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Small-Molecule Negative Modulators of Adrenomedullin: Design, Synthesis, and 3D-QSAR Study

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In memory of Ángel R. Ortíz

Adrenomedullin (AM) is a peptidic hormone that was isolated in 1993, the function of which is related to several diseases such as diabetes, hypertension, and cancer. Compound **1** is one of the first nonpeptidic small-molecule negative modulators of AM, identified in a high-throughput screen carried out at the National Cancer Institute. Herein we report the synthesis of a series of analogues of **1**. The ability of the synthesized compounds to disrupt the binding between AM and its monoclonal antibody has been measured, together with surface plasmon resonance (SPR)-based binding assays as implemented with Biacore technology. These data were used to derive a three-dimensional quantitative structure–activity relationship (3D-QSAR) model, with a q^2 (LOO) value of 0.8240. This study has allowed us to identify relevant features for effective binding to AM: the presence of a hydrogen-bond donor group and an aromatic ring. Evaluation of the ability of selected compounds to modify cAMP production in Rat2 cells showed that the presence of a free carboxylic acid is essential for negative AM modulation.

Introduction

Adrenomedullin (AM) is a 52-amino acid peptide identified and isolated from a human pheochromocytoma[1] and belongs to the calcitonin/calcitonin gene-related peptide (CGRP)/amylin/AM superfamily. In humans this peptide is expressed by many cell types, and exerts a variety of physiological roles, including vasodilation, bronchodilation, and regulation of hormone secretion.[2–6]

AM levels are dysregulated in many human pathologies such as hypertension, heart failure, sepsis, cancer, and diabetes.[7] This observation, together with experimental data in animal model systems, suggests this molecule is involved in the pathophysiology of such diseases. Modifications of AM levels seem to have paradoxical effects on a patient's health. For instance, elevated AM expression exerts a protective role in renal[8–10] and cardiovascular diseases[9,11,12] and central nervous system ischemia.[13] However, elevated AM expression worsens the progression of type 2 diabetes[14] and cancer.[15]

The finding that AM may promote tumor development by regulating angiogenesis is of particular interest. AM has been recently characterized as a pro-angiogenic factor with the help of ex vivo and in vivo models.[16–19] In addition to inducing angiogenesis, AM behaves as an autocrine growth factor in cancer cells, enhances thymidine incorporation, reduces apoptosis, and is induced by hypoxia, therefore suggesting that this peptide may be an important tumor cell survival factor and a potential target for antitumor therapy.[20]

The development of nonpeptidic small molecules that regulate the physiological effects of AM is of great interest, as they may constitute attractive pharmacological tools for the treatment of the above-mentioned diseases. Several peptidic AM antagonists have been reported, such as monoclonal antibodies and inhibitory peptide fragments.[21–23] However, these molecules have significant limitations as potential drugs, given the lack of humanized blocking antibodies and the short biological half-life of fragmentary peptides.

More recently, Martínez et al. reported a fast and efficient high-throughput screening method to detect nonpeptidic modulators of AM from the library of small molecules of the National Cancer Institute (NCI).[24] The first phase involved a screen to search for compounds that disrupt the binding between AM and its monoclonal antibody. All the compounds that gave a positive response in this assay were subjected to a secondary screen that analyzed their ability to modify the production of cAMP, a second messenger elicited by the specific receptor system. This assay allowed classification of the molecules in positive or negative modulators, depending on their ability to elevate or decrease cAMP levels in the presence of

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AM, respectively. On the other hand, we reported a study in which a series of nonpeptidic positive modulators detected in the NCI library, together with new analogues synthesized by us, were used in a three-dimensional quantitative structure–activity relationship (3D-QSAR) study that elucidated some of the structural requirements to bind AM.[25]

We selected one of the negative hit compounds (compound **1**) detected by Martínez et al.[24] in the screen carried out with the NCI library, as a starting point for the development of AM negative modulators with an interest as antiangiogenic and antitumor compounds. Herein we report the preparation of piperazine derivatives of type A, in which R is an ethyl or benzyl group. Moreover, we synthesized a series of piperazines of type B, allowing a wider scope for substitution on the nitrogen atom (Figure 1). We tested the ability of the synthesized compounds to disrupt the binding between AM and its monoclonal antibody. All compounds were tested for their ability to bind AM by using surface plasmon resonance (SPR) based on a biosensor (Biacore instruments). These data were used to derive a 3D-QSAR model that proved to be useful in proposing new candidates to be synthesized. Selected compounds were tested for their ability to modify cAMP production in Rat2 cells, allowing us to confirm the behavior of type A piperazines as negative AM modulators. The results of these studies are discussed below.

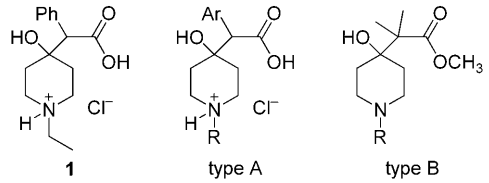
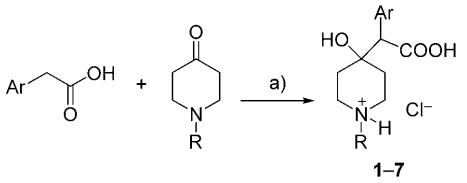


Figure 1. Compound **1** and structures of type A and type B piperazine derivatives.

Results and Discussion

Chemistry

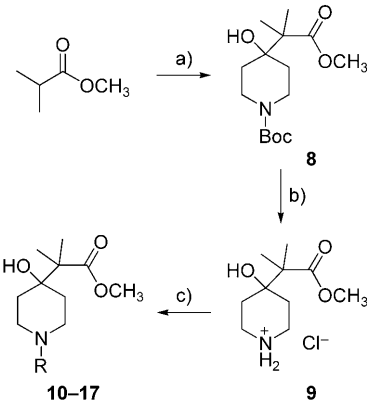
Piperazine derivatives of type A were prepared following the method described by Blicke and Zinnes[26] for the synthesis of **1**. Thus, 1-ethyl-4-piperidone or 1-benzyl-4-piperidone was treated with the corresponding aryl acetic acid in the presence of *i*PrMgCl as base (Scheme 1). The aromatic moiety was selected to include electron-withdrawing and electron-donating substituents as well as thiophene and naphthalene rings.



Scheme 1. Reagents: a) *i*PrMgCl, benzene.

Purification of the synthesized compounds proved troublesome, and although a broader series of compounds was attempted, only in the cases shown in Scheme 1 was it possible to isolate, by successive extractions with hot nitromethane, the desired amino acid with sufficient purity (microanalysis) to be tested.

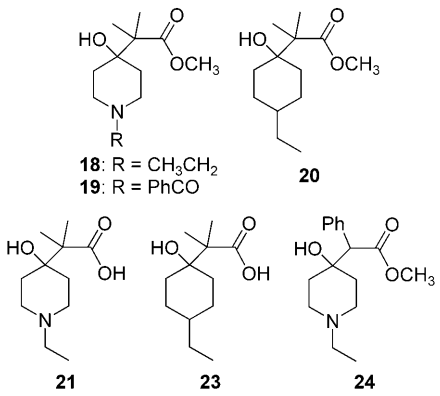
An alternative route for the synthesis of compounds of type A consists of a Reformatsky condensation of the corresponding methyl α-bromoaryl acetate with 1-ethyl-4-piperidone or 1-benzyl-4-piperidone, followed by hydrolysis of the formed α-hydroxyester. However, the Reformatsky reaction between ethyl α-bromophenyl acetate and 1-ethyl-4-piperidone failed to give the desired α-hydroxyester. An alternative to the Reformatsky reaction is the formation of the ester enolate with a base (lithium cyclohexylisopropylamide (LiCA) is usually the base of choice) followed by addition of the corresponding ketone.[27] Unfortunately, although this procedure gave the desired α-hydroxyester, the yield was less than 10%, and the whole route was very inefficient. However, this method proved useful in the synthesis of piperazines of type B (Scheme 2).



Scheme 2. Reagents and conditions: a) LiCA, *tert*-butyl-4-oxopiperidine-1-carboxylate, THF, −78 °C; b) HCl(g), EtOAc, 0 °C; c) RBr, K_2CO_3, DMF, room temperature.

These compounds were designed in order to avoid the stereogenic center present in type A piperazines. In this case, *N*-Boc-4-piperidone was treated with the enolate of ethyl isobutyrate to give **8**. Removal of the Boc protecting group with HCl gave amine **9**, which was a useful intermediate for the synthesis of a wide variety of derivatives with various substituents on the nitrogen atom.

Compounds **18** and **19**, respectively bearing an ethyl and a benzoyl group on the nitrogen atom, were synthesized by direct condensation of the enolate of ethyl isobutyrate with the corresponding commercially available ketone. Compound **20**, in which the nitrogen atom has been removed, was synthesized by a similar method, starting from the commercially available 4-ethylcyclohexanone. Saponification of the ethyl



esters **18** and **20** gave the corresponding carboxylic acids **21** and **23**.

Competitive monoclonal antibody assays

The affinity of the various compounds for AM was qualitatively evaluated by their ability to interfere in the binding between the peptide and its blocking monoclonal antibody, following the same methodology described by Martínez et al.[24] A neutralizing monoclonal antibody will bind to an epitope on a region of the peptide that is critical for receptor recognition. Thus, molecules that disrupt peptide–antibody binding may be good candidates as modulators of peptide physiology. Internal controls were placed in every plate as described in the Experimental Section (wells without coating, wells in which no potential modulators were added, and wells with a positive inhibition control). The results are shown in Tables 1–3.

Although compound **1** was among the 121 compounds reported to cause a statistically significant inhibition of color intensity in this assay,[24] in our hands this compound showed 99.2% absorbance relative to the negative control, allowing us to classify it as a very modest AM binder. Introduction of chemical modifications led to an incremental increase in affinity for 11 compounds, as listed in Tables 1–3. It should be taken into account that we have not introduced drastic structural modifications, and therefore, small variations in this assay were expected. However, it seems that the presence of a free carboxylic group is not essential for AM binding, as demonstrated by the type B series of compounds. Moreover, the absence of the stereogenic center does not lead to a significant loss of binding affinity, as exemplified by ester **12**. The results obtained in this preliminary assay suggested that a more accurate technique, such as SPR, would be necessary to quantitatively evaluate the binding of these compounds to AM.

Table 2. Chemical structures and biological data for type B piperazines **8**–**19.**

Compd	R	$A \pm$ SD [%][a]	RU $\pm$ SD[b]	cAMP $\pm$ SD [%][c]
8	Boc	93.0 $\pm$ 1.5	n.b.	
9	H	92.5 $\pm$ 4.6	2.4	
10	allyl	103.9 $\pm$ 4.9	1.5 $\pm$ 0.7	
11	Benzyl	91.1 $\pm$ 8.5	4.9 $\pm$ 2.3	122.4 $\pm$ 3.2 (*)
12	*p*-nitrobenzyl	88.1 $\pm$ 11.9	6.6 $\pm$ 1.4	
13	*p*-(trifluoromethyl)benzyl	100.7 $\pm$ 2.1	3.5 $\pm$ 1.7	118.2 $\pm$ 5.2 (*)
14	2-hydroxyethyl	106.8 $\pm$ 2.8	n.b.	
15	2-(diethylamino)ethyl	96.4 $\pm$ 4.9	1.1 $\pm$ 0.1	89.9 $\pm$ 5.1 (*)
16	*p*-methoxybenzyl	105.5 $\pm$ 9.0	7.6 $\pm$ 1.4	104.2 $\pm$ 0.6 (n.s.)
17	propargyl	99.1 $\pm$ 7.6	2.2	
18	ethyl	94.4 $\pm$ 1.7	n.b.	92.5 $\pm$ 5.1 (n.s.)
19	benzoyl	109.4 $\pm$ 5.3	n.b.	

[a] Percent absorbance at primary screening. [b] M_r-adjusted SPR response units at a compound concentration of 200 μM; n.b. = no binding. [c] Percent cAMP production evaluated with the [^{125}I]cAMP Biotrack Assay System; n.s. = no significant difference; *$p < 0.05$.

Surface plasmon resonance assays

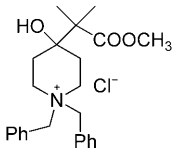
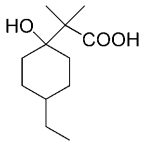
Binding of all synthesized compounds to immobilized AM was evaluated by SPR. AM was immobilized on the dextran matrix of a sCM5 sensor chip, and the binding of small molecules was measured as relative response units (RU), which depend on the mass of compound bound to immobilized AM. This effect is dose dependent, as illustrated in Figure 2d. Tables 1–3 summarize the equilibrium responses at a concentration 200 μM for all compounds, normalized as M_r-adjusted responses (RU $\times$ 100/M_r). In accordance with the results obtained by us for compound **1** in the evaluation of the interference in the binding between the peptide and its blocking monoclonal antibody, the RU value (3.1 $\pm$ 1.5) indicates that it behaves as a modest AM binder. This result disagrees with the binding affinity previously reported for this compound ($K_d = 1.30 \times 10^8 \pm 1.57 \times$

Table 1. Chemical structures and biological data for type A piperazines **1**–**7.**

Compd	Ar	R	$A \pm$ SD [%][a]	RU $\pm$ SD[b]	cAMP $\pm$ SD [%][c]
1	phenyl	CH_3CH_2	99.2 $\pm$ 5.6	3.1 $\pm$ 1.5	73.1 $\pm$ 6.9 (***)
2	*p*-bromophenyl	CH_3CH_2	106.4 $\pm$ 0.0	n.b.	
3	*m*-hydroxyphenyl	CH_3CH_2	85.4 $\pm$ 17.8	8.3 $\pm$ 1.3	78.1 $\pm$ 2.4 (**)
4	2-thienyl	CH_3CH_2	94.1 $\pm$ 5.0	92.1 (aggr.)	93.2 $\pm$ 8.9 (n.s.)
5	2-thienyl	$PhCH_2$	102.3 $\pm$ 1.8	19.0	79.9 $\pm$ 3.8 (**)
6	1-naphthyl	CH_3CH_2	105.4 $\pm$ 7.8	2.8 $\pm$ 0.3	97.0 $\pm$ 6.4 (n.s.)
7	1-naphthyl	$PhCH_2$	92.1 $\pm$ 0.0	14.4 $\pm$ 2.4	84.3 $\pm$ 6.4 (*)

[a] Percent absorbance at primary screening. [b] M_r-adjusted SPR response units at a compound concentration of 200 μM; n.b. = no binding; aggr. = probably aggregated. [c] Percent cAMP production evaluated with the [^{125}I]cAMP Biotrack Assay System; n.s. = no significant difference; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 3. Chemical structures and biological data for **22** and **23.**

Compd	Structure	$A \pm$ SD [%][a]	RU $\pm$ SD[b]	cAMP $\pm$ SD [%][c]
22		97.6 $\pm$ 7.7	11.4	112.4 $\pm$ 4.1 (*)
23		111.5 $\pm$ 8.2	n.b.	

[a] Percent absorbance at primary screening. [b] M_r-adjusted SPR response units at a compound concentration of 200 μM; n.b. = no binding. [c] Percent cAMP production evaluated with the [^{125}I]cAMP Biotrack Assay System; *$p < 0.05$.

3D-QSAR studies

To rationalize all these results, a 3D-QSAR study was undertaken. On the one hand, this methodology increased our knowledge of the chemical aspects required to bind AM, and is useful in the further design of more potent analogues; on the other hand, it helped us to scrutinize the anchoring points that compose the binding site.

All calculations were done by means of the software Almond

10^7).[24] Structural modifications of the lead compound allowed us to obtain compounds with an increased binding affinity toward AM, such as **3**, **5**, **7**, **16** and **22**, that showed RU values two- to threefold higher than that of compound **1**. Figure 2 shows the sensorgrams obtained for compounds **2**, **6**, **7**, and **23**.

Importantly, in the evaluation of the interference of the compounds in the binding between AM and its blocking monoclonal antibody, the neutralizing monoclonal antibody binds to an epitope on the peptide that is critical for receptor recognition. Thus, a negative result in this assay should not always be in accordance with a negative response in the SPR experiments, because the ligand could bind to a different part of the peptide, explaining the lack of correlation between absorbance values at primary screening and SPR responses. Such could be the case of compound **16**, which, according to the primary assay, should be a poor AM binder, whereas the SPR response (7.6 RU) indicates AM binding.

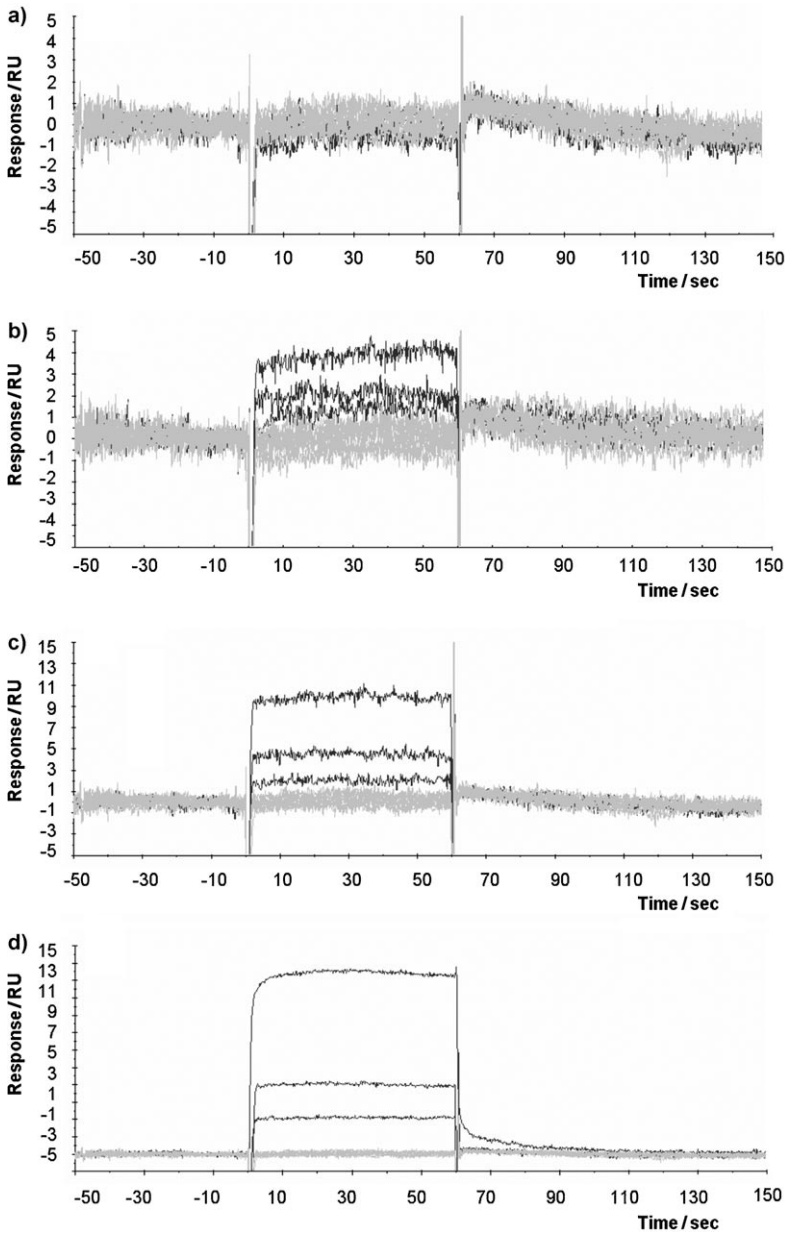


Figure 2. SPR binding curves corresponding to 60-s injections of compounds a) **23**, b) **2**, c) **6**, and d) **7** at concentrations of 0 (gray), 50, 100, and 200 μM (black) on surface-immobilized AM.

3.3.0,[28] following the *gr*id-*in*dependent *d*escriptors (GRIND) approach.[29] A matrix of 500 variables and 13 objects was obtained by using four GRID[30] probes: DRY (which represents hydrophobic interactions), O (sp^2 carbonyl oxygen, representing an H-bond acceptor), N1 (neutral flat NH, such as an amide, an H-bond donor), and the TIP probe (molecular shape descriptor). Ten correlograms of 50 variables each were obtained, thus producing a matrix of 500 variables and 13 objects. A variable selection was applied to decrease the variable number using fractional factorial design (FFD) variable selection implemented in the Almond program. Default values suggested by the program were used. The partial least squares (PLS) analysis resulted in a three-latent-variables model with $r^2 = 0.93$. The cross-validation of the model for three PCs was carried out using the leave-one-out (LOO), the random groups, and the leave-two-out methods, yielding q^2 values of 0.82, 0.79, and 0.76, respectively. The quality of the obtained model was tested by predicting the binding of compound **13** that was not included in the training set. The predicted binding value was very similar to the experimentally measured one. Figure 3 shows the plot of the experimental versus calculated AM binding values, including the binding prediction for **13** as well.

The inspection of the correlograms was carried out. The DRY–DRY and DRY–O correlograms clearly show that the intensity of the interaction is correlated with binding (Figure 4). The variables represent pairs of nodes at different distances where the DRY and O probes have favorably interacted. The greatest interactions (red and magenta, Figure 4) are exhibited by compounds **5** and **7**, which belong to the type A ($R = PhCH_2$) series, and bear an aromatic ring at Cα that might interact with a hydrophobic pocket at the receptor, in agreement with favorable interactions with the DRY probe. On the other hand, these compounds have a hydroxy group that could act as a hydrogen bond donor in the interaction with the receptor, in agreement with favorable interactions with the O probe. Remarkably, these two features were also identified as relevant for binding AM in the 3D-QSAR model derived for the positive modulators.[25]

Among the type B derivates, none behaved as a strong AM binder (RU value > 10), suggesting that the presence of an aromatic substituent at Cα may be crucial for AM binding. However, ester **22**, with a similar substitution pattern at Cα, but with a second benzyl group attached to the piperazine nitrogen atom, showed an RU value of 11.4. It seems that the extra benzyl group could counterbalance the lack of an aromatic ring at Cα, although the presence of a positive charge in the nitrogen atom could also explain its enhanced affinity. Figure 5 shows the geometrical relationships between some regions of favorable interaction with the DRY probe and some regions of favorable interaction with the O probe, identifying putative pockets of the binding site where hydrophobic and hydrogen bond interactions are favorable for binding. Notably, the inclusion of the variables from TIP correlograms considerably enhanced the quality of the model. This fact highlights the importance of the shape description in 3D-QSAR models applied to drug design, as an inappropriate shape complementarity might prevent some ligands from binding purely for steric reasons.

Analysis of second messengers

As mentioned before, it is possible that not all molecules that prevent binding between the peptide and its antibody would also modify binding between the peptide and its receptor.[24] Therefore, selected compounds were analyzed for their ability to modify the levels of cAMP, an intracellular second messenger, in Rat2 cells. The Rat2 cell line contains specific AM receptors and reacts to AM addition by elevating intracellular cAMP concentration.[31] This cell line was obtained from the American Type Culture Collection (Manassas, VA, USA), and the cAMP contents were measured using a cAMP [^{125}I] Biotrack Assay System (Amersham Biosciences) as previously described.[32] Results are summarized in Tables 1–3. All free carboxylic acids (type A compounds and **23**) decreased cAMP levels in accordance with negative modulation of the peptide functions. In the absence of AM, none of the compounds elicited any response.

Surprisingly, all the esters tested in this assay produced an elevation of cAMP, and therefore behaved as positive modulators. This fact suggests that the presence of a free carboxylic group

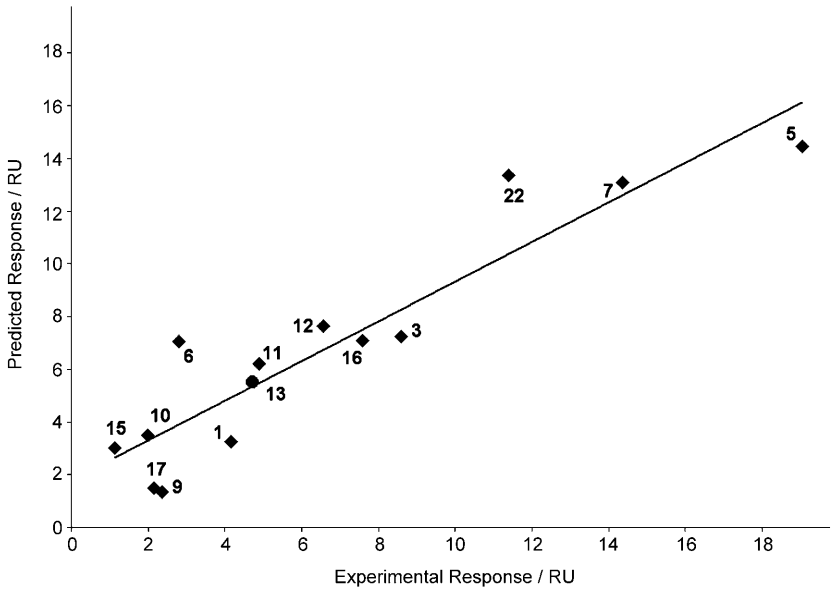


Figure 3. Plot of experimental versus calculated AM binding values; the binding value predicted for **13** is shown as a circle.

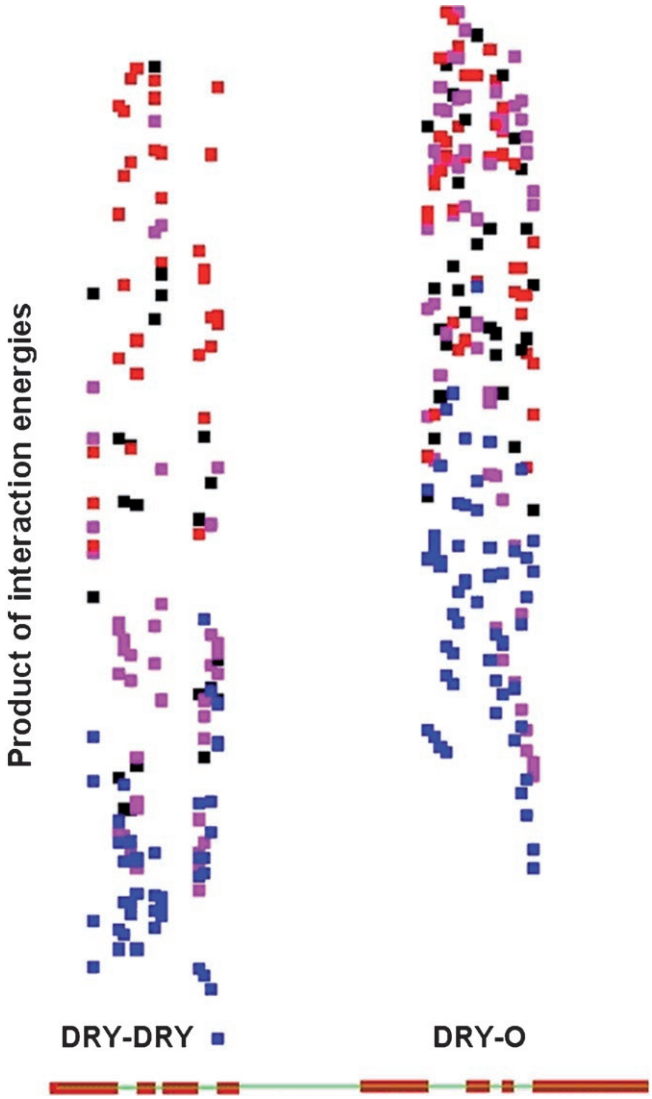


Figure 4. Set of superimposed correlograms corresponding to the DRY–DRY and DRY–O correlograms, representing interactions for ligand hydrophobic regions (DRY–DRY) and ligand hydrogen-bond donor and hydrophobic regions (DRY–O). Every point in the correlogram represents the product of two particular nodes for a certain compound. Points are color-coded according to the binding SPR response of the corresponding compound; the strongest AM binders are in red, medium in magenta, intermediate in black, and weak in blue. A simple visual inspection shows that the strength of the interaction is positively correlated with biological affinity.

is essential for negative modulation. However, there are other structural features, such as the lack of aryl substitution at Cα, which could somehow influence this change in behavior. To discard this possibility, the methyl ester derived from **1** was synthesized and analyzed for comparison with the free acid. We observed that the corresponding ester **24** maintained affinity for AM (percent affinity = 78.1 % ± 3.7 in the competitive monoclonal antibody assay), but behaved as a positive modulator, with percent cAMP of 116.8 % ± 1.3. A similar conclusion can be obtained from comparison of ester **18** and the corresponding acid **21**. Whereas the former has little influence in the levels of cAMP, the latter produces a decrease of 71.4 % ± 3.7, as expected for an effective negative modulator.

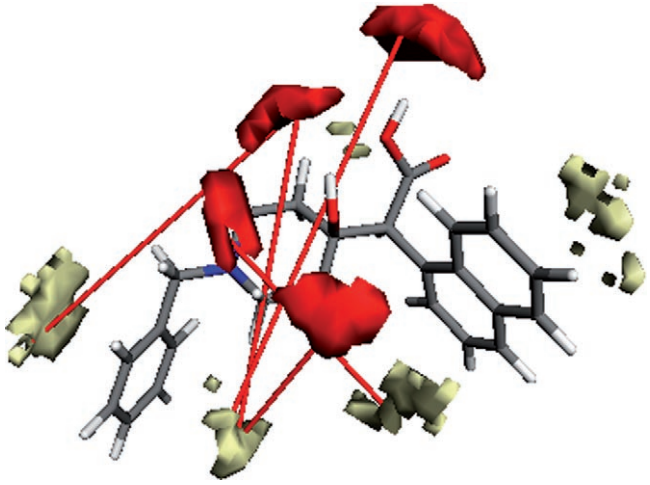


Figure 5. Interactions present in compound **7**; the fields represent interactions of the probe DRY (yellow) and O (red).

Conclusions

Herein we describe the synthesis of a series of analogues of **1** and the use of a competitive AM monoclonal antibody assay and SPR measurements to evaluate the affinity of these compounds toward AM. Some of them showed higher affinity relative to **1**, such as **5**, **7**, **16**, and **22**. A 3D-QSAR study has highlighted essential features for AM binding such as the presence of a hydrogen bond donor and an aromatic ring. This model will be a valuable tool for the design of new derivatives with increased affinity toward AM. The ability to modify cAMP production in Rat2 cells has been evaluated for selected compounds, showing that the presence of a free carboxylic group seems to be essential to obtain negative modulators with potential interest as antiangiogenic and anticancer agents.

Experimental Section

Chemical procedures

Melting points (uncorrected) were determined on a Stuart Scientific SMP3 apparatus. Infrared spectra were recorded with a PerkinElmer 1330 IR spectrophotometer. ^{1}H and ^{13}C NMR data were recorded on a Bruker 300-AC instrument. Chemical shifts (δ) are expressed in parts per million (ppm) relative to internal tetramethylsilane (TMS); coupling constants (J) are reported in Hz. Mass spectra were run on a Bruker Esquire 3000 spectrometer. Elemental analyses (C, H, N) were performed on LECO CHNS-932 equipment at the Microanalyses Service of the University Complutense of Madrid. Thin-layer chromatography (TLC) was run on Merck silica gel 60 F_{254} plates. Unless stated otherwise, starting materials used were high-grade commercial products.

General procedure for the preparation of 1-alkyl-4-[carboxy-(phenyl)methyl]-4-hydroxypiperidinium chlorides (1–7). A solution of the corresponding acid (1.5 equiv) in anhydrous toluene was added to a stirred solution of *i*PrMgCl in Et_2O (2 M) under argon atmosphere at room temperature, and the reaction mixture was held at reflux for 24 h. A solution of 1-ethyl-4-piperidone or 1-benzyl-4-piperidone (1 equiv) in anhydrous toluene was then added, and the suspension was heated at reflux until completion

of the reaction, as monitored by TLC (1–5 h). The reaction mixture was poured into an ice-cold solution of concentrated HCl. The organic layer was extracted with 10% aqueous HCl (3×20 mL). The combined aqueous layers were washed with Et_2O and evaporated to dryness under reduced pressure. The residue was held at reflux in nitromethane for 30 min, and the hot mixture was filtered through a sintered glass funnel. After this extraction procedure had been repeated three times, the combined nitromethane solutions were concentrated under reduced pressure to afford the corresponding hydrochloride.

4-[Carboxy(phenyl)methyl]-1-ethyl-4-hydroxypiperidinium chloride (1). From phenylacetic acid (2.00 g, 15.0 mmol) in toluene (20 mL), *N*-ethyl-4-piperidone (1.27 g, 10.0 mmol) in toluene (20 mL), and *i*PrMgCl (2 M, 15 mL) was obtained **1** (2.42 g, 81%) as a brown solid; mp: 80–85 °C; IR (neat): $\tilde{\nu}_{max}$ = 1690, 3400 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ = 1.31 (t, J = 7.3 Hz, 3H, CH_3), 1.84–2.16 (m, 4H, $2CH_2$), 3.09–3.43 (m, 6H, $2NCH_2$ and CH_2CH_3), 3.72 (s, 1H, CH), 7.30–7.37 (m, 3H, ArH), 7.43–7.46 ppm (m, 2H, ArH); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 9.7, 32.6, 34.0, 53.1, 61.4, 69.5, 128.9, 129.4, 131.0, 135.8, 175.6 ppm; MS (EI): *m/z* 264 $[M+H]^+$; elemental analysis calcd (%) for $C_{15}H_{22}ClNO_3$: C 60.10, H 7.40, N 4.67, found: C 60.32, H 7.25, N 4.33.

4-[(4-Bromophenyl)(carboxy)methyl]-1-ethyl-4-hydroxypiperidinium chloride (2). From (4-bromophenyl)acetic acid (1.00 g, 5.0 mmol) in toluene (11 mL), *N*-ethyl-4-piperidone (0.38 g, 3.0 mmol) in toluene (4 mL), and *i*PrMgCl (2 M, 5 mL) was obtained **2** (0.75 g, 66%) as a yellow solid; mp: 80–82 °C; IR (neat): $\tilde{\nu}_{max}$ = 1720, 3400; ^1H NMR (300 MHz, [D_6]DMSO): δ = 1.30 (t, J = 7.3 Hz, 3H, CH_3), 1.81–2.13 (m, 4H, $2CH_2$), 3.13 (q, J = 7.3 Hz, 2H, CH_2CH_3), 3.19–3.43 (m, 4H, $2NCH_2$), 3.71 (s, 1H, CH), 7.38 (d, J = 8.5 Hz, 2H, ArH), 7.51 ppm (d, J = 8.5 Hz, 2H, ArH); ^{13}C NMR (75.4 MHz, [D_6]DMSO): δ = 9.0, 31.0, 31.5, 47.0, 50.8, 60.0, 120.8, 130.9, 132.1, 134.5, 172.7 ppm; MS (EI): *m/z* 344 $[M+H+2]^+$, 342 $[M+H]^+$; elemental analysis calcd for $C_{15}H_{21}BrClNO_3$: C 45.41, H 5.84, N 3.53, found: C 45.72, H 5.56, N 3.50.

4-[Carboxy(3-hydroxyphenyl)methyl]-1-ethyl-4-hydroxypiperidinium chloride (3). From (3-hydroxyphenyl)acetic acid (1.50 g, 9.8 mmol) in toluene (7 mL), *N*-ethyl-4-piperidone (0.84 g, 6.6 mmol) in toluene (8 mL), and *i*PrMgCl (2 M, 11 mL) was obtained **3** (0.44 g, 25%) as a yellow oil; IR (neat): $\tilde{\nu}_{max}$ = 1750, 3500; ^1H NMR (300 MHz, CD_3OD): δ = 1.30 (t, J = 7.3 Hz, 3H, CH_3), 1.87–2.07 (m, 4H, $2CH_2$), 3.12 (q, J = 7.3 Hz, 2H, CH_2CH_3), 3.29–3.31 (m, 4H, $2NCH_2$), 3.64 (s, 1H, CH), 6.72–6.75 (d, J = 8.5 Hz, 1H, ArH), 6.85–6.91 (m, 2H, ArH), 7.15 ppm (t, J = 7.9 Hz, 1H, ArH); ^{13}C NMR (75.4 MHz, [D_6]DMSO): δ = 9.1, 31.0, 47.1, 50.9, 56.0, 60.8, 114.4, 116.7, 120.7, 128.9, 136.2, 157.0, 173.2 ppm; MS (EI): *m/z* 280 $[M+H]^+$.

4-[Carboxy(2-thienyl)methyl]-1-ethyl-4-hydroxypiperidinium chloride (4). From 2-thienylacetic acid (2.00 g, 14.1 mmol) in toluene (10 mL), *N*-ethyl-4-piperidone (1.20 g, 9.4 mmol) in toluene (10 mL), and *i*PrMgCl (2 M, 14.1 mL) was obtained **4** (3.23 g, 92%) as a brown solid; mp: 106 °C (dec); IR (neat): $\tilde{\nu}_{max}$ = 1720, 3400 cm^{-1}; ^1H NMR (300 MHz, [D_6]DMSO): δ = 1.19 (t, J = 7.3 Hz, 3H, CH_3), 1.70–2.10 (m, 4H, $2CH_2$), 3.01 (bs, 4H, $2NCH_2$), 3.37 (bs, 2H, CH_2CH_3), 3.99 (s, 1H, CH), 5.25 (bs, 1H, OH), 7.00 (m, 1H, thiophene-H), 7.06 (d, J = 3.1 Hz, 1H, thiophene-H), 7.46 ppm (d, J = 4.9 Hz, 1H, thiophene-H); ^{13}C NMR (75.4 MHz, [D_6]DMSO): δ = 9.0, 30.9, 31.3, 47.0, 50.8, 56.9, 126.2, 128.0, 136.1, 172.4 ppm; MS (EI): *m/z* 270 $[M+H]^+$; elemental analysis calcd for $C_{13}H_{20}ClNO_3S$: C 51.06, H 6.59, N 4.58, S 10.49, found: C, 51.29, H 6.32, N 4.62, S 10.27.

4-[Carboxy(2-thienyl)methyl]-1-benzyl-4-hydroxypiperidinium chloride (5). From 2-thienylacetic acid (2.00 g, 14.1 mmol) in toluene (10 mL), *N*-benzyl-4-piperidone (1.80 g, 9.5 mmol) in toluene (10 mL), and *i*PrMgCl (2 M, 14.1 mL) was obtained **5** (2.50 g, 71%) as a brown solid; mp: 115 °C (dec); IR (neat): $\tilde{\nu}_{max}$ = 1720, 3400 cm^{-1}; ^1H NMR (300 MHz, [D_6]DMSO): δ = 1.68–2.17 (m, 4H, $2CH_2$), 3.13 (bs, 4H, $2NCH_2$), 3.98 (s, 1H, CH), 4.25 (bs, 2H, CH_2Ph), 5.23 (bs, 1H, OH), 6.97–7.04 (m, 2H, thiophene-H), 7.43–7.44 (m, 4H, thiophene-H and ArH), 7.60–7.67 ppm (m, 2H, ArH); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 30.7, 31.2, 47.1, 56.9, 58.7, 126.2, 126.6, 128.0, 128.7, 129.4, 129.9, 131.6, 136.1, 172.4 ppm; MS (EI): *m/z* 332 $[M+H]^+$; elemental analysis calcd for $C_{18}H_{22}ClNO_3S$: C 58.77, H 6.03, N 3.81, S 8.72, found: C 58.41, H 6.14, N 3.77, S 8.89.

4-[Carboxy(1-naphthyl)methyl]-1-ethyl-4-hydroxypiperidinium chloride (6). From 1-naphthylacetic acid (2.00 g, 11.0 mmol) in toluene (7 mL), *N*-ethyl-4-piperidone (0.89 g, 7.0 mmol) in toluene (8 mL), and *i*PrMgCl (2 M, 11 mL) was obtained **6** (1.25 g, 51%) as a white solid; mp: 124 °C (dec); IR (neat): $\tilde{\nu}_{max}$ = 1710, 3400 cm^{-1}; ^1H NMR (300 MHz, [D_6]DMSO): δ = 1.15 (t, J = 7.3 Hz, 3H, CH_3), 1.83–2.01 (m, 4H, $2CH_2$), 2.96 (q, J = 7.9 Hz, 2H, CH_2CH_3), 3.19–3.26 (m, 2H, NCH_2), 3.38–3.44 (m, 2H, NCH_2), 4.69 (s, 1H, CH), 7.48–7.59 (m, 3H, ArH), 7.61–7.90 (m, 2H, ArH), 8.6 ppm (d, J = 8.6 Hz, 2H, ArH); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 9.0, 30.5, 32.3, 47.0, 50.8, 53.4, 69.0, 123.8, 125.2, 125.6, 126.5, 126.9, 128.0, 128.9, 131.4, 132.5, 133.7, 173.7 ppm; MS (EI): *m/z* 350 $[M+H]^+$; elemental analysis calcd for $C_{19}H_{24}ClNO_3 \cdot 1H_2O$: C 62.03, H 7.12, N 3.81, found: C 62.39, H 6.84, N 4.32.

4-[Carboxy(1-naphthyl)methyl]-1-benzyl-4-hydroxypiperidinium chloride (7). From 1-naphthylacetic acid (1.12 g, 6.0 mmol) in toluene (7 mL), *N*-benzyl-4-piperidone (0.75 g, 4.0 mmol) in toluene (8 mL), and *i*PrMgCl (2 M, 11 mL) was obtained **7** (0.18 g, 11%) as a white solid; mp: 204 °C (dec); IR (neat): $\tilde{\nu}_{max}$ = 1690, 3460 cm^{-1}; ^1H NMR (300 MHz, [D_6]DMSO): δ = 1.74–1.88 (m, 4H, $2CH_2$), 3.18–3.31 (m, 4H, $2NCH_2$), 4.25 (s, 2H, CH_2), 4.72 (s, 1H, CH), 7.45–7.47 (m, 8H, ArH), 7.79–7.90 (m, 2H, ArH), 8.22 ppm (d, J = 8.6 Hz, 2H, ArH); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 30.4, 32.5, 47.6, 49.2, 59.2, 68.7, 124.1, 125.4, 126.0, 126.9, 127.1, 128.4, 129.1, 129.2, 129.7, 129.9, 131.4, 131.7, 132.6, 134.0, 173.8 ppm; MS (EI): *m/z* 376 $[M+H]^+$; elemental analysis calcd for $C_{24}H_{26}ClNO_3 \cdot 2H_2O$: C 64.35, H 6.75, N 3.13, found: C 65.94, H 6.27, N 4.10.

***tert*-Butyl-4-hydroxy-4-(2-methoxy-1,1-dimethyl-2-oxoethyl)piperidine-1-carboxylate (8)**. Lithium *N*-isopropyl-*N*-cyclohexylamide was prepared at −78 °C under argon by adding *n*BuLi (1.6 M, 15.75 mL) in hexane dropwise to isopropylcyclohexylamine (4.1 mL) in anhydrous THF (20 mL). After addition was complete and the solution was stirred for 30 min, ethyl isobutyrate (2.6 mL, 25 mmol) in anhydrous THF (5 mL) was added dropwise to the mixture at −78 °C. 30 min later, *tert*-butyl-4-oxopiperidine-1-carboxylate (3.3 g, 17 mmol) in anhydrous THF (5 mL) was added dropwise, and the mixture was stirred at −78 °C for 24 h. The crude reaction was diluted with EtOAc and washed with H_2O (3×20 mL) and brine. The organic layer was dried (Na_2SO_4), filtered, and concentrated to dryness. Flash chromatography of the residue using EtOAc/hexane (1:1) as eluent gave **8** (4.45 g, 87%) as a white solid; mp: 80–81 °C; IR (neat): $\tilde{\nu}_{max}$ = 1650, 1720, 3480 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ = 1.23 (s, 6H, $2CH_3$), 1.46 (s, 9H, $3CH_3$), 1.41–1.50 (m, 2H, CH_2), 1.62 (td, J = 12.6, 4.9 Hz, 2H, CH_2), 3.10 (td, J = 12.6, 2.7 Hz, 2H, CH_2), 3.72 (s, 3H, OCH_3), 3.92–3.97 ppm (m, 2H, CH_2); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 20.7, 28.7, 31.3, 39.0, 39.7, 49.6, 52.2, 72.6, 79.3, 154.8, 178.9 ppm; MS (EI): *m/z* 324 $[M+Na]^+$; elemental analysis calcd for $C_{15}H_{27}NO_5$: C 59.78, H 9.03, N 4.65, found: C 59.40, H 8.79, N 4.68.

4-Hydroxy-4-(2-methoxy-1,1-dimethyl-2-oxoethyl)piperidinium chloride (9). HCl gas was bubbled for 15 min through a solution of **8** (4.46 g, 15.0 mmol) in EtOAc (40 mL) at 0 °C. The solution was concentrated to dryness to give **9** (2.70 g, 75%) as a white solid; mp: 176–177 °C; IR (neat): $\tilde{\nu}_{max}$ = 1710, 2700, 3300 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ = 1.24 (s, 6H, $2CH_3$), 1.41–1.46 (m, 2H, CH_2), 1.65 (td, J = 12.6, 4.9 Hz, 2H, CH_2), 2.85–2.90 (m, 2H, CH_2), 3.03 (td, J = 12.9, 2.7 Hz, 2H, CH_2), 3.72 ppm (s, 3H, OCH_3); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 20.8, 29.8, 41.4, 51.4, 52.5, 71.5 ppm; MS (EI): m/z 202 $[M+H]^+$; elemental analysis calcd for $C_{10}H_{20}ClNO_3$: C 50.52, H 8.58, Cl 14.91, N 5.89, O 20.19, found: C 50.06, H 8.39, N 5.95.

General procedure for the preparation of methyl 2-(1-alkyl-4-hydroxypiperidin-4-yl)-2-methylpropanoates (10–17 and 22). K_2CO_3 (2 equiv) followed by the corresponding alkylating agent (1 equiv) were added to a stirred solution of amine hydrochloride **9** (1 equiv) in *N*,*N*-dimethylformamide (DMF, 2 mL) at room temperature, and the mixture was stirred until the reaction was complete (4–24 h). H_2O (10 mL) was added, and the mixture was extracted with EtOAc (3×15 mL). The combined organic extracts were washed with H_2O and brine, dried ($MgSO_4$), filtered, and concentrated to dryness. The residue was purified by flash column chromatography on silica gel.

Methyl 2-(1-allyl-4-hydroxypiperidin-4-yl)-2-methylpropanoate (10). From **9** (0.10 g, 0.4 mmol) in DMF (2 mL), K_2CO_3 (0.14 g, 0.8 mmol), and allyl bromide (0.043 mL, 0.4 mmol), and after chromatography of the crude reaction using EtOAc as eluent, compound **10** (0.042 g, 41%) was obtained as a yellow oil; IR (neat): $\tilde{\nu}_{max}$ = 1720, 3500 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ = 1.24 (s, 6H, $2CH_3$), 1.44–1.49 (m, 2H, CH_2), 1.79 (td, J = 12.6, 4.9 Hz, 2H, CH_2), 2.30 (td, J = 12.1, 2.2 Hz, 2H, CH_2), 2.73–2.76 (m, 2H, CH_2), 3.02 (d, J = 6.6 Hz, 2H, CH_2), 3.42 (s, 1H, OH), 3.71 (s, 3H, OCH_3), 5.13–5.22 (m, 2H, C=CH_2), 5.82–5.85 ppm (m, 1H, C=CH); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 20.8, 31.4, 48.9, 49.5, 52.0, 61.8, 72.3, 117.8, 135.2, 178.9 ppm; MS (EI): m/z 242 $[M+H]^+$.

Methyl 2-(1-benzyl-4-hydroxypiperidin-4-yl)-2-methylpropanoate (11). From **9** (0.50 g, 2.1 mmol) in DMF (2 mL), K_2CO_3 (0.58 g, 4.2 mmol), and benzyl bromide (0.25 mL, 2.11 mmol), and after chromatography of the crude reaction using EtOAc as eluent, compound **11** (0.23 g, 38%) was obtained as a yellow oil; IR (neat): $\tilde{\nu}_{max}$ = 1700–1720, 3500 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ = 1.22 (s, 6H, $2CH_3$), 1.42 (bd, J = 11.5 Hz, 2H, CH_2), 1.77 (bt, J = 13.2 Hz, 2H, CH_2), 2.37–2.44 (m, 2H, CH_2), 2.70–2.74 (m, 2H, CH_2), 3.36 (s, 1H, OH), 3.51 (s, 2H, CH_2Ph), 3.67 (s, 3H, OCH_3), 7.27–7.33 ppm (m, 5H, ArH); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 20.6, 31.3, 48.8, 49.4, 51.7, 62.9, 72.1, 126.6, 127.9, 128.9, 138.4, 178.5 ppm; MS (EI): m/z 292 $[M+H]^+$.

Methyl 2-(4-hydroxy-1-(4-nitrobenzyl)piperidin-4-yl)-2-methylpropanoate (12). From **9** (0.10 g, 0.4 mmol) in DMF (2 mL), K_2CO_3 (0.12 g, 0.8 mmol), and 4-nitrobenzyl bromide (0.09 g, 0.4 mmol), and after chromatography of the crude reaction using EtOAc as eluent, compound **12** (0.07 g, 48%) was obtained as a yellow solid; mp: 77–78 °C; IR (neat): $\tilde{\nu}_{max}$ = 1700, 3460 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ = 1.25 (s, 6H, $2CH_3$), 1.43–1.47 (m, 2H, CH_2), 1.80 (td, J = 12.6, 4.4 Hz, 2H, CH_2), 2.40–2.51 (m, 2H, CH_2), 2.63–2.67 (m, 2H, CH_2), 3.45 (s, 1H, OH), 3.61 (s, 2H, CH_2Ph), 3.72 (s, 3H, OCH_3), 7.52 (d, J = 8.8 Hz, 2H, Ar-H), 8.17 ppm (d, J = 8.8 Hz, 2H, Ar-H); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 20.8, 31.4, 41.2, 52.1, 53.0, 61.9, 72.0, 123.5, 123.7, 129.2, 129.7, 178.9 ppm; MS (EI): m/z 337 $[M+H]^+$; elemental analysis calcd for $C_{17}H_{24}N_2O_5$: C 60.70, H 7.19, N 8.33, found: C 59.87, H 7.05, N 8.25.

Methyl 2-(4-hydroxy-1-[4-(trifluoromethyl)benzyl)]piperidin-4-yl)-2-methylpropanoate (13). From **9** (0.15 g, 0.6 mmol) in DMF (2 mL), K_2CO_3 (0.18 g, 1.3 mmol), and 4-(trifluoromethyl)benzyl bromide (0.15 g, 0.6 mmol), and after chromatography of the crude reaction using EtOAc as eluent, compound **13** (0.21 g, 94%) was obtained as a yellow oil; IR (neat): $\tilde{\nu}_{max}$ = 1340, 1700, 3500 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ = 1.24 (s, 6H, $2CH_3$), 1.42–1.47 (m, 2H, CH_2), 1.79 (td, J = 13.2, 4.4 Hz, 2H, CH_2), 2.37–2.46 (m, 2H, CH_2), 2.65–2.68 (m, 2H, CH_2), 3.43 (s, 1H, OH), 3.57 (s, 2H, CH_2Ph), 3.70 (s, 3H, OCH_3), 7.46 (d, J = 8.3 Hz, 2H, Ar-H), 7.56 ppm (d, J = 8.3 Hz, 2H, Ar-H); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 20.8, 31.4, 49.1, 49.6, 52.1, 62.4, 72.3, 122.4, 125.0, 125.1, 125.1, 125.2, 126.0, 129.2, 178.2 ppm; MS (EI): m/z 360 $[M+H]^+$; elemental analysis calcd for $C_{18}H_{24}F_3NO_3$: C 60.16, H 6.73, N 3.90, found: C 59.77, H 6.69, N 3.94.

Methyl 2-[4-hydroxy-1-(2-hydroxyethyl)piperidin-4-yl]-2-methylpropanoate (14). From **9** (0.20 g, 0.8 mmol) in DMF (2 mL), K_2CO_3 (0.23 g, 1.7 mmol), and 2-bromoethanol (0.11 g, 0.8 mmol), and after chromatography of the crude reaction using EtOAc/MeOH (9:1) as eluent, compound **14** (0.07 g, 34%) was obtained as a yellow oil; IR (neat): $\tilde{\nu}_{max}$ = 1720, 3400 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ = 1.23 (s, 3H, CH_3), 1.24 (s, 3H, CH_3), 1.43–1.48 (m, 2H, CH_2), 1.74–1.84 (m, 2H, CH_2), 2.38–2.45 (m, 2H, CH_2), 2.56–2.58 (m, 2H, CH_2), 2.71–2.77 (m, 2H, CH_2), 3.45 (d, J = 6.6 Hz, 1H, 1/2CH_2), 3.65 (d, J = 6.6 Hz, 1H, 1/2CH_2), 3.72 ppm (s, 3H, OCH_3); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 20.8, 31.5, 48.8, 49.5, 52.1, 57.8, 59.2, 72.2, 178.9 ppm; MS (EI): m/z 246 $[M+H]^+$.

Methyl 2-[1-(2-diethylaminoethyl)-4-hydroxypiperidin-4-yl]-2-methylpropanoate (15). From **9** (0.20 g, 0.8 mmol) in DMF (2 mL), K_2CO_3 (0.23 g, 1.7 mmol), and 2-chloro-*N*,*N*-diethylamine (0.15 g, 0.8 mmol), and after chromatography of the crude reaction using EtOAc/MeOH/NH_3 (9:1:0.1) as eluent, compound **15** (0.086 g, 34%) was obtained as a yellow oil; IR (neat): $\tilde{\nu}_{max}$ = 1720, 3300 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ = 1.05 (t, J = 7.2 Hz, 6H, $2CH_3$), 1.24 (s, 6H, $2CH_3$), 1.44–1.48 (m, 2H, CH_2), 1.85 (td, J = 13.2, 4.4 Hz, 2H, CH_2), 2.48 (bt, J = 11.0 Hz, 4H, CH_2), 2.60 (q, J = 14.3, 7.1 Hz, 4H, $2CH_2$), 2.80–2.83 (m, 2H, CH_2), 3.48 (s, 1H, OH), 3.71 ppm (s, 3H, OCH_3); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 11.6, 20.8, 31.4, 47.4, 49.6, 50.2, 52.0, 56.5, 72.3, 178.9 ppm; MS (EI): m/z 301 $[M+H]^+$.

Methyl 2-[4-hydroxy-1-(4-methoxybenzyl)piperidin-4-yl]-2-methylpropanoate (16). From **9** (0.20 g, 0.8 mmol) in DMF (2 mL), K_2CO_3 (0.23 g, 1.7 mmol), and 4-methoxybenzyl bromide (0.17 g, 0.8 mmol), and after chromatography of the crude reaction using EtOAc/MeOH/NH_3 (1:1:0.1) as eluent, compound **16** (0.05 g, 20%) was obtained as a white solid; mp: 65–67 °C; IR (neat): $\tilde{\nu}_{max}$ = 1690, 1720, 3500 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ = 1.23 (s, 6H, $2CH_3$), 1.40–1.44 (m, 2H, CH_2), 1.76 (td, J = 12.6, 4.4 Hz, 2H, CH_2), 2.29–2.38 (m, 2H, CH_2), 2.66–2.70 (m, 2H, CH_2), 3.34 (s, 1H, OH), 3.46 (s, 2H, CH_2Ph), 3.70 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3), 6.85 (d, J = 8.8 Hz, 2H, Ar-H), 7.24 ppm (d, J = 8.8 Hz, 2H, Ar-H); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 20.8, 31.4, 48.8, 49.6, 52.0, 55.2, 62.4, 72.4, 113.4, 130.3, 158.5, 178.9 ppm; MS (EI): m/z 322 $[M+H]^+$; elemental analysis calcd for $C_{28}H_{27}NO_4$: C 67.26, H 8.47, N 4.36, found: C 66.74, H 8.31, N 4.46.

Methyl 2-(4-hydroxy-1-prop-2-yn-1-ylpiperidin-4-yl)-2-methylpropanoate (17). From **9** (0.20 g, 0.8 mmol) in DMF (2 mL), K_2CO_3 (0.23 g, 1.7 mmol), and propargyl bromide (0.13 g, 0.8 mmol), and after chromatography of the crude reaction using EtOAc/MeOH/NH_3 (9:1:0.1) as eluent, compound **17** (0.09 g, 45%) was obtained as a yellow solid; mp: 66–67 °C; IR (neat): $\tilde{\nu}_{max}$ = 1730, 3260, 3490 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ = 1.24 (s, 6H, $2CH_3$), 1.50 (dd, J = 13.2, 2.2 Hz, 2H, CH_2), 1.82 (td, J = 12.6, 4.4 Hz, 2H, CH_2),

2.27 (t, $J=2.2$ Hz, 1H, CH), 2.53–2.61 (m, 2H, CH_2), 2.75 (dt, $J=11.0$, 2.2 Hz, 2H, CH_2), 3.28 (d, $J=2.8$ Hz, 2H, CH_2), 3.44 (s, 1H, OH), 3.72 (s, 3H, OCH_3); ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta=20.7$, 31.4, 46.9, 48.1, 49.5, 52.0, 71.9, 72.9, 79.2, 178.8 ppm; MS (EI): *m/z* 240 $[M+H]^+$; elemental analysis calcd for $C_{13}H_{21}NO_3$: C 65.25, H 8.84, N 5.85, found: C 64.71, H 8.63, N 5.68.

1,1-Dibenzyl-4-hydroxy-4-(2-methoxy-1,1-dimethyl-2-oxoethyl)-piperidinium bromide (22). From **9** (0.50 g, 2.1 mmol) in DMF (10 mL), K_2CO_3 (0.58 g, 4.2 mmol), and benzyl bromide (0.25 mL, 2.1 mmol), and after chromatography of the crude reaction using EtOAc/MeOH (9:1) as eluent, compound **22** (0.53 g, 54%) was obtained as a white solid; mp: 87–88 °C, IR (neat): $\tilde{\nu}_{max}=1710$, 3400 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=1.29$ (s, 6H, CH_3), 1.84–1.88 (d, 2H, CH_2), 2.36–2.45 (t, 2H, CH_2), 3.56–3.64 (t, 2H, CH_2), 3.72 (s, 3H, OCH_3), 3.72–3.81 (t, 2H, CH_2), 4.00 (s, 1H, OH), 4.77 (s, 2H, CH_2), 5.22 (s, 2H, CH_2), 7.28–7.24 (m, 8H, Ar-H), 7.71–7.74 ppm (d, 2H, Ar-H); ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta=20.83$, 27.80, 50.82, 52.65, 61.79, 67.68, 70.38, 79.49, 128.24, 128.71, 130.24, 130.67, 131.75, 132.00, 134.18, 134.88, 178.24 ppm; MS (EI): *m/z* 382.33 $[M]^+$; elemental analysis calcd for $C_{24}H_{32}BrNO_3\cdot 1H_2O$: C 60.00, H 7.13, N 3.03, found: C 59.77, H 6.92, N 2.92.

Methyl 2-(1-ethyl-4-hydroxypiperidin-4-yl)-2-methylpropanoate (18). The procedure described above for **8** was used for the synthesis of **18**. From *n*BuLi (1.6 M, 15.8 mL) in hexane, isopropylcyclohexylamine (4.1 mL) in anhydrous THF (20 mL), ethyl isobutyrate (2.6 mL, 25.0 mmol) in anhydrous THF (5 mL), and *N*-ethyl-4-piperidone (2.29 mL, 17.0 mmol) in anhydrous THF (5 mL), and after chromatography of the crude reaction using CH_2Cl_2/MeOH (9:1) as eluent, compound **18** (3.59 g, 92%) was obtained as a yellow oil; IR (neat): $\tilde{\nu}_{max}=1720$, 1740, 3500 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=1.09$ (t, $J=7.1$ Hz, 3H, CH_3), 1.24 (s, 6H, $2CH_3$), 1.47 (dd, $J=13.7$, 2.8 Hz, 2H, CH_2), 1.80 (td, $J=13.2$, 4.4 Hz, 2H, CH_2), 2.26–2.35 (m, 2H, CH_2), 2.43 (q, $J=7.1$ Hz, 2H, CH_2), 2.74–2.78 (m, 2H, CH_2), 3.42 (s, 1H, OH), 3.72 (s, 3H, OCH_3); ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta=11.8$, 20.6, 31.2, 48.4, 49.4, 51.8, 52.1, 72.2, 178.7 ppm; MS (EI): *m/z* 230 $[M+H]^+$.

Methyl 2-(1-benzoyl-4-hydroxypiperidin-4-yl)-2-methylpropanoate (19). The procedure described above for **8** was used for the synthesis of **19**. From *n*BuLi (1.6 M, 3.15 mL) in hexane, isopropylcyclohexylamine (0.825 mL, 5.0 mmol) in anhydrous THF (10 mL), ethyl isobutyrate (0.57 mL, 5.0 mmol) in anhydrous THF (2 mL), and *N*-benzoyl-4-piperidone (0.676 g, 3.3 mmol) in anhydrous THF (5 mL), and after chromatography of the crude reaction using hexane/EtOAc (9:1) as eluent, compound **19** (0.39 g, 39%) was obtained as a white solid; mp: 88–89 °C; IR (neat): $\tilde{\nu}_{max}=1640$, 1740, 3450 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=1.17$ (s, 6H, CH_3), 1.31–1.68 (m, 4H, CH_2), 3.07–3.42 (m, 2H, CH_2), 3.65 (s, 3H, OCH_3), 7.32 ppm (s, 5H, Ar-H); ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta=20.7$, 31.3, 32.2, 37.9, 43.5, 49.4, 52.2, 72.8, 126.8, 128.4, 129.5, 136.1, 170.2, 178.8 ppm; MS (EI): *m/z* 306 ($[M+H]^+$); elemental analysis calcd for $C_{17}H_{23}NO_4\cdot 1H_2O$: C 63.14, H 7.79, N 4.33, found: C 62.98, H 7.56, N 4.45.

Methyl 2-(4-ethyl-1-hydroxycyclohexyl)-2-methylpropanoate (20). The procedure described above for **8** was used for the synthesis of **20**. From *n*BuLi (1.6 M, 14.9 mL) in hexane, isopropylcyclohexylamine (3.95 mL, 23.8 mmol) in anhydrous THF (10 mL), ethyl isobutyrate (2.73 mL, 23.8 mmol) in anhydrous THF (2 mL), and 4-ethylcyclohexanone (2.00 g, 15.9 mmol) in anhydrous THF (5 mL), and after chromatography of the crude reaction using hexane/EtOAc (9:1) as eluent, compound **20** (4.54 g, 87%) was obtained as a colorless oil; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=0.87$ (t, $J=7.1$ Hz, 3H, CH_3), 0.95–1.10 (m, 2H, CH_2), 1.22 (s, 6H, $2CH_3$), 1.24–1.57 (m, 9H, $4CH_2$ and CH), 3.13 (bs, 1H, OH), 3.70 ppm (s, 3H, OCH_3); ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta=11.36$, 20.76, 27.60, 29.46, 31.41, 38.84, 49.91, 51.71, 73.82, 178.91 ppm; MS (EI): *m/z* 306 ($[M+H]^+$); elemental analysis calcd for $C_{13}H_{24}O_3$: C 66.98, H 10.59, found: C 67.31, H 10.23.

2-(1-Ethyl-4-hydroxypiperidin-4-yl)-2-methylpropanoic acid (21). An aqueous solution of NaOH (5 mL, 0.5 M) was added to **18** (0.20 g, 0.9 mmol) in THF (7.5 mL), and the mixture was held at reflux for 2 days. Then, 10% aqueous HCl was added until the appearance of a white solid, which was filtered and washed with H_2O, giving compound **21** (0.07 g, 37%); mp: 189 °C (dec); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=1.24$ (s, 3H, CH_3), 1.25 (s, 3H, CH_3), 1.36 (td, $J=7.2$, 1.7 Hz, 3H, CH_3), 1.94 (d, $J=14.9$ Hz, 2H, CH_2), 2.14 (td, $J=13.2$, 2.7 Hz, 2H, CH_2), 3.14–3.47 (m, 6H, $3CH_2$), 1.24 (s, 3H, CH_3), 1.25 (s, 3H, CH_3), 1.36 (td, $J=7.2$, 1.7 Hz, 3H, CH_3), 1.94 (d, $J=14.9$ Hz, 2H, CH_2), 2.14 (td, $J=13.2$, 2.7 Hz, 2H, CH_2), 3.14–3.47 (m, 6H, $3CH_2$); ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta=0.22$, 24.17, 30.95, 49.51, 49.37, 52.80, 71.83, 180.37 ppm; MS (EI): *m/z* 217 $[M+H]^+$.

2-(4-Ethyl-1-hydroxycyclohexyl)-2-methylpropionic acid (23). An aqueous solution of NaOH (11 mL, 0.5 M) was added to **20** (0.30 g, 1.3 mmol) in THF (9 mL) and stirred at room temperature for 24 h. Then, 10% aqueous HCl was added, and the mixture was extracted with Et_2O (3×20 mL). The extracts were dried (Na_2SO_4), filtered, and concentrated to dryness. Flash column chromatography of the residue using EtOAc as eluent gave **23** (0.25 g, 88%) as a white solid; mp: 70–71 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=0.87$ (t, $J=7.1$ Hz, 3H, CH_3), 0.95–1.10 (m, 2H, CH_2), 1.22 (s, 6H, $2CH_3$), 1.24–1.57 (m, 9H, $4CH_2$ and CH), 3.13 (bs, 1H, OH), 3.70 ppm (s, 3H, OCH_3); ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta=11.43$, 20.75, 27.54, 29.63, 31.22, 38.72, 50.11, 74.67, 183.23 ppm; MS (EI): *m/z* 237.05 $[M+Na]^+$.

Methyl (1-ethyl-4-hydroxypiperidin-4-yl)(phenyl)acetate (24). The procedure described above for **8** was used for the synthesis of **24**. From *n*BuLi (1.6 M, 2.1 mL) in hexane, isopropylcyclohexylamine (0.55 mL) in anhydrous THF (3 mL), ethyl phenylacetate (0.50 g, 3.3 mmol) in anhydrous THF (2 mL), and *N*-ethyl-4-piperidone in anhydrous THF (5 mL), and after chromatography using $CHCl_3$/CH_3OH (9:1) as eluent, compound **24** (0.11 g, 12%) was obtained as a yellow oil; ^{1}H NMR (300 MHz, CD_3OD): $\delta=1.09$ (t, $J=7.3$ Hz, 3H, CH_3), 1.59–1.70 (m, 4H, $2NCH_2$), 2.36–2.64 (m, 4H, $2NCH_2$), 2.50 (q, $J=7.3$ Hz, 2H, CH_2CH_3), 3.65 (s, 3H, OCH_3), 3.72 (s, 1H, CH), 7.24–7.33 (m, 3H, ArH), 7.42–7.44 ppm (m, 2H, ArH); ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta=11.7$, 34.0, 36.8, 48.2, 48.4, 52.1, 52.2, 59.7, 70.2, 127.7, 128.4, 129.5, 134.1, 174.6 ppm; MS (EI): *m/z* 278 ($[M+H]^+$).

Competitive monoclonal antibody assay

Human AM was attached to PVC 96-well plates by adsorption, which involved incubating 50 μL AM (at 100 μg μL) per well for 1 h. After discarding the coating solution, wells were blocked with 200 μL 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). After 1 h, this solution was aspirated off, and 50 μL of one of the compounds (1 μM) in PBS were added. After 1 h, 50 μL peroxidase-labeled antibody (at 1.2 μg mL^{-1}) were added, and the solution was allowed to react for 1 h. Following thorough washes with PBS, peroxidase activity was developed using *o*-phenylenediamine dihydrochloride (Sigma) as a substrate. The reaction product was quantified with a plate reader (Titertek Multiskan PLUS) at 450 nm. Each plate contained several internal controls, including wells without coating, which are used to calculate nonspecific

binding; wells with no potential antagonists were added, which provided maximum binding; and wells in which the unlabeled antibody (5.0 $\mu g\,mL^{-1}$) substituted for the small molecule as a positive inhibition control. Each compound was added to duplicate wells in the same plate. A positive hit was defined as a compound that significantly decreases the amount of reaction product in three independent plates. The intra-assay variation was 6%, and the inter-assay variation was 13%. The sensitivity of the assay, as calculated with the cold antibody, was 12 nM, and the dynamic range was between 12 and 54 nM.

Surface plasmon resonance studies

Real-time biomolecular interaction analysis was performed with a Biacore T100 instrument (GE Healthcare Biacore, Uppsala, Sweden). All experiments were performed at 25 °C. Briefly, 1800 response units (RU) of AM were immobilized on flow cell 2 (Fc 2) of a sensor chip (sCM5) using the amine coupling chemistry according to the manufacturer's instructions (BIAapplications Handbook). Fc 1 was treated as Fc 2 except that peptide injection was omitted, for use as reference. Binding studies were conducted with HBS-DMSO (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 5% DMSO) as running buffer at a flow rate of 30 $\mu L\,min^{-1}$. Samples containing the compounds at concentrations ranging between 10 and 200 μM in HBS-DMSO were injected for 1 min over reference and peptide surfaces, followed by a post-injection period of 2 min. Regeneration was performed by two successive injections (30 s each) of NaCl (0.5 M), followed by a stabilization period of 2 min. Typically, the first 10 cycles consisted of HBS-DMSO injections to ensure that the surface was fully equilibrated. HBS-DMSO was also injected before each new compound, for double referencing. All biosensor data were analyzed using the Biacore T100 Evaluation software, version 1.1.1 (GE Healthcare). Binding curves were obtained by subtracting data recorded on Fc 1 from those recorded on other flow cells to correct for bulk refractive index changes, and also for each flow cell, by subtracting an average of the HBS-DMSO injection data from compound injection data (double referencing). DMSO generates large shifts in refractive index (RI) relative to the small signal expected from compound binding. Therefore, data were also solvent-corrected to account for possible differences in DMSO-generated RI between flow cells.[33]

3D-QSAR analysis

3D structure compounds were built on their neutral form, and the bulky substituent at piperidine C4 was placed in the equatorial position. The same absolute configuration of the stereogenic center was considered for all compounds. Geometry was optimized at the AM1 level using ChemDraw Ultra 10.0 and Gaussian98 software.[34] Data from SPR studies were used as the *y* variable (Table 1). The conformations obtained were then analyzed using the GRIND methodology with the program Almond 3.3.0. DRY, O, and N1 probes were chosen in order to represent potentially important groups of the binding site. Molecular shape field (TIP "probe") was also included. The grid spacing was set to 0.5 Å, and the smoothing window, to 0.8. The number of filtered nodes was set to 100 with 50% relative weights. Ten groups of variables were produced by Almond: four autocorrelograms and six cross-correlograms. The baseline was removed for scaling. Cross-validation was done by using the leave-one-out (LOO) method or by assigning the compounds randomly to five groups, performing cross-validation on these groups, and then repeating the whole procedure 20 times. No relevant differences were found between both validation methods. All computations were carried out on an Intel Pentium 4 using the Linux RedHat 9 operating system.

Analysis of second messengers (cAMP assay)

The Rat2 cell line contains specific AM receptors and reacts to AM addition by elevating its intracellular cAMP content. Rat2 fibroblasts were grown in RPMI 1640 containing 10% fetal bovine serum (Life Technologies). Cells were seeded into 24-well plates at 1×10^5 cells per well and incubated for 24 h at 37 °C in 5% CO_2. Before the assay, cells were incubated in TIS medium (RPMI 1640 plus 10 $mg\,mL^{-1}$ transferrin, 10 $mg\,mL^{-1}$ insulin, and 50 nM sodium selenite) for 15 min. The cells were then treated for 5 min with TIS medium containing 1% BSA, 1 $mg\,mL^{-1}$ bacitracin, and 100 mM isobutylmethylxanthine. Test compounds were then added to obtain a final concentration of 100 nM. The reaction was terminated after 5 min by adding an equal volume of ice-cold EtOH. cAMP contents were measured using a [125I]cAMP RIA kit (Biotrack, Amersham Pharmacia, Piscataway, NJ, USA) following the manufacturer's instructions.

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