nitrophenyl carbonate (**19d-f**). The final compound **20g** was achieved by hydrogenolysis of the benzyloxy precursor **20f**.

Compounds **27-29** were obtained by condensation of 5-(aminoalkoxy)-*N*-acetyltryptamines **24-26** with the (4-nitrophenyl)carbonate derivative **19d** as outlined in Scheme 3. The intermediate amines **24-25** were prepared by O-alkylation of *N*-acetylserotonin with *tert*-butyl (6-bromohexyl)carbamate (**14b**) or *tert*-butyl (8-bromooctyl)carbamate (**14c**) in the presence of K_2CO_3, and subsequent *N*-Boc deprotection with trimethylsilyl bromide. The 2-bromoindole derivative **23** was obtained by

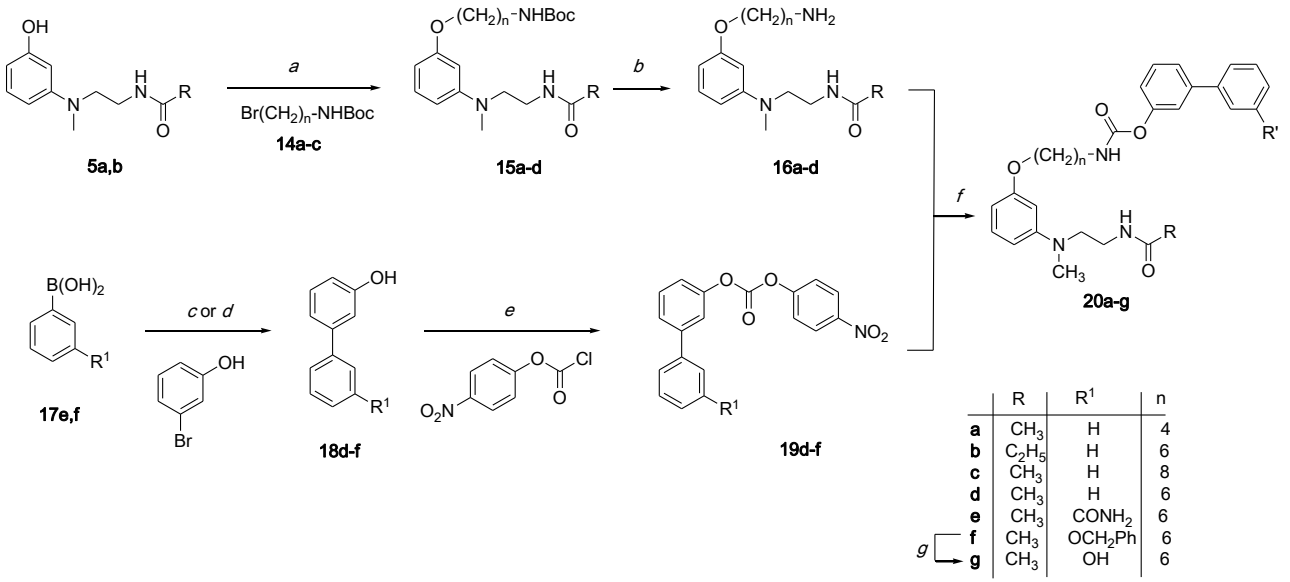
bromination of **21** with trimethylphenylammonium tribromide in THF, before submitting it to N-Boc deprotection to give **26**.

Scheme 1[a]



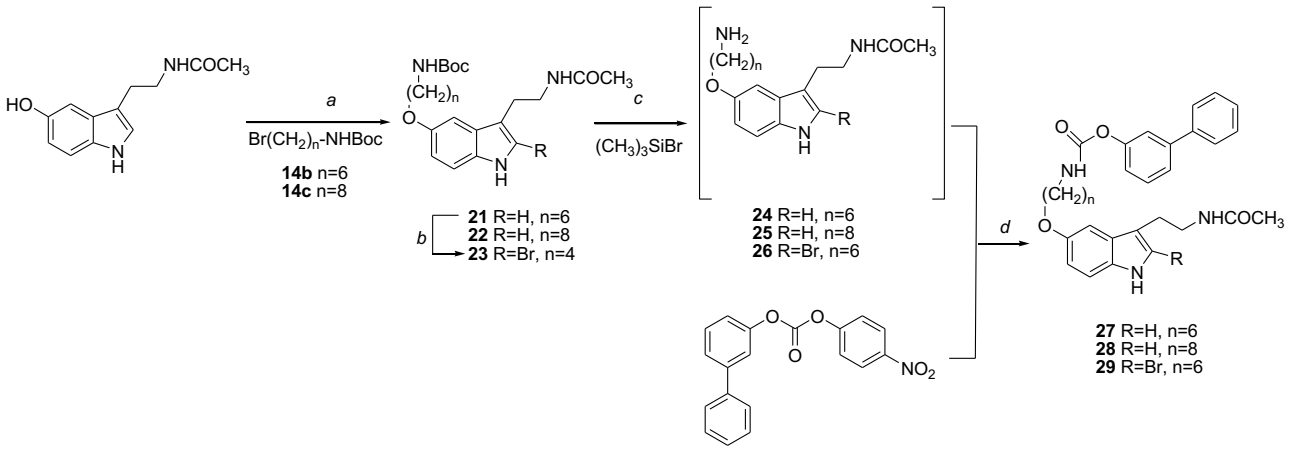
[a]Reagents and conditions: a) $(RCO)_2O$, Et_3N, CH_2Cl_2, rt, 2 h, yield 90-93%; b) 3-methoxy-*N*-methylaniline, TES, TFA, CH_2Cl_2, rt, 20 h, yield 51-52%; c) BBr_3, CH_2Cl_2, 0 °C to rt, 20 h, yield 68-76%; d) K_2CO_3, acetone, 50 °C, 24 h, yield 68-82%; e) NaH, DMF, -10 °C to rt, 16 h, yield 85-89%; f) H_2 (1 atm), 10% Pd-C, EtOH/EtOAc, rt, 16 h, yield 78-92%; g) Et_3N, EtOH/ CH_3CN, rt, 18 h, yield 65-95%.

Scheme 2[a]



[a]Reagents and conditions: a) K_2CO_3, NaI, acetone, 60 °C, 24 h, yield 20-44%; b) TFA, CH_2Cl_2, rt, 16 h, yield 60-64%; c) for **18e** $Pd(PPh_3)_4$, Na_2CO_3, CH_3CN, 90 °C, 1.5 h, yield 97%; d) for **18f** $Pd(OAc)_2$, Na_2CO_3, H_2O, acetone, 35 °C, 40 min, yield 81%; e) DIPEA, CH_3CN, rt, 1 h, yield 47-74%; f) Et_3N, CH_2Cl_2, rt, 4 h, yield 38-90%; g) H_2 (1 atm), 10% Pd-C, EtOH/EtOAc, rt, 20 h, yield 76%.

Scheme 3[a]



[a]Reagents and conditions: a) K_2CO_3, NaI, CH_3CN, reflux, 20 h, yield 47-59%; b) $(CH_3)_3N(Br_3)C_6H_5$, THF, rt, 30 min, yield 59%; c) CH_3CN, rt, 1 h; d) Et_3N, CH_2Cl_2/DMF, rt, 3 h, two steps (c, d) yield 83-95%.

Results and discussion

Design of new compounds

The accommodation of putative dual-acting agents into MLT receptor binding site and FAAH catalytic pocket was previously evaluated by docking simulations. Due to the lack of crystal structures of MLT receptors, docking studies were performed within a homology model of the MT_1 receptor in its active conformation previously developed and tested by us.[21] Preceding docking models for melatonergic ligands had proposed that substituent originating from the aromatic oxygen atom (e.g., the methoxy oxygen of MLT), which is important for receptor affinity, can be accommodated within a lipophilic channel outlined by transmembrane (TM) helices 3, 4 and 5, open toward the extracellular space in its upper portion. The presence of some smaller amino acids lining this channel in the MT_1 receptor subtype, compared to the corresponding residues in the MT_2 subtype, has been proposed as the structural element conferring MT_1 selectivity to ligands with bulkier substituents at the oxygen atom (e.g., a phenylbutyl group).[21] As both receptor subtypes are responsible for MLT activity on IOP, in order to obtain compounds with similar activity at MT_1 and MT_2 receptors an alkyl linker was inserted to connect the MLT receptor binding portion to the FAAH inhibiting one, with the idea that the slim alkyl chain could be tolerated into both receptors, allowing the carbamate portion to interact with the extracellular region of the receptor. Docking models showed that both orientations of the carbamate portion, corresponding to the two series of compounds **12-13** and **20**, could be accommodated in the extracellular lobby, provided that at least four-methylene long spacers were inserted. The binding pose into the MT_1 receptor obtained for compound **20e**, which has a six-methylene spacer, is represented in Figure 3, where the bulkier

amino acids lining the lipophilic channel of the MT_2 receptor are also shown. The carbamoyl fragment of **20e** protrudes out of the TM portion of the receptor and interacts with amino acids belonging to extracellular loop 2. The N-anilinoethylamide portion undertakes the pattern of interactions already proposed for melatonergic ligands, comprising hydrogen bonds between Tyr285 in TM7 and the amide oxygen and between Tyr187 in TM5 and the alkoxy oxygen.

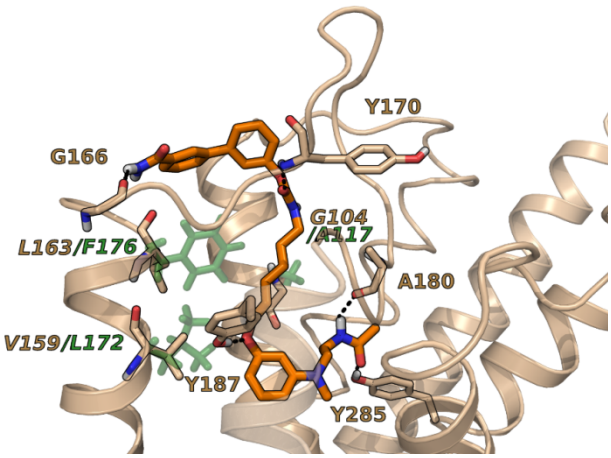


Figure 3. Induced-fit docking solution obtained for compound **20e** into the homology model of the MT_1 receptor. Side chains of bulkier amino acids belonging to the MT_2 receptor are represented in shaded green (MT_1: Gly104$^{3.29}$, Val159$^{4.57}$, Leu163$^{4.61}$; MT_2: Ala117$^{3.29}$, Leu172$^{4.57}$, Phe176$^{4.61}$).

Fitting of dual-acting compounds into the active site of FAAH was also evaluated. Compounds belonging to the N-cyclohexyl-O-phenylcarbamate series, exemplified by compound **13** in Figure 4 (left), place their cyclohexyl ring in the acyl chain binding (ACB) channel, consistent with its location in the crystal structure of FAAH carbamoylated by URB597.[25] The long substituent at carbamate oxygen atom occupies the cytoplasmic access (CA) channel taking polar interactions with Lys263 and Gln273. The lower FAAH-inhibitory potency observed for this series (see below) was attributed to possible repulsive interaction of the anilinoethylamide fragment with the dimer interface of FAAH which could be detrimental for compound affinity.

The second class of compounds (**20a-d**) generally gave docking solutions placing the melatonergic portion within the ACB channel, which looked more tolerant to structural alternatives. For this reason, and because preliminary data indicated this class as more promising than the first one, structural variants were introduced while conserving the O-biphenyl-3-yl-carbamate scaffold. In particular, small polar groups were introduced at the 3' position of the biphenyl nucleus (**20e**, **20g**), trying to improve FAAH inhibitory potency and the length of the spacer was increased to eight methylenes (**20c**) to exploit the steric tolerance of the ACB channel of FAAH. At the melatonergic portion, the acetylamino group was replaced by a propionylamino one (**20b**) and the N-anilinoethylamide scaffold by the N-indolylethylamide one of MLT to favour interactions at the melatomergic receptors. A representation of the docking solution obtained for the bulkiest inhibitor **29** is provided in Figure 4 (right). The carbamate portion occupies the catalytic site and its NH group is hydrogen-bonded to the carbonyl oxygen of Met191. The carbamate oxygen is stabilized into the oxyanion hole, interacting with the backbone NH groups of Ile238 and Gly239. The biphenyl portion is accommodated into the CA channel, while the long substituent at carbamate nitrogen extends inside the ACB channel. The bulky 2-bromolindole terminal portion of **29** has room enough to be properly accommodated: while the acetylamide takes a hydrogen bond with Thr488, the bromine atom fits into a lipophilic pocket lined by amino acids Ala377, Leu380, Phe381 and Phe432.

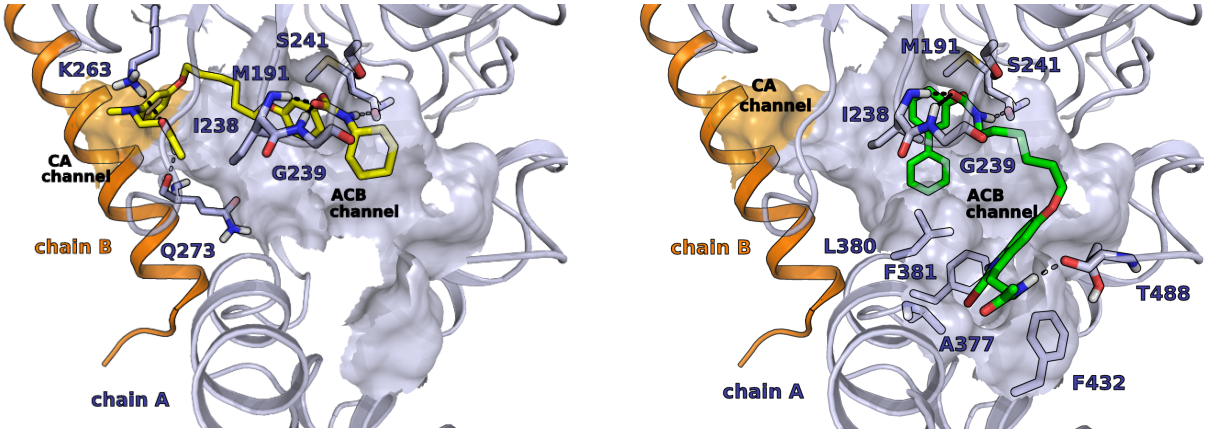
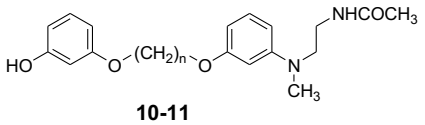


Figure 4. Compounds **13** (left) and **29** (right) docked into FAAH substrate binding site. Gray ribbons and surfaces refer to the monomer of FAAH which includes the catalytic site were inhibitors were docked; orange ribbons and surfaces refer to the other FAAH monomer associated with the first one. Docked inhibitors are represented with yellow (**13**) or green (**29**) carbons.

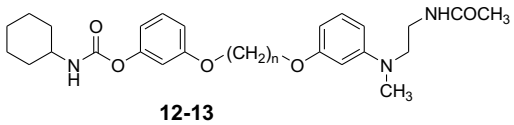
In vitro potency and structure-activity relationships

Binding affinity and intrinsic activity at MT_1 and MT_2 receptors and FAAH inhibitory potency obtained for the newly synthesized compounds are reported in Table 1.

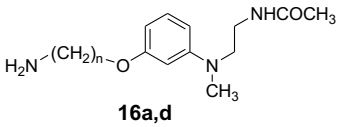
Table 1. Binding Affinity and Intrinsic Activity of Newly Synthesized Compounds at Human Melatonin Receptors MT_1 and MT_2 and Inhibition Potency (IC_{50}) of Rat FAAH Activity.



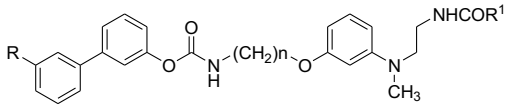
10-11



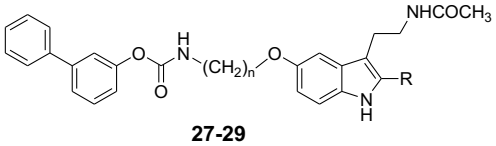
12-13



16a,d



20a-e, 20g



27-29

Cpd				**MLT receptors**				**rFAAH**
	n	**R**	**R^1**	**hMT_1**		**hMT_2**		
				$pK_i \pm SD^a$	$IA_r \pm SD^b$	$pK_i \pm SD^a$	$IA_r \pm SD^b$	IC_{50} (nM) $\pm$ SEM[c]
1 MLT				9.69 ± 0.09	1.00 ± 0.01	9.54 ±0.06	1.00 ± 0.03	nd[d]
2				nd	nd	nd	nd	4.6 ± 1.6[e]

URB 597								
12	4	-	-	7.46 ± 0.10	0.07 ± 0.07	7.27 ± 0.01	0.05 ±0.05	184±3
13	6	-	-	7.56 ± 0.09	0.71 ± 0.04	6.53 ± 0.06	0.71 ± 0.05	197±6
10	4	-	-	6.73 ± 0.08	0.27 ± 0.07	6.22 ± 0.07	0.18 ± 0.09	nd
11	6	-	-	7.23 ± 0.11	0.62 ± 0.14	6.48 ± 0.04	0.57 ± 0.08	nd
20a	4	H	CH_3	7.89 ± 0.03	0.64 ±0.07	7.35 ± 0.03	0.39 ± 0.07	1.45±0.01
20d	6	H	CH_3	7.67 ± 0.01	0.70 ± 0.01	7.48 ± 0.02	0.75 ± 0.03	1.34±0.03
20c	8	H	CH_3	7.50 ± 0.01	0.85 ± 0.08	7.49 ± 0.02	0.96 ± 0.03	0.63±0.04
20b	6	H	CH_2CH_3	7.79 ± 0.04	0.77 ± 0.08	7.72 ± 0.02	0.88 ± 0.02	3.07±0.08
20e	6	$CONH_2$	CH_3	7.41 ± 0.03	0.79 ± 0.06	7.81 ± 0.05	1.01 ± 0.02	0.43±0.01
20g	6	OH	CH_3	7.53 ± 0.02	0.89 ±0.08	7.57 ±0.01	0.94 ± 0.02	0.36±0.01
16a	4	-	-	5.51 ± 0.08	0.32 ± 0.09	4.89 ± 0.50	0.35 ± 0.04	nd
16d	6	-	-	6.35 ± 0.03	0.34 ± 0.05	6.40 ± 0.04	0.08 ± 0.01	nd
27	6	H	-	8.22 ± 0.01	0.73 ±0.08	8.34 ± 0.09	1.02 ± 0.02	2.38±0.16
28	8	H	-	8.31 ± 0.70	0.73 ± 0.08	8.20 ± 0.02	0.53 ± 0.02	4.00±0.10
29	6	Br	-	9.11 ± 0.10	0.73 ± 0.08	8.77 ± 0.03	0.97 ± 0.02	0.85±0.01

[a] pK_i values were calculated from IC_{50} values, obtained from competition curves by the method of Cheng and Prusoff,[26] and are the mean of at least three determinations performed in duplicate. [b] The relative intrinsic activity values were obtained by dividing the maximum analogue-induced G-protein activation by that of MLT. Measurements were performed in triplicate. [c] IC_{50} values are the mean of at least two determinations. [d] nd: not determined. [e] Ref. 23.

N-Cyclohexylcarbamic acid O-phenyl esters **12** and **13** showed only moderate binding affinity at human MT_1 and MT_2 receptors, about one hundred times lower than that of MLT. These

compounds, as well as all the other agents in Table 1, showed no receptor subtype selectivity, consistent with our design strategy. Compounds **10** and **11**, which could be produced in vivo from hydrolysis of carbamates **12** and **13**, showed reduced binding affinity, that may be attributed to lower lipophilicity of their chains. In fact, the detrimental role played by hydrophilic substituents on the side chain stemming from the *meta*-oxygen of N-anilinoethylamide derivatives has already been observed.[27] Compounds **12** and **13** also showed poor potency on FAAH. Compared to the biphenyl substituent of compound **2** the longer phenoxyalkyl chain significantly impacted on the ability to inhibit enzyme activity.

O-biphenylcarbamates **20a-e**, **20g** showed binding affinities at MT_1 and MT_2 receptors similar or slightly higher than the previous N-cyclohexylcarbamates **12** and **13**. The length of the alkyl spacer did not influence binding affinity and similar potencies were observed for compounds **20a** and **20d**, and for the longest compound **20c**. However, chain lengthening was correlated with an increase in intrinsic activity, with compound **20c** behaving as an agonist. Also the replacement of the acetylamino group of **20d** with a propionylamino one (**20b**) produced the expected slight increase in binding affinity and intrinsic activity, consistent with SAR profile of melatonergic ligands.[4,20] These compounds showed remarkable ability to inhibit FAAH activity, with a subnanomolar IC_{50} value observed for compound **20c**. Substituents known to be accommodated in FAAH active site were inserted in meta position of the distal phenyl ring.[23] As expected, the aminocarbonyl and hydroxyl derivatives **20e** and **20g** showed improved potencies compared to the unsubstituted **20d**. These substituents were tolerated at MLT receptors and led to an increase of intrinsic activity at both receptor subtypes. MT_1 and MT_2 binding affinity of the amino derivatives **16a** and **16d**, formally deriving from carbamate hydrolysis was also tested. Consistently with the previously cited SAR for N-anilinoethylamide ligands, no significant affinity could be measured for these basic and hydrophilic derivatives. Therefore activity of O-biphenyl carbamates at MLT receptors has to be ascribed to the compounds *per se*, and not to metabolites deriving from hydrolysis reactions performed by FAAH or other hydrolases.

O-Biphenylcarbamates demonstrated that it is possible to fuse the structural elements required for FAAH inhibition and for MLT receptor binding and activation in the same molecule. However, activity at the two targets of compounds **20a-e** and **20g** appeared rather unbalanced as FAAH inhibitory potency is about 10-100 times higher than binding affinity at MLT receptors. To increase MT_1 and MT_2 binding affinity the N-anilinoethylamide portion was replaced by the N-indolylethylamide scaffold of MLT. This bioisosteric replacement allowed to increase MLT receptor binding affinity of about one order of magnitude as observed for compounds **27** and **28**, with maintenance of FAAH inhibitory potency in the nanomolar range. Moreover, introduction of a bromine atom in position 2 of the indole ring in compound **29** allowed a further increase of MT_1 and MT_2 binding affinity, consistent with SAR for indole derivatives.[28] The bromine atom slightly improved FAAH inhibitory potency leading to a subnanomolar IC_{50} value, consistently with our hypothesis that this substituent can be favorably positioned in the lipophilic acyl chain binding pocket of FAAH (see previous modelling paragraph). Compound **29** represents therefore a potent dual-acting melatonergic agonist and FAAH inhibitor with balanced potency at the two targets.

Ocular pressure lowering activity

The ability of the two best dual-acting compounds **20e** and **29**, with N-anilinoethylamide and N-indolylethylamide portion, respectively, to reduce IOP was evaluated *in vivo* in an animal model of glaucoma. Their behavior was compared with that of compounds **1** and **2** and of the clinically used IOP lowering agent dorzolamide (**30**). 50 μL of a solution of the test compounds at 1 mM concentration were topically administered to male New Zealand albino rabbits with elevated IOP, induced by the injection of 0.1 mL of sterile hypertonic saline solution (5% in distilled water) into the vitreous body. Reference compound **30** was tested at 1% concentration (w/v) of dorzolamide hydrochloride. IOP was measured 60, 120 and 240 min after pressure stabilization following hypertonic saline injection.

Efficacy of compounds was monitored after 60 and 120 min, as after 4 hours IOP was returned to basal, pre-injection values (Figure 5). Compound **1** had a prompt effect, visible at 60 min which was maintained also at 120 min, even if with lower efficacy. A similar behavior was observed for the reference FAAH inhibitor **2**. Reduction of IOP by cannabinoid receptor ligands, both endocannabinoids and synthetic compounds, had been widely described, but this is the first time that efficacy as an IOP lowering agent is reported for a compound which acts indirectly, by increasing the local availability of anandamide and other fatty acid ethanolamides. Compounds **20e** and **29** had a limited effect after 60 min, which significantly increased at 120 min, greatly exceeding those measured for compounds **1** and **2**. Reference agent **30** had a slightly lower efficacy than compounds **1** and **2** after 60 min. At 120 min it reduced IOP as compound **29**, while compound **20e** had greater efficacy than dorzolamide itself. This is a remarkable result as dorzolamide was administered at significantly higher concentration.

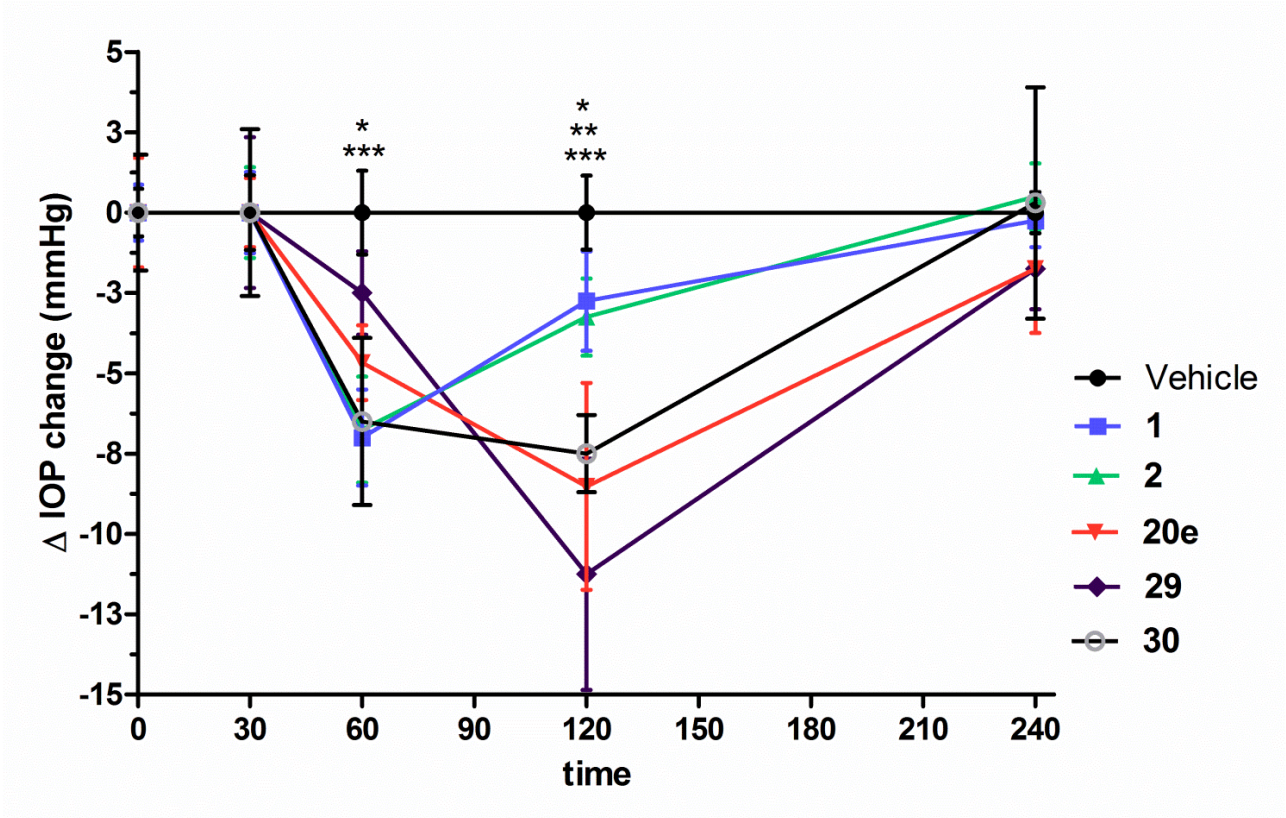


Figure 5. Drop of intraocular pressure (ΔIOP, mmHg) versus time (min) in hypertonic saline-induced ocular hypertension in rabbits. Data are analyzed with 2way Anova followed by Bonferroni multiple comparison test. * p<0.05, **20e** *vs* vehicle and **1** *vs* **29** at 60'; **1** *vs* **30** at 120'; ** p< 0.01, **1** and **2** *vs* **20e** at 120'; *** p<0.001, **1**, **2** and **30** *vs* vehicle at 60'; **20e**, **29** and **30** *vs* vehicle at 120'; **1** and **2** *vs* **29** at 120'.

Discussion and conclusions

Novel compounds targeting two physiological systems involved in the control of IOP were devised. Exploitation of SAR for melatonin receptor agonists and carbamate FAAH inhibitors led to dual-acting agents with remarkable and balanced activity at both targets. In the rabbit model of high IOP these compounds were more effective at longer times (120 min) compared to reference single-acting agents **1**, **2** and **30**. This behavior might be due to the synergistic effect of activity at the two targets or to the higher lipophilicity of the dual-acting compounds, which could assure a longer residence time at the site of action. In any case, these compounds represent a promising alternative for the treatment of glaucoma due to their IOP lowering effect and also in light of their potential neuroprotective effect. In fact, the endocannabinoid system is involved in cell survival mechanisms of RGC[29] and endocannabinoids and FAAH inhibitors have been described as able to minimize the retinal damage and reduce RGC loss in a model of high intraocular pressure-induced ischemia.[30] On the other hand, the neuroprotective activity of melatonin is widely known and its protective role has been demonstrated also in ocular tissues.[31] A neuroprotective role has been described also for synthetic melatonin receptor ligands. In addition, reduction of RGC death may positively modulate pineal melatonin production that could improve circadian rhythm-related functions, thus ameliorating conditions often present in glaucomatous patients such as sleep disorders and depression.[32]

Experimental section

General procedures

Melting points were determined on a Buchi B-540 capillary melting point apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE 400 instrument, using $CDCl_3$ as solvent unless stated otherwise. Chemical shifts (δ scale) are reported in parts per million (ppm) relative to the central peak of the solvent. Coupling constants (*J*) are given in hertz (Hz). ESI MS spectra were taken on a Waters Micromass ZQ instrument; molecular ions $[M+1]^+$ or $[M-1]^-$ are given. High-resolution mass spectroscopy was performed on a Micromass Q-ToF Micro mass spectrometer (Micromass, Manchester, UK) using an ESI source. The purity of all tested compounds, determined by LC-MS using a Waters HPLC/UV/MS system (separation module Alliance HT2795, Photo Diode Array Detector 2996, mass detector Micromass ZQ; software: MassLynx 4.1), was greater than 95% (see Supporting Information). Column chromatography purifications were performed under "flash" conditions using Merck 230−400 mesh silica gel. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel 60 F_{254} plates. *terz*-Butil (4-bromobutyl)carbamate, *terz*-butil (6-bromohexyl)carbamate, [1,1'-biphenyl]-3-ol, (3-carbamoylphenyl)boronic acid, [3-(benzyloxy)phenyl]boronic acid, 3-(benzyloxy)phenol, 2,2-dimethoxyethanamine and *N*-acetylserotonin were purchased from commercial suppliers and used without further purification.

General Procedure for the synthesis of *N*-(2,2-Dimethoxyethyl)acylamines 3a,b. Acetic or propionic anhydride (12 mmol) and Et_3N (1.65 mL, 12 mmol) were added to a solution of 2,2-dimethoxyethanamine (1.08 mL, 10 mmol) in dry CH_2Cl_2 (16 mL) and the resulting mixture was stirred at room temperature for 2 h. The mixture was neutralized with a saturated solution of $NaHCO_3$ and extracted with CH_2Cl_2. The combined organic phases were washed with brine, dried

(Na_2SO_4), filtered and concentrated in vacuo affording a crude residue that was purified by silica gel column chromatography.

***N*-(2,2-Dimethoxyethyl)acetamide (3a).** Flash chromatography (CH_2Cl_2-MeOH 98:2 as eluent). Oil; 90% yield. Chemical physical data were identical to those previously reported.[24]

***N*-(2,2-Dimethoxyethyl)propionamide (3b).** Flash chromatography (CH_2Cl_2-EtOAc 95:5 as eluent). Oil; 93% yield. ^{1}H NMR ($CDCl_3$) δ: 1.16 (t, 3H, *J* = 7.5), 2.23 (q, 2H, *J* = 7.5), 3.40 (s, 6H), 3.41 (m, 2H), 4.38 (t, 1H, *J* = 5.0), 5.70 (brs, 1H). ESI MS (*m/z*): 184 $[M+Na]^+$.

General Procedure for the synthesis of (anilinoethyl)amido derivatives 4a,b. Trifluoroacetic acid (2.2 mL, 29 mmol) and triethylsilane (0.9 mL, 5.6 mmol) were added to a solution of 3-methoxy-*N*-methylaniline (0.3 mL, 2.3 mmol) and the suitable acetal **3a,b** (3.1 mmol) in CH_2Cl_2 (5 mL), and the resulting mixture was stirred at room temperature for 20 h under a nitrogen atmosphere. After cooling to 0 °C, the mixture was carefully neutralized with a saturated aqueous solution of $NaHCO_3$ and the aqueous layer was extracted with CH_2Cl_2. The combined organic phases were washed with brine, dried over Na_2SO_4 and concentrated under reduce pressure to give a crude residue that was purified by column chromatography.

***N*-{2-[(3-Methoxyphenyl)methylamino]ethyl}acetamide (4a).** Purification by silica gel flash chromatography (EtOAc as eluent) and crystallization. White solid (diethyl ether/petroleum ether), 51% yield. Chemical physical data were identical to those previously reported.[24]

***N*-{2-[(3-Methoxyphenyl)methylamino]ethyl}propionamide (4b).** Purification by silica gel chromatography (EtOAc-cyclohexane 7:3 as eluent). Oil, 52% yield. ^{1}H NMR ($CDCl_3$) δ: 1.12 (t, 3H, *J* = 7.5), 2.16 (q, 2H, *J* = 7.5), 2.95 (s, 3H), 3.47 (m, 4H), 3.80 (s, 3H), 5.70 (brs, 1H), 6.31-6.33

(m, 2H), 6.42 (dd, 1H, J = 2.0), 7.16 (dd, 1H, $J_1 = J_2$ = 8.5). ^{13}C NMR ($CDCl_3$) δ: 174.0, 160.9, 150.6, 130.0, 105.8, 102.1, 99.3, 55.2, 52.0, 38.6, 37.2, 29.6, 9.7. ESI MS (*m/z*): 237 $[M+H]^+$.

General Procedure for the synthesis of (3-hydroxyanilino)ethylamido derivatives 5a,b. A 1M solution of BBr_3 in CH_2Cl_2 (2.5 mL, 2.5 mmol) diluted with dry CH_2Cl_2 (9 mL) was added dropwise to an ice-cooled solution of the suitable amide **4a,b** (1.23 mmol) in dry CH_2Cl_2 (9 mL) and the resulting mixture was stirred at room temperature for 20 h. After neutralization with a 2N aqueous solution of Na_2CO_3, the mixture was extracted with CH_2Cl_2, and the combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure to give a crude residue that was purified by column chromatography.

***N*-{2-[(3-Hydroxyphenyl)methylamino]ethyl}acetamide (5a).** Purification by silica gel flash chromatography (CH_2Cl_2-MeOH 98:2 as eluent). Oil, 76% yield. Chemical physical data were identical to those previously reported.[21]

***N*-{2-[(3-Hydroxyphenyl)methylamino]ethyl}propionamide (5b).** Purification by silica gel chromatography (EtOAc-cyclohexane 7:3 as eluent). Oil, 68% yield. ^{1}H NMR ($CDCl_3$) δ: 1.13 (t, 3H, J = 7.5), 2.18 (q, 2H, J = 7.5), 2.94 (s, 3H), 3.46 (m, 4H), 5.71 (brs, 1H), 6.01 (brs, 1H), 6.25 (d, 1H, J = 7.5), 6.32-6.36 (m, 2H), 7.09 (dd, 1H, $J_1 = J_2$ = 8.0). ESI MS (*m/z*): 223 $[M+H]^+$.

General Procedure for the synthesis of bromoalkylphenoxy derivatives 6 and 7. K_2CO_3 (0.31 g, 2.25 mmol) and the suitable dibromoalkane (2.25 mmol) were added to a solution of 3-(benzyloxy)phenol (0.3 g, 1.5 mmol) in dry acetone (4 mL), and the resulting mixture was stirred at 50°C for 24 h. After addition of water the mixture was extracted with EtOAc and the combined organic phases were dried (Na_2SO_4). After removing the solvent by distillation under reduced

pressure, the crude residue was purified by silica gel column chromatography (cyclohexane-EtOAc 95:5 as eluent).

1-(Benzyloxy)-3-(4-bromobutyloxy)benzene (6). Oil, 68% yield. Analytical data were in agreement to those previously reported.[33]

1-(Benzyloxy)-3-(6-bromohexyloxy)benzene (7). Oil, 82% yield. ^{1}H NMR ($CDCl_3$) δ: 1.53 (m, 4H), 1.87 (m, 4H), 3.45 (t, 2H, J = 6.5 Hz), 3.96 (t, 2H, J = 6.5 Hz), 5.07 (s, 2H), 6.58-6.62 (m, 3H), 7.19 (dd, 1H, $J_1 = J_2$ = 8.0 Hz), 7.28-7.45 (m, 5H); ^{13}C NMR ($CDCl_3$) δ: 160.3, 160.0, 137.0, 129.9, 128.6, 128.0, 127.5, 107.1, 106.9, 101.8, 70.0, 67.7, 33.9, 32.7, 29.1, 27.9, 25.3. ESI MS (*m/z*): 363-365 $[M+H]^+$.

General Procedure for the synthesis of derivatives 8 and 9. NaH (60% in mineral oil, 0.026 g, 0.65 mmol) was added to a solution of **5a** (0.13 g, 0.63 mmol) in dry DMF (1 mL) and the resulting mixture was stirred at -10 °C for 10 min under a nitrogen atmosphere. Then a solution of the suitable bromoalkylphenoxy derivative **6** or **7** (0.65 mmol) in dry DMF (1.5 mL) was added and the resulting mixture was stirred at room temperature for 16 h. After addition of water the mixture was extracted with EtOAc, the combined organic phases were dried (Na_2SO_4), filtered and concentrated under reduced pressure to afford a crude product that was purified by silica gel column chromatography (EtOAc as eluent).

***N*-[2-({3-[4-(3-Benzyloxy)phenoxy]butoxy}phenyl)methylaminoethyl]acetamide (8).** Oil, 89% yield. ^{1}H NMR ($CDCl_3$) δ: 1.94 (s, 3H), 1.97 (m, 4H), 2.94 (s, 3H), 3.46 (m, 4H), 4.03 (m, 4H), 5.05 (s, 2H), 5.61 (brs, 1H), 6.35 (m, 3H), 6.55 (m, 3H), 7.14 (m, 2H), 7.32-7.46 (m, 5H). ESI MS (*m/z*): 463 $[M+H]^+$.

***N*-{2-([3-[6-(3-Benzyloxy)phenoxy)hexyloxy]phenyl(methylamino)ethyl}acetamide (9).** Oil, 85% yield. ^{1}H NMR ($CDCl_3$) δ: 1.54 (m, 4H), 1.82 (m, 4H), 1.94 (s, 3H), 2.93 (s, 3H), 3.46 (m, 4H), 3.96 (m, 4H), 5.05 (s, 2H), 5.59 (brs, 1H), 6.35 (m, 3H), 6.54 (m, 3H), 7.16 (m, 2H), 7.32-7.46 (m, 5H). ESI MS (*m/z*): 491 $[M+H]^+$.

General Procedure for the synthesis of 3-hydroxyphenoxy derivatives 10 and 11. A solution of the suitable benzyloxy derivative **8** or **9** (0.63 mmol) in EtOH (5 mL) and EtOAc (5 mL) was hydrogenated (1 atm) at room temperature in the presence of 10% Pd-C (0.05 g) for 16 h. The catalyst was removed by filtration on Celite and the filtrate was concentrated under reduced pressure to afford a crude residue that was purified by silica gel column chromatography (EtOAc as eluent) and crystallization.

***N*-{2-((3-(4-(3-Hydroxyphenoxy)butoxy)phenyl)(methylamino)ethyl}acetamide (10).** White solid, mp 113-4 °C (EtOAc-petroleum ether), 92% yield. ^{1}H NMR ($CDCl_3$) δ: 1.95 (s, 3H), 1.97 (m, 4H), 2.94 (s, 3H), 3.48 (m, 4H), 4.04 (m, 4H), 5.75 (brs, 1H), 6.33-6.49 (m, 6H), 7.00 (brs, 1H), 7.09-7.18 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 171.1, 160.2, 157.5, 151.0, 130.0, 107.9, 106.7, 105.4, 102.5, 101.8, 99.7, 67.7, 51.8, 38.3, 37.5, 29.7, 26.4, 26.1, 23.1. ESI MS (m/z): 373 [M+H]+. HRMS (ESI): *m/z* calculated for $C_{21}H_{29}N_2O_4$, $[M + H]^+$ 373.2127. Found: 373.2154.

***N*-(2-((3-(6-(3-Hydroxyphenoxy)hexyloxy)phenyl)(methylamino)ethyl)acetamide (11).** White solid, mp 108-9 °C; (EtOAc-petroleum ether), 78% yield. ^{1}H NMR ($CDCl_3$) δ: 1.56 (m, 4H), 1.81 (m, 4H), 1.95 (s, 3H), 2.94 (s, 3H), 3.50 (m, 4H), 3.97 (m, 4H), 5.80 (brs, 1H), 6.31-6.48 (m, 6H), 6.72 (brs, 1H), 7.07-7.19 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 170.8, 160.4, 160.3, 157.2, 130.0, 107.8, 106.6, 105.5, 102.5, 102.1, 99.9, 67.7, 67.6, 51.9, 38.1, 37.5, 29.1, 28.9, 25.8, 23.2. ESI MS (*m/z*): 401 $[M+H]^+$. HRMS (ESI): *m/z* calculated for $C_{23}H_{33}N_2O_4$, $[M + H]^+$ 401.2440. Found: 401. 2417.

General Procedure for the synthesis of cyclohexyl carbamates 12 and 13. Cyclohexyl isocyanate (62 µl, 0.48 mmol) and Et_3N (5 µL) were added to a solution of the appropriate phenol **10** or **11** (0.21 mmol) in absolute EtOH (0.6 mL) and dry CH_3CN (0.6 mL), and the resulting mixture was stirred at room temperature for 18 h under a nitrogen atmosphere. After removing the solvent by distillation under reduced pressure, the residue was purified by silica gel column chromatography (EtOAc-cyclohexane 7:3 as eluent) and crystallization.

3-(4-{3-[(2-Acetamidoethyl)methylamino]phenoxy}butyloxy)phenylcyclohexylcarbamate (12). White solid, mp 115-6 °C; (EtOAc-petroleum ether), 65% yield. ^{1}H NMR ($CDCl_3$) δ:1.14-1.50 (m, 6H), 1.58-1.80 (m, 4H), 1.93 (s, 3H), 1.92-2.05 (m, 4H), 2.94 (s, 3H), 3.45 (m, 4H), 3.54-3.58 (m, 1H), 4.02 (m, 4H), 4.98 (brd, 1H), 5.72 (brs, 1H), 6.30-6.40 (m, 3H), 6.70-6.74 (m, 3H), 7.10-7.23 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 170.4, 160.2, 159.8, 153.5, 152.0, 150.9, 130.0, 129.6, 113.7, 111.6, 108.1, 105.5, 102.3, 99.7, 67.6, 67.3, 51.7, 50.1, 38.4, 37.3, 33.2, 26.0, 25.4, 24.7, 23.2. ESI MS (*m/z*): 498 $[M+H]^+$. HRMS (ESI): *m/z* calculated for $C_{28}H_{40}N_3O_5$, $[M + H]^+$ 498.2968. Found: 498.2961.

3-(6-{3-[(2-Acetamidoethyl)methylamino]phenoxy}hexyloxy)phenylcyclohexylcarbamate (13). White solid, mp 99-101°C; (EtOAc-petroleum ether), 95% yield. ^{1}H NMR ($CDCl_3$) δ:1.17-1.46 (m, 6H), 1.48-1.65 (m, 4H), 1.72-2.04 (m, 8H), 1.94 (s, 3H), 2.94 (s, 3H), 3.46 (m, 4H), 3.54-3.59 (m, 1H), 3.92-4.01 (m, 4H), 4.95 (brd, 1H), 5.66 (brs, 1H), 6.31-6.40 (m, 3H), 6.69-6.75 (m, 3H), 7.10-7.24 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 170.4, 160.4, 159.9, 153.5, 152.0, 150.9, 130.0, 129.5, 113.6, 111.6, 108.1, 105.4, 102.3, 99.8, 67.9, 67.7, 51.8, 50.1, 38.4, 37.3, 33.2, 29.3, 29.1, 25.9, 25.8, 25.4, 24.7, 23.2. ESI MS (*m/z*): 526 $[M+H]^+$. HRMS (ESI): *m/z* calculated for $C_{30}H_{44}N_3O_5$, $[M + H]^+$ 526.3281. Found: 526.3251.

***tert*-Butyl (ω-bromoalkyl)carbamates (14a-c).** *tert*-Butyl (8-bromooctyl)carbamate (**14c**) was prepared according to literature procedure and chemical physical data were identical with those previously reported.[34] *tert*-Butyl (4-bromobutyl)carbamate (**14a**) and *tert*-butyl (6-bromohexyl)carbamate (**14b**) are commercially available.

General Procedure for the synthesis of *N*-Boc-aminoalkoxyphenyl derivatives 15a-d. The suitable *tert*-butyl (ω-bromoalkyl)carbamate **14a-c** (1.50 mmol), K_2CO_3 (0.315 g, 2.25 mmol) and a catalytic amount of NaI were added to a solution of the appropriate (3-hydroxyanilino)ethylamido derivative **5a** or **5b** (1.50 mmol) in dry acetone (8 mL), and the resulting mixture was stirred at 60 °C for 24 h under a nitrogen atmosphere. After water addition addition the mixture was extracted with EtOAc, the combined organic phases were dried (Na_2SO_4) and the solvent was removed by distillation under reduced pressure to afford a crude residue that was purified by column chromatography.

***tert*-Butyl {4-[3-((2-acetamidoethyl)methylamino)phenoxy]butyl}carbamate (15a).** This product was obtained following the above general procedure by reacting **5a** with **14a**. Purification by silica gel chromatography (EtOAc as eluent). Oil, 37% yield. ^{1}H NMR ($CDCl_3$) δ: 1.45 (s, 9H), 1.65-1.71 (m, 2H), 1.77-1.83 (m, 2H), 1.94 (s, 3H), 2.94 (s, 3H), 3.17-3.21 (m, 2H), 3.46 (m, 4H), 3.98 (t, 2H, J = 6.5), 4.66 (brs, 1H), 5.71 (brs, 1H), 6.28-6.38 (m, 3H), 7.13 (dd, 1H, $J_1 = J_2 =$ 8.0). ESI MS (*m/z*): 380 $[M+H]^+$.

***tert*-Butyl {4-[3-((2-propionamidoethyl)methylamino)phenoxy]butyl}carbamate (15b).** This product was obtained following the above general procedure by reacting **5b** with **14b**. Purification by silica gel chromatography (EtOAc-cyclohexane 7:3 as eluent). Oil, 20% yield. ^{1}H NMR ($CDCl_3$) δ: 1.12 (t, 3H, J = 7.5), 1.45 (s, 9H), 1.38-1.52 (m, 6H), 1.74-1.81 (m, 2H), 2.16 (q, 2H, J = 7.5), 2.94 (s, 3H), 3.10-3.15 (m, 2H), 3.47 (m, 4H), 3.94 (t, 2H, J = 6.5), 4.55 (brs, 1H), 5.65 (brs, 1H),

6.29-6.38 (m, 3H), 7.13 (dd, 1H, $J_1 = J_2 = 8.0$). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 174.0, 160.3, 156.0, 130.0, 105.5, 102.2, 99.7, 67.6, 51.8, 40.5, 38.4, 37.2, 30.0, 29.6, 29.2, 28.4, 26.5, 25.8, 9.7. ESI MS (*m/z*): 422 $[M+H]^+$.

***tert*-Butyl {8-[3-((2-acetamidoethyl)methylamino)phenoxy]octyl}carbamate (15c).** This product was obtained following the above general procedure by reacting **5a** with **14c**. Purification by silica gel chromatography (EtOAc as eluent). Oil, 37% yield. ^{1}H NMR ($CDCl_3$) δ: 1.22-1.37 (m, 6H), 1.43 (s, 9H), 1.39-1.48 (m, 4H), 1.70-1.77 (m, 2H), 1.93 (s, 3H), 2.92 (s, 3H), 3.05-3.13 (m, 2H), 3.44 (m, 4H), 3.93 (t, 2H, $J = 6.5$), 4.52 (brs, 1H), 5.69 (brs, 1H), 6.27-6.37 (m, 3H), 7.12 (dd, 1H, $J_1 = J_2 = 8.0$). ESI MS (*m/z*): 436 $[M+H]^+$.

***tert*-Butyl {6-[3-((2-acetamidoethyl)methylamino)phenoxy]hexyl}carbamate (15d).** This product was obtained following the above general procedure by reacting **5a** with **14b**. Purification by silica gel chromatography (EtOAc as eluent). Oil, 44% yield. ^{1}H NMR ($CDCl_3$) δ: 1.36-1.55 (m, 6H), 1.45 (s, 9H), 1.73-1.82 (m, 2H), 1.95 (s, 3H), 2.94 (s, 3H), 3.08-3.16 (m, 2H), 3.46 (m, 4H), 3.95 (t, 2H, $J = 6.5$), 4.55 (brs, 1H), 5.67 (brs, 1H), 6.27-6.40 (m, 3H), 7.14 (dd, 1H, $J_1 = J_2 = 8.0$). ESI MS (*m/z*): 408 $[M+H]^+$.

General Procedure for *N*-Boc deprotection: synthesis of aminoalkoxyphenyl derivatives 16a-d. Trifluoroacetic acid (0.8 mL, 10 mmol) was added to an ice-cooled solution of the suitable *N*-Boc derivative **15a-d** (0.3 mmol) in dry CH_2Cl_2 (3.5 mL), and the resulting mixture was stirred at room temperature for 16 h under a nitrogen atmosphere. The reaction mixture was basified by drop-wise addition of a 2N aqueous solution of Na_2CO_3 and extracted with CH_2Cl_2. The combined organic phases were washed with brine, dried (Na_2SO_4) and the solvent removed by distillation under reduced pressure to give the desired crude amines that were used without further purification or purified by filtration through a pad of silica gel.

***N*-{2-[(3-(4-Aminobutoxy)phenyl)methylamino]ethyl}acetamide (16a).** Purification by silica gel plug filtration (EtOAc-MeOH-conc.NH_3 9:1:1 as eluent). Oil, 60% yield. ^{1}H NMR ($CDCl_3$) δ: 1.64-1.71 (m, 2H), 1.77-1.85 (m, 2H), 1.93 (s, 3H), 2.81 (t, 2H, J = 7.0), 2.93 (s, 3H), 3.43 (m, 4H), 3.97 (t, 2H, J = 6.0), 5.95 (brs, 1H), 6.26-6.31 (m, 2H), 6.35-6.37 (m, 1H), 7.12 (dd, 1H, $J_1 = J_2$ = 8.0). ^{13}C NMR ($CDCl_3$) δ : 170.7, 160.5, 150.4, 130.1, 105.8, 102.5, 99.5, 67.9, 51.7, 42.0, 38.5, 37.1, 29.6, 26.7, 23.3. ESI MS (*m/z*): 280 $[M+H]^+$. HRMS (ESI): *m/z* calculated for $C_{15}H_{26}N_3O_2$, $[M + H]^+$ 280.2020. Found: 280.2031.

***N*-{2-[(3-(6-Aminohexyloxy)phenyl)methylamino]ethyl}propionamide (16b).** This intermediate was used for the next step without any further purification. ESI MS (*m/z*): 322 $[M+H]^+$.

***N*-{2-[(3-(8-Aminooctyloxy)phenyl)methylamino]ethyl}acetamide (16c).** This intermediate was used for the next step without any further purification. ESI MS (*m/z*): 336 $[M+H]^+$.

***N*-{2-[(3-(6-Aminohexyloxy)phenyl)methylamino]ethyl}acetamide (16d).** Purification by silica gel flash filtration (EtOAc-MeOH-conc.NH_3 9:1:1 as eluent). Oil, 64% yield. ^{1}H NMR ($CDCl_3$) δ: 1.35-1.51 (m, 6H), 1.72-1.79 (m, 2H), 1.91 (s, 3H), 2.69 (t, 2H, J = 7.0), 2.91 (s, 3H), 3.42 (m, 4H), 3.92 (t, 2H, J = 6.5), 5.90 (brs, 1H), 6.23-6.28 (m, 2H), 6.33-6.36 (m, 1H), 7.11 (dd, 1H, $J_1 = J_2$ = 8.0). ^{13}C NMR ($CDCl_3$) δ : 170.6, 160.3, 150.8, 129.9, 105.4, 102.2, 99.5, 67.6, 51.7, 41.7, 38.4, 37.2, 29.7, 29.2, 26.5, 25.9, 23.1. ESI MS (*m/z*): 308 $[M+H]^+$. HRMS (ESI): *m/z* calculated for $C_{17}H_{30}N_3O_2$, $[M + H]^+$ 308.2338. Found: 308.2321.

3'-Hydroxy-(1,1'-biphenyl)-3-carboxamide (18e). 3-Bromophenol (0.25 g, 1.44 mmol) and a 0.4M aqueous solution of Na_2CO_3 (7.5 mL) were added to a solution of 3-carbamoylphenylboronic acid (**17e**) (0.25 g, 1.5 mmol) in CH_3CN (7.5 mL); the reaction mixture was degassed by bubbling

N_2 for 10 min and then added of $Pd(PPh_3)_4$ (10 mg). After stirring at 90°C for 1.5 h, the reaction mixture was filtered on Celite, the filtrated poured in H_2O and extracted with EtOAc. The combined organic phases were dried over Na_2SO_4, filtered and concentrated in vacuo to yield a crude residue that was purified by silica gel column chromatography (EtOAc-cyclohexane 8:2 as eluent). White solid, 97% yield. Chemical physical data were identical to those previously reported.[35]

3'-(Benzyloxy)-[1,1'-biphenyl]-3-ol (18f). Na_2CO_3 (0.11 g, 2 mmol), $Pd(OAc)_2$ (0.001 g) and a solution of 3-bromophenol (0.17, 1 mmol) in H_2O (0.7 mL) were added to a solution of 3-(benzyloxy)phenylboronic acid **17f** (0.336 g, 2 mmol) in acetone (3 mL), and the resulting mixture was stirred at 35 °C for 40 min. The mixture was extracted with EtOAc, the combined organic phases were dried (Na_2SO_4) and the solvent removed by distillation under reduced pressure to yield a crude residue that was purified by silica gel column chromatography (cyclohexane-EtOAc 9:1 as eluent). Oil, 81% yield. ^{1}H NMR ($CDCl_3$) δ: 5.14 (s, 2H), 5.43 (brs, 1H), 6.84 (ddd, 1H, J = 1.0, 2.5 and 8.0), 7.00 (ddd, 1H, J = 1.0, 2.5 and 8.0), 7.06 (m, 1H), 7.16-7.23 (m, 3H), 7.30-7.50 (m, 7H). ESI MS (*m/z*): 275 $[M-H]^-$.

General Procedure for the synthesis of carbonates 19d-f. DIPEA (0.15 mL; 0.8 mmol) and a solution of 4-nitrophenyl chloroformate (0.16 g, 0.8 mmol) in dry CH_3CN (4 mL) were added dropwise to a solution of the suitable phenol **18d-f** (0.8 mmol) in dry CH_3CN (2.5 mL), and the resulting mixture was stirred at room temperature for 1 h under a nitrogen atmosphere. Upon completion, the mixture was poured into water and filtered to separate the desired solid precipitate (for **19e**) or extracted with EtOAc (for cpds **19d** and **19f**). The combined organic phases were washed with brine, dried (Na_2SO_4) and the solvent removed by distillation under reduced pressure to give a crude residue that was purified by silica gel column chromatography (cyclohexane-EtOAc 9:1 as eluent) and crystallization.

[1,1'-Biphenyl]-3-yl-(4-nitrophenyl)carbonate (19d). White solid, mp 84-5 °C (EtOAc-petroleum ether); 47% yield. ^{1}H NMR ($CDCl_3$) δ: 7.28 (m, 1H), 7.37-7.67 (m, 8H), 7.51 (d, 2H, *J* = 9.0), 8.34 (d, 2H, *J* = 9.0).

3'-Carbamoyl-[1,1'-biphenyl]-3-yl-(4-nitrophenyl)carbonate (19e). Amorphous solid, 74% yield. ^{1}H NMR (DMSO-d_6) δ: 7.45-7.47 (m, 2H), 7.57-7.64 (m, 2H), 7.73-7.76 (m, 1H), 7.74 (d, 2H, *J* = 9.0), 7.83-7.92 (m, 3H), 8.12 (brs, 1H), 8.21 (brs, 1H), 8.38 (d, 2H, *J* = 9.0). ESI MS (*m/z*): 379 $[M+H]^+$.

3'-(Benzyloxy)-[1,1'-biphenyl]-3-yl (4-nitrophenyl)carbonate (19f). Amorphous solid, 50% yield. ^{1}H NMR ($CDCl_3$) δ: 5.14 (s, 2H), 7.01 (dd, 1H, *J* = 2.5 and 8.0), 7.20-7.29 (m, 3H), 7.36-7.53 (m, 9H), 7.48 (d, 2H, *J* = 9.0), 8.34 (d, 2H, *J* = 9.0).

General Procedure for the synthesis of *O*-biphenyl carbamate derivatives 20a-f. A solution of the appropriate amine **16a-d** (0.23 mmol) in dry CH_2Cl_2 (2 mL, dry DMF for **20e**) and Et_3N (0.14 mL, 1.05 mmol) was added to an ice-cooled solution (room temperature for **20e**) of the suitable (4-nitrophenyl)carbonate **19d-f** (0.11 g, 0.33 mmol) in dry CH_2Cl_2 (2 mL) under a nitrogen atmosphere, and the resulting mixture was stirred for 4 h at room temperature. Upon completion of the reaction, a saturated solution of $NaHCO_3$ was added and the aqueous phase was extracted with CH_2Cl_2 (EtOAc for **20e**). The combined organic phases were washed with brine, dried (Na_2SO_4) and the solvent removed by distillation under reduced pressure to afford a crude residue that was purified by silica gel column chromatography (EtOAc-cyclohexane 8:2 as eluent or EtOAc-MeOH 95:5 for **20e**).

[1,1'-Biphenyl]-3-yl{4-[3-((2-acetamidoethyl)methylamino)phenoxy]butyl}carbamate (20a). This product was obtained starting from amine **16a** and carbonate **19d**. Amorphous solid, 75%

yield. ^{1}H NMR ($CDCl_3$) δ: 1.64-1.94 (m, 4H), 1.87 (s, 3H), 2.93 (s, 3H), 3.36-3.43 (m, 6H), 4.04 (t, 2H, *J* = 6.0), 5.38 (brs, 1H), 5.71 (brs, 1H), 6.29-6.38 (m, 3H), 7.11-7.16 (m, 2H), 7.34-7.44 (m, 6H), 7.55-7.57 (m, 2H). ^{13}C NMR ($CDCl_3$) δ : 170.5, 160.1, 154.7, 151.4, 142.6, 140.3, 130.1, 129.5, 128.7, 128.0, 127.6, 127.1, 127.0, 124.0, 120.4, 105.6, 102.2, 99.6, 67.4, 51.7, 40.9, 38.4, 37.0, 26.7, 26.4, 23.1. ESI MS (*m/z*): 476 $[M+H]^+$. HRMS (ESI): *m/z* calculated for $C_{28}H_{34}N_3O_4$, $[M + H]^+$ 476.2549. Found: 476. 2557.

[1,1'-Biphenyl]-3-yl{6-[3-(methyl(2-propionamidoethyl)amino)phenoxy]hexyl}carbamate (20b). This product was obtained starting from amine **16b** and carbonate **19d**. Oil, 79% yield. ^{1}H NMR ($CDCl_3$) δ: 1.11 (t, 3H, *J* = 7.5), 1.47-1.54 (m, 4H), 1.61-1.66 (m, 2H), 1.79-1.83 (m, 2H), 2.11-2.18 (m, 2H) 2.94 (s, 3H), 3.28-3.34 (m, 2H), 3.46 (m, 4H), 3.97 (t, 2H, *J* = 6.5), 5.14 (brs, 1H), 5.64 (brs, 1H), 6.28-6.38 (m, 3H), 7.11-7.13 (m, 2H), 7.35-7.45 (m, 6H), 7.54-7.59 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 174.1, 160.3, 154.6, 151.4, 150.8, 142.6, 140.3, 130.0, 129.5, 128.7, 127.5, 127.2, 124.0, 120.4, 105.4, 102.1, 99.6, 67.6, 51.7, 41.2, 38.4, 37.2, 29.7, 29.6, 29.2, 26.4, 25.8, 9.7. ESI MS (*m/z*): 518 $[M+H]^+$. HRMS (ESI): *m/z* calculated for $C_{31}H_{40}N_3O_4$, $[M + H]^+$ 518.3019. Found: 518.3015.

[1,1'-Biphenyl]-3-yl{8-[3-((2-acetamidoethyl)methylamino)phenoxy]octyl}carbamate (20c). This product was obtained starting from amine **16c** and carbonate **19d**. Amorphous solid, 90% yield. ^{1}H NMR ($CDCl_3$) δ: 1.34-1.47 (m, 6H), 1.48-1.52 (m, 2H), 1.58-1.64 (m, 2H), 1.71-1.82 (m, 2H), 1.93 (s, 3H), 2.94 (s, 3H), 3.25-3.31 (m, 2H), 3.45 (m, 4H), 3.95 (t, 2H, *J* = 6.5), 5.10 (brs, 1H), 5.63 (brs, 1H), 6.27-6.41 (m, 3H), 7.09-7.17 (m, 2H), 7.32-7.47 (m, 6H), 7.55-7.61 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 170.4, 160.4, 154.6, 151.4, 150.9, 142.6, 140.3, 130.0, 129.5, 128.7, 127.5, 127.2, 124.0, 120.4, 105.5, 102.3, 99.7, 67.7, 51.8, 41.3, 38.4, 37.4, 29.8, 29.3, 29.2, 29.1, 26.6, 26.0, 23.2. ESI MS (*m/z*): 532 $[M+H]^+$. HRMS (ESI): *m/z* calculated for $C_{32}H_{42}N_3O_4$, $[M + H]^+$ 532.3175. Found: 532.3183.

[1,1'-Biphenyl]-3-yl{6-[3-((2-acetamidoethyl)methylamino)phenoxy]hexyl}carbamate (20d). This product was obtained starting from amine **16d** and carbonate **19d**. Oil, 85% yield. ^{1}H NMR ($CDCl_3$) δ: 1.58-1.65 (m, 6H), 1.71-1.89 (m, 2H), 1.93 (s, 3H), 2.95 (s, 3H), 3.26-3.37 (m, 2H), 3.45 (m, 4H), 3.98 (t, 2H, *J* = 6.0), 5.18 (brs, 1H), 5.70 (brs, 1H), 6.26-6.49 (m, 3H), 7.11-7.18 (m, 2H), 7.28-7.47 (m, 6H), 7.56-7.61 (m, 2H). ^{13}C NMR ($CDCl_3$) δ: 170.5, 160.1, 154.7, 151.4, 142.6, 140.3, 130.1, 129.5, 128.7, 128.0, 127.6, 127.1, 127.0, 124.0, 120.4, 105.6, 102.2, 99.6, 67.5, 51.7, 41.2, 38.5, 37.1, 29.7, 29.3, 26.4, 25.7, 23.1. ESI MS (*m/z*): 504 $[M+H]^+$. HRMS (ESI): *m/z* calculated for $C_{30}H_{38}N_3O_4$, $[M + H]^+$ 504.2862. Found: 504.2856

3'-Carbamoyl-[1,1'-biphenyl]-3-yl{6-[3-((2-acetamidoethyl)methylamino)phenoxy]hexyl}carbamate (20e). This product was obtained starting from amine **16a** and carbonate **19e**. Purification by silica gel chromatography (EtOAc-MeOH 95:5 as eluent). Amorphous solid, 38% yield. ^{1}H NMR ($CDCl_3$) δ: 1.47-1.54 (m, 4H), 1.60-1.67 (m, 2H), 1.77-1.84 (m, 2H), 1.88 (s, 3H), 2.91 (s, 3H), 3.29-3.38 (m, 2H), 3.40 (m, 4H), 3.97 (t, 2H, *J* = 6.5), 5.27 (brs, 1H), 5.73 (brs, 1H), 5.84 (brs, 1H), 6.28-6.35 (m, 3H), 6.46 (brs, 1H), 7.11-7.15 (m, 2H), 7.36-7.54 (m, 4H), 7.37 (d, 1H, *J* = 8.0), 7.81 (d, 1H, *J* = 8.0), 8.00 (m, 1H). ^{13}C NMR ($CDCl_3$) δ: 170.6, 169.3, 160.3, 154.7, 153.9, 151.5, 141.6, 140.8, 134.0, 130.6, 130.0, 129.7, 129.1, 126.5, 126.2, 124.1, 121.0, 120.6, 105.5, 102.2, 99.6, 67.6, 51.7, 41.1, 38.4, 37.2, 29.6, 29.1, 26.3, 25.7, 23.2. ESI MS (*m/z*): 547 $[M+H]^+$. HRMS (ESI): *m/z* calculated for $C_{31}H_{39}N_4O_5$, $[M + H]^+$ 547.2920. Found: 547.2940.

3'-(Benzyloxy)-[1,1'-biphenyl]-3-yl {6-[3-((2-acetamidoethyl)methylamino)phenoxy]hexyl}carbamate (20f). This product was obtained starting from amine **16a** and carbonate **19f**. Oil, 57% yield. ^{1}H NMR ($CDCl_3$) δ: 1.47-1.66 (m, 6H), 1.77-1.83 (m, 2H), 1.92 (s, 3H), 2.93 (s, 3H), 3.29-3.34 (m, 2H), 3.44 (m, 4H), 3.97 (t, 2H, *J* = 6.5),

5.12 (s, 2H), 5.13 (brs, 1H), 5.66 (brs, 1H), 6.29-6.41 (m, 3H), 6.97 (dd, 1H, *J* = 2.0 and 8.0), 7.10-7.22 (m, 4H), 7.32-7.48 (m, 9H). ESI MS (*m/z*): 610 $[M+H]^+$.

3'-Hydroxy-[1,1'-biphenyl]-3-yl {6-[3-((2-acetamidoethyl)methylamino)phenoxy]hexyl}carbamate (20g). A solution of the benzyloxy derivative **20f** (0.13 g, 0.21 mmol) in EtOH (0.5 mL) and EtOAc (2.5 mL) was hydrogenated (1 atm) at room temperature in the presence of 10% Pd-C (0.03 g) for 20 h. The catalyst was removed by filtration on Celite and the filtrate concentrated under reduced pressure to afford a crude product which was purified by silica gel chromatography (EtOAc as eluent). Amorphous solid, 76% yield. ^{1}H NMR ($CDCl_3$) δ: 1.44-1.57 (m, 4H), 1.58-1.66 (m, 2H), 1.76-1.83 (m, 2H), 1.93 (s, 3H), 2.91 (s, 3H), 3.28-3.33 (m, 2H), 3.43 (m, 4H), 3.98 (t, 2H, *J* = 6.5), 5.18 (brs, 1H), 5.81 (brs, 1H), 6.32-6.37 (m, 3H), 6.84 (dd, 1H, *J* = 2.0 and 8.0), 7.03 (m, 1H), 7.07-7.18 (m, 3H), 7.25-7.29 (m, 2H), 7.30-7.32 (m, 1H), 7.38-7.40 (m, 2H). ^{13}C NMR ($CDCl_3$) δ: 170.1, 160.3, 156.7, 154.9, 151.3, 142.5, 141.7, 130.1, 129.9, 129.5, 124.0, 120.4, 120.3, 119.0, 114.8, 114.2, 105.6, 102.7, 99.9, 67.8, 51.9, 41.1, 38.5, 37.2, 29.6, 29.0, 26.3, 25.7, 23.1. ESI MS (*m/z*): 520 $[M+H]^+$. HRMS (ESI): *m/z* calculated for $C_{30}H_{38}N_3O_5$, $[M + H]^+$ 520.2811. Found: 520.2798.

General Procedure for the Synthesis of 5-(*N*-Boc-aminoalkoxy)-*N*-acetyltryptamines 21-22. K_2CO_3 (0.19 g, 1.4 mmol) was added to a solution of *N*-acetylserotonin (0.10 g, 0.46 mmol) in dry CH_3CN (3 mL) and the resulting mixture was stirred under reflux for 1 h under a nitrogen atmosphere. Then a solution of the suitable *N*-Boc-derivative **14b** or **14c** (0.46 mmol) in dry CH_3CN (2 mL) and a catalytic amount of NaI were added and the resulting mixture was refluxed for 20 h. Water was added to quench the reaction and the aqueous phase was extracted with EtOAc. The combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure to give a crude residue that was purified by silica gel column chromatography (EtOAc as eluent).

***tert*-Butyl (6-{[3-(2-acetamidoethyl)-1*H*-indol-5-yl]oxy}hexyl)carbamate (21).** Amorphous solid; 59% yield. ^{1}H NMR ($CDCl_3$) δ: 1.38-1.56 (m, 4H), 1.48 (s, 9H), 1.78-1.85 (m, 4H), 1.96 (s, 3H), 2.95 (t, 2H, *J* = 6.5), 3.13-3.15 (m, 2H), 3.59-3.61 (m, 2H), 4.00 (t, 2H, *J* = 6.5), 4.55 (brs, 1H), 5.75 (brs, 1H), 6.87 (dd, 1H, *J* = 2.0 and 8.5), 7.04 (m, 2H), 7.26 (d, 1H, *J* = 8.5), 7.97 (brs, 1H). ESI MS (*m/z*): 418 $[M+H]^+$. ESI MS (*m/z*): 416 $[M-H]^-$.

***tert*-Butyl (8-{[3-(2-acetamidoethyl)-1*H*-indol-5-yl]oxy}octyl)carbamate (22).** White solid, mp 75-6 °C; (EtOAc-petroleum ether), 47% yield. ^{1}H NMR ($CDCl_3$) δ: 1.29–1.41 (m, 6H), 1.42–1.54 (m, 4H), 1.45 (s, 9H), 1.78-1.82 (m, 2H), 1.95 (s, 3H), 2.95 (t, 2H, *J* = 6.5), 3.08-3.11 (m, 2H), 3.58-3.61 (m, 2H), 4.00 (t, 2H, *J* = 6.5), 4.52 (brs, 1H), 5.75 (brs, 1H), 6.88 (dd, 1H, *J* = 2.0 and 8.5), 7.03 (m, 2H), 7.27 (d, 1H, *J* = 8.5), 8.01 (brs, 1H). ESI MS (*m/z*): 446 $[M+H]^+$ · ESI MS (*m/z*): 444 $[M-H]^-$.

***tert*-Butyl (6-{[3-(2-acetamidoethyl)-2-bromo-1*H*-indol-5-yl]oxy}hexyl)carbamate (23).** Trimethyl phenyl ammonium tribromide (0.050 g, 0.132 mmol) was added to a solution of **21** (0.055 g, 0.132 mmol) in dry THF (2.5 mL) and the resulting mixture was stirred at room temperature for 30 min under a nitrogen atmosphere. After removing the solvent by distillation under reduced pressure, the residue was partitioned between water and EtOAc. The combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure to afford a crude residue that was purified by silica gel column chromatography (EtOAc-cyclohexane 6:4 as eluent). Oil, 59% yield. ^{1}H NMR ($CDCl_3$) δ: 1.34-1.52 (m, 6H), 1.45 (s, 9H), 1.76-1.80 (m, 2H), 1.94 (s, 3H), 2.90 (t, 2H, *J* = 6.5), 3.11-3.15 (m, 2H), 3.51-3.54 (m, 2H), 3.96 (t, 2H, *J* = 6.5), 4.63 (brs, 1H), 5.72 (brs, 1H), 6.81 (dd, 1H, *J* = 2.0 and 8.5), 6.95 (m, 1H), 7.19 (d, 1H, *J* = 8.5), 8.71 (brs, 1H). ESI MS (*m/z*): 496 $[M+H]^+$.

General Procedure for the synthesis of indole target compounds 27-29. $(CH_3)_3SiBr$ (48 µl, 0.36 mmol) was added to a solution of the suitable *N*-Boc-derivative **21-23** (0.12 mmol) in dry CH_3CN (1.2 mL) and the resulting mixture was stirred at room temperature for 1 h under a nitrogen atmosphere. Upon completion of the reaction, MeOH was added and the resulting mixture was concentrated in vacuo to give the corresponding crude amine that was used for the next step without any further purification.

Et_3N (0.075 mL, 0.53 mmol) and a solution of the suitable above crude amine (**24-26**) in dry CH_2Cl_2 (2 mL) and DMF (0.2 mL) were added to an ice-cooled solution of carbonate **19d** (0.06 g, 0.17 mmol) in dry CH_2Cl_2 (1 mL) under a nitrogen atmosphere, and the resulting mixture was stirred at room temperature for 3 h. The reaction mixture was neutralized by addition of an aqueous saturated solution of $NaHCO_3$ and extracted with CH_2Cl_2. The combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure to afford a crude residue that was purified by column chromatography.

[1,1'-Biphenyl]-3-yl(6-((3-(2-aminoethyl)-1*H*-indol-5-yl)oxy)hexyl)carbamate (27). Purification by silica gel chromatography (EtOAc as eluent) and crystallization. White solid, mp 100-1 °C (EtOAc-petroleum ether); 83% yield. ^1H NMR ($CDCl_3$) δ: 1.43-1.70 (m, 6H), 1.81-1.88 (m, 2H), 1.93 (s, 3H), 2.93 (t, 2H, *J* = 6.5), 3.31-3.34 (m, 2H), 3.57-3.60 (m, 2H), 4.03 (t, 2H, *J* = 6.5), 5.20 (brs, 1H), 5.70 (brs, 1H), 6.89 (dd, 1H, *J* = 2.0 and 8.5), 7.00 (m, 1H), 7.05 (d, 1H, *J* = 2.0), 7.10-7.13 (m, 1H), 7.25 (m, 1H), 7.33-7.37 (m, 2H), 7.41-7.45 (m, 4H), 7.55-7.59 (m, 2H), 8.02 (brs, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 170.8, 154.6, 153.5, 151.4, 142.6, 140.3, 131.5, 129.5, 128.7, 127.8, 127.5, 127.2, 127.1, 124.0, 122.7, 120.4, 113.0, 112.7, 111.9, 101.8, 68.7, 41.2, 39.8, 29.8, 29.3, 26.5, 25.8, 25.3, 23.3. ESI MS (*m/z*): 514 $[M+H]^+$. HRMS (ESI): *m/z* calculated for $C_{31}H_{36}N_3O_4$, $[M + H]^+$ 514.2706. Found: 514.2700.

[1,1'-Biphenyl]-3-yl(8-((3-(2-aminoethyl)-1*H*-indol-5-yl)oxy)octyl)carbamate (28). Purification by silica gel chromatography (EtOAc as eluent) and crystallization. White solid, mp 108-9 °C (EtOAc-petroleum ether); 95% yield. ^{1}H NMR ($CDCl_3$) δ: 1.26-1.67 (m, 10H), 1.80-1.85 (m, 2H), 1.93 (s, 3H), 2.94 (t, 2H, *J* = 6.5), 3.27-3.32 (m, 2H), 3.57-3.62 (m, 2H), 4.01 (t, 2H, *J* = 6.5), 5.10 (brs, 1H), 5.68 (brs, 1H), 6.88 (dd, 1H, *J* = 2.0 and 8.5), 7.02 (m, 1H), 7.04 (d, 1H, *J* = 2.0), 7.10-7.14 (m, 1H), 7.25 (m, 1H), 7.35-7.38 (m, 2H), 7.41-7.46 (m, 4H), 7.57-7.60 (m, 2H), 7.96 (brs, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 170.8, 154.6, 153.5, 151.4, 142.6, 140.3, 131.5, 129.5, 128.7, 127.8, 127.5, 127.2, 124.0, 122.7, 120.4, 113.0, 112.7, 111.9, 101.7, 68.8, 41.3, 39.8, 29.8, 29.4, 29.2, 29.1, 26.6, 26.0, 25.2, 23.3. ESI MS *(m/z)*: 542 $[M+H]^+$. HRMS (ESI): *m/z* calculated for $C_{33}H_{40}N_3O_4$, $[M + H]^+$ 542.3019. Found: 542.3044.

[1,1'-Biphenil]-3-yl(6-((3-(2-aminoethyl)-2-bromo-1*H*-indol-5-yl)oxy)hexyl)-carbamate (29). Purification by silica gel chromatography (cyclohexane-EtOAc 7:3 as eluent). Amorphous solid; 83% yield. ^{1}H NMR ($CDCl_3$) δ: 1.44-1.57 (m, 4H), 1.62-1.67 (m, 2H), 1.80-1.84 (m, 2H), 1.94 (s, 3H), 2.89 (t, 2H, *J* = 6.5), 3.30-3.31 (m, 2H), 3.48-3.52 (m, 2H), 4.01 (t, 2H, *J* = 6.5), 5.27 (brs, 1H), 6.18 (brs, 1H), 6.83 (dd, 1H, *J* = 2.0 and 8.5), 6.98 (m, 1H), 7.11-7.15 (m, 1H), 7.13 (d, 1H, *J* = 8.5), 7.37-7.39 (m, 2H), 7.40-7.43 (m, 4H), 7.54-7.57 (m, 2H), 8.18 (brs, 1H). ^{13}C NMR ($CDCl_3$) δ : 171.0, 154.8, 153.8, 151.4, 142.6, 140.3, 131.3, 129.6, 128.7, 128.1, 127.6, 127.2, 124.0, 120.4, 120.4, 112.8, 111.9, 111.5, 108.9, 101.2, 68.7, 41.2, 39.6, 29.7, 29.2, 26.5, 25.8, 24.7, 22.9. ESI MS (*m/z*): 592 $[M+H]^+$. HRMS (ESI): *m/z* calculated for $C_{31}H_{35}N_3O_4Br$, $[M + H]^+$ 592.1811. Found: 592.1844.

Molecular modelling

Protein preparation. The X-ray structure of rFAAH in its covalent adduct with methylarachidonyl phosphonate (MAP; PDB: 1MT5[36]) was used for docking studies. The complete structure of chain A and a portion of chain B, including the α-helix involved in the dimerization surface (Pro438-

Asn466), were prepared using the Protein Preparation Wizard tool[37] of the Schrodinger 2015-4 software suite and modeled using the OPLS2005 force field.[38,39] The bound inhibitor was removed and missing hydrogen atoms were added. Basic and acid amino acids were modeled in their charged form, except for K142, which is part of the catalytic site and was maintained neutral. The orientation of thiol and hydroxyl groups, the conformations of asparagine, glutamine and histidine residues were adjusted to optimize the overall hydrogen bonding network. The model was first submitted to a minimization in which the heavy atoms were restrained, leaving the hydrogen atoms free to move. A second minimization was performed, restraining the position of the heavy atoms to an RMSD value of 0.3 Å.

A previously reported homology model of the MT_1 receptor[21] was used for induced-fit docking studies.

rFAAH docking studies. Ligands were built using Maestro 10.4[40] and optimized using LigPrep.[41] Docking was performed with Glide 6.9.[42,43] The docking grid was centered on the position of the covalent inhibitor, with enclosing and bounding box dimensions set to 24 Å and 33 Å, respectively. Hydrogen bond constraints were imposed between the carbamate oxygen of the ligands and the backbone nitrogen atoms of Ile238 and Gly239, and between the carbamate nitrogen of the ligands and the backbone oxygen atom of Met191. Ligand docking was performed in standard precision mode with default settings. The resulting binding poses were ranked according to their Emodel score. The best-ranked poses were merged into the protein structure and the complexes were minimized with the OPLS2005 force field implemented in MacroModel 11.0,[44] applying the Polak-Ribiere conjugate gradient method to a convergence threshold of 0.05 kJ mol^{-1} $Å^{-1}$. During energy minimization the ligands and residues within 10 Å from them were free to move, while the backbone of the other residues was kept fixed.

MT_1 induced-fit docking studies. The docking grid was centered on the putative binding site of the MT_1 receptor, comprising Tyr187$^{5.38}$, Trp251$^{6.48}$ and Tyr285$^{7.43}$, setting the dimension of the enclosing and bounding boxes to 15 Å and 30 Å, respectively. As a first step, a softened-potential

docking run was performed applying van der Waals radii scaling factors of 0.7 and 0.5 on protein and ligand non-polar atoms, respectively. Residues Asn169 and Tyr175, which are situated on extracellular loop 2, were temporarily mutated to alanine. To reproduce the polar contacts that have been postulated for melatonergic agonists, hydrogen bonds constraints were imposed between the hydroxyl group of Tyr187 and the phenolic oxygen of the ligands, and between the hydroxyl group of Tyr285 and the amide C=O group of the ligands. The resulting complexes were then submitted to a protein refinement procedure. In this stage, once the amino acid side chains previously mutated to alanine (i.e., Asn169 and Tyr175) were rebuilt, all residues within a shell of 5 Å from the ligand poses, except for Tyr187 and Tyr285, were refined through a conformational search using Prime.[45] During the last step of the induced-fit docking protocol, the ligand structures were optimized in the field of the refined receptor binding site, and finally scored using the default Glide settings. The ligand-protein complexes obtained were ranked according to their induced-fit docking score, which is a composite score accounting for ligand-receptor interaction energy, receptor strain and solvation terms. The best-ranked complexes were finally minimized with the OPLS2005 force field implemented in MacroModel, applying the Polak-Ribiere conjugate gradient method to a convergence threshold of 0.05 kJ mol^{-1} $Å^{-1}$. During the minimization ligands and residues within 10 Å from them were free to move, while the backbone of other residues was kept fixed.

Pharmacology

Melatonin receptor binding and intrinsic activity evaluation. Binding affinities were determined using 2-[^{125}I]iodomelatonin as the labeled ligand in competition experiments on cloned human MT_1 and MT_2 receptors expressed in NIH3T3 rat fibroblast cells. The characterization of NIH3T3-MT_1 and –MT_2 cells had been already described in detail.[46,47] Membranes were incubated for 90 min at 37 °C in binding buffer (Tris-HCl, 50 mM, pH 7.4). The final membrane concentration was 5−10 μg of protein per tube. The membrane protein level was determined in accordance with a previously reported method.[48] 2-[^{125}I]Iodomelatonin (100 pM) and different concentrations of MLT (10^{-10}–10^{-6}

M) or of the new compounds were incubated with the receptor preparation for 90 min at 37 °C. Nonspecific binding was assessed with 10 μM MLT; IC_{50} values were determined by nonlinear fitting strategies with the program PRISM (GraphPad SoftWare Inc., San Diego, CA). The pK_i values were calculated from the IC_{50} values in accordance with the Cheng−Prusoff equation.[26] The pK_i values are the mean of at least three independent determinations performed in duplicate.

To define the functional activity of the new compounds at MT_1 and MT_2 receptor subtypes, [^{35}S]GTPγS binding assays in NIH3T3 cells expressing human-cloned MT_1 or MT_2 receptors were performed. The amount of bound [^{35}S]GTPγS is proportional to the level of the analogue-induced G-protein activation and is related to the intrinsic activity of the compound under study. The detailed description and validation of this method were reported elsewhere.[47,49] Membranes (15−25 μg of protein, final incubation volume 100 μL) were incubated at 30 °C for 30 min in the presence and in the absence of MLT 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). Nonspecific binding was defined using [^{35}S]GTPγS (10 μM). 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 receptors, respectively. Basal stimulation is the amount of [^{35}S]GTPγS specifically bound in the absence of compounds, and it 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 was equivalent to 100 nM 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 100 nM MLT. All of the measurements were performed in triplicate. The relative intrinsic activity (IAr) 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. By convention, the natural ligand MLT has an efficacy

(E_{max}) of 100%. Full agonists stimulate [^{35}S]GTPγS binding with a maximum efficacy, close to that of MLT itself. If E_{max} is between 30% and 70% that of MLT (0.3 < IAr < 0.7), the compound is considered a partial agonist, whereas if E_{max} is lower than 30% (IAr < 0.3), the compound is considered an antagonist.[50]

FAAH activity assay. Wistar rat was anesthetized with isoflurane and decapitated. The brain was removed and homogenized in ice-cold Tris buffer (10 volumes, 50 mM, pH 7.5) containing sucrose (0.32 M). The homogenates were centrifuged at 1,000 × *g* for 10 min at 4 °C, and FAAH activity was measured using the supernatant (homogenate). Protein concentrations were determined with the BCA protein assay kit (Thermo Scientific, Rockford, USA). FAAH activity was measured by using [^{3}H]anandamide (anandamide[ethanolamine-^{3}H], 60 Ci mmol^{-1}, American Radiolabeled Chemicals, St. Louis, USA) as substrate. Homogenates (50 μg protein) were incubated for 30 min at 37 °C in Tris buffer (50 mM, pH 7.5, 0.5 mL) containing fatty-acid-free bovine serum albumin (BSA, 0.05 %, w/v), 10 μM anandamide and [^{3}H]anandamide (20,000 cpm), and varying concentrations of test compounds. The reactions were stopped with 1 mL $CHCl_3$/MeOH (1:1) and centrifugation at 2,000 × *g* for 10 min at 4 °C. Radioactivity in the aqueous layer ([^{3}H]ethanolamine) was measured by liquid scintillation counting. For in vitro experiments, the drugs were dissolved in DMSO and final DMSO concentration was 1 %.

Rabbit IOP lowering studies. The experimental procedures were carried out in New Zealand albino rabbits. We followed the Resolution of the Association for Research in Vision and Ophthalmology, the Good Laboratory Practice for the use of animals upon the authorization of Italian regulation on protection of animals (DM 116/1992)) and from the Italian Health Ministry (Authorization N° 1179/2015-PR), in agreement with the European Union Regulations (OJ of ECL 358/1, 12/12/1986). Male albino rabbits (body weight 2-2.5 kg) were kept in individual cages, food and water was provided ad libitum. The animals were maintained on a 12-12 h light/dark cycle in a temperature controlled room (22-23 °C). Male albino SPF (specific pathogen free) New Zealand

rabbits were used for the procedures. Animals were identified with a tattoo in the ear, numbered consecutively and examined before the beginning of the study to verify the good general and ophthalmic health condition. Tested compounds were dissolved in pyrogen free sterile 0.9% NaCl solution (i.e., physiologic solution) and 0.1% DMSO, 0.1% ETOH at 1mM concentration. Vehicle was 0.9% NaCl + 0.1% DMSO and 0.1% ETOH. The reference compound dorzolamide (**30**) was used at 1% concentration (w/v). The viability of tested compounds was always evaluated after repeated administration using the Draize Eye Test.[51] Topical delivery into the conjunctival cul-de-sac is the most common route of ocular drug delivery. All the compounds were given prior to saline injection and the IOP was measured at the very beginning of the experimental session to establish basal IOP. Four different animals were used for each tested compounds. One eye was treated with 0.05 mL of drug solution and the contralateral eye received the same volume of vehicle. Ocular hypertension was induced by the injection of 0.05 mL of sterile hypertonic saline (5%) into the vitreous bilaterally with local anaesthesia provided by one drop of 0.2% oxybuprocaine hydrochloride in each eye one minute before. IOP was measured using using a Model 30™ Pneumatonometer (Reichert Inc. Depew, NY, USA) after hypertonic saline injection after stabilization (normally 10 minutes) to verify the rise of IOP into the suitable experimental range (IOP > 30 and < 40 mm Hg) and after 60, 90, 120, 240 minutes in all groups after drug or vehicle treatment. One drop of 0.2% oxybuprocaine hydrochloride was instilled in each eye immediately before each set of pressure measurements.

Supporting information

The Supporting Information is available free of charge on the ACS Publications website.

Molecular formula strings data (CSV)

Purity of target compounds (PDF).

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Notes

The authors declare no competing financial interest.

Abbreviations

DIPEA, *N,N*-diisopropylethylamine; FAAH, fatty acid amide hydrolase; GTPγS, guanosine 5′-O-(3-thiotriphosphate); Emax, maximum activation of receptor; HPLC, high-performance liquid chromatography; IOP, intraocular pressure; MLT, melatonin; MT_1, melatonin receptor subtype 1; MT_2, melatonin receptor subtype 2; THF, tetrahydrofuran.

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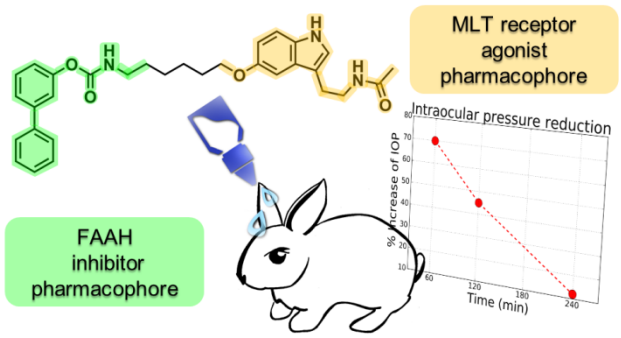
Table of Contents Graphic


MLT receptor agonist pharmacophore
FAAH inhibitor pharmacophore
Intraocular pressure reduction
% decrease of IOP
Time (min)