DOI: 10.1002/cmdc.201200246

2-Aminopyridine Derivatives as Potential σ_2 Receptor Antagonists

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σ_2 Receptor research is receiving increasing interest with regard to the potential of σ_2 proteins as targets for tumor therapy and diagnosis. Nevertheless, knowledge about the σ_2 receptor is far from conclusive. The paucity and modest affinity of known σ_2 antagonists represent one of the limitations to σ_2 receptor research. Previous studies of the high-affinity σ_2 agonist 1-cyclohexyl-4-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)-*n*-propyl]piperazine **4** (PB28) suggested that a decrease in lipophilicity might lead to σ_2 ligands devoid of antiproliferative activity (potential σ_2 antagonists). With the aim of producing σ_2 receptor antagonists, we replaced the tetralin nucleus of compound **4** with a 2-aminopyridine moiety. A series of compounds with high affinity for both σ subtypes and with no antiproliferative activity in various cells (mouse HT-22, human SK-N-SH, MCF-7wt, and MCF-7σ_1) were obtained. The effect on Ca^{2+} mobilization was investigated for high-affinity compounds **18** and **4**, which showed opposite effects. All of the data support the new 2-aminopyridines as high-affinity σ ligands with σ_2 antagonist and σ_1 agonist activity, and, despite the lack of significant σ_2 versus σ_1 selectivity, these novel compounds may be better tools for σ receptor research than the known low-affinity σ_2 antagonists.

Introduction

The two subtypes of sigma (σ) receptors, namely σ_1 and σ_2, represent potential and interesting targets for the diagnosis and therapy of different kinds of tumors and a number of central nervous system (CNS) diseases.[1,2] Since their discovery, impressive progress has been made in σ receptor research, although several pieces of information are still missing for comprehensive knowledge about their mechanisms of action. Of the two subtypes, only σ_1 has been cloned from different sources.[3] Increasing evidence links this protein to neuroprotective and neuroregulatory functions and to CNS pathologies such as schizophrenia, depression, and Alzheimer's and Parkinson's diseases.[4–6] Recently, it has been shown that juvenile amyotrophic lateral sclerosis is caused by a mutation to the gene encoding the σ_1 receptor.[7] Diverse mechanisms of action have been proposed for the σ_1 protein, which has been assigned a chaperone function for cross-talk between the endoplasmic reticulum (ER) and the mitochondrion through Ca^{2+} signaling, as well as a role in lipid compartmentalization.[8] The lesser-known σ_2 subtype has yet to be cloned. Attempts to isolate σ_2 receptors led to the hypothesis that they are related to histone proteins.[9,10] Nevertheless, later studies showed accumulation of σ_2 fluorescent ligands in diverse organelles except the nucleus.[11,12] Very recently, the σ_2 receptor has been identified as the progesterone receptor membrane component 1 (PGRMC1).[13] Increasing interest in σ_2 receptor research is mostly due to the diagnostic and therapeutic potentials that σ_2 ligands possess. This subtype is overexpressed in a number of cancer tissues, thus σ_2 radioligands and fluorescent ligands have been developed for the imaging of these proteins as biomarkers of tumors. Recently, a σ_2 receptor ^{18}F-labeled radioligand entered a phase I clinical trial for application in positron emission tomography (PET) imaging of three kinds of tumors.[14] In addition, diverse fluorescent σ_2 receptor ligands have been employed to clarify the pathways activated by σ_2 proteins in tumor cells.[11,12] As for therapeutic potential, σ_2 ligands are under investigation for cancer treatment, as activation of σ_2 receptors leads to tumor cell death, and encouraging results have been shown for in vivo studies with σ_2 agonists for the treatment of tumors.[15,16]

On the other hand, it has been suggested that σ_2 antagonists mitigate many cocaine induced behaviors.[17] However, only a few σ_2 receptor antagonists are known in the literature, such as 1-(2-phenylethyl)piperidine (**1**, AC927, Figure 1),[18] 1-(2-phenylethyl)-4-(2-pyridyl)piperazine (**2**, UMB24, Figure 1),[17] and (±)-3α-tropanyl-2-(4-chlorophenoxy)butyrate (**3**, (±)-SM21, Figure 1).[19] In addition, these antagonists display low affinity for the σ_2 receptor (K_i values $\geq$100 nM), so results obtained

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201200246.

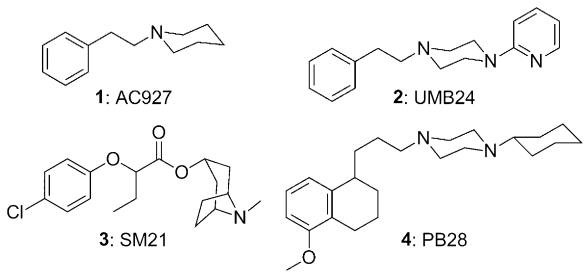


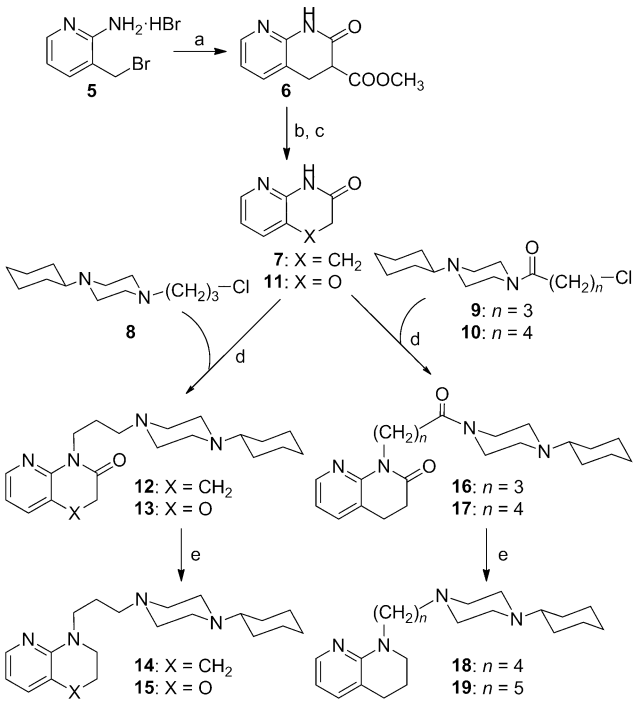
Figure 1. Known σ_2 receptor antagonists and agonist PB28.

with these ligands are hardly conclusive.[20,21] Therefore, there is a need for higher-affinity σ_2 antagonists as pharmacological tools to clarify the role of the σ_2 subtype in both in vitro and in vivo studies. In our previous work, we studied several analogues of 1-cyclohexyl-4-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)-*n*-propyl]piperazine (**4**, PB28, Figure 1),[18] which is one of the highest-affinity σ_2 receptor ligands known, displaying potent agonist activity in diverse tumor cells.[22,23] According to these structure–affinity relationship (SAfiR) studies, the *N*-cyclohexylpiperazine moiety was confirmed as an important feature for conferring high σ_2 receptor affinity.[24–26] Moreover, the σ_2-mediated antiproliferative activity of several analogues of compound **4** appeared to be correlated to lipophilicity. In fact, less lipophilic compounds do not exert antiproliferative activity and may therefore have an antagonist effect at the σ_2 receptor.[27,28] Keeping this hypothesis in mind, we developed a series of *N*-cyclohexylpiperazines linked to a less lipophilic nucleus than tetralin with the aim of producing high-affinity potential σ_2 receptor antagonists. A tetrahydro-1,8-naphthyridine nucleus was used, in spite of the tetralin, to keep the bicyclic structure while the lipophilicity of the ligands was decreased. The corresponding monocyclic structure was also investigated, and a series of *N*-cyclohexylpiperazine derivatives in which the tetralin was replaced by the *N*-(pyridin-2-yl)ethylamino moiety was obtained. Both in the bicycle-bearing and monocycle-bearing cyclohexylpiperazine derivatives, the alkyl chain length was varied from three to five methylene moieties. Also, the effect of the insertion of an oxygen atom was investigated both in the bicyclic and monocyclic rings, generating the pyrido-oxazine and the 4-methoxy-2-aminopyridine moieties, respectively. The nitrogen atom adjacent to the pyridine was made into an amide to provide diverse electron lone pair availabilities.

Results and Discussion

Chemistry

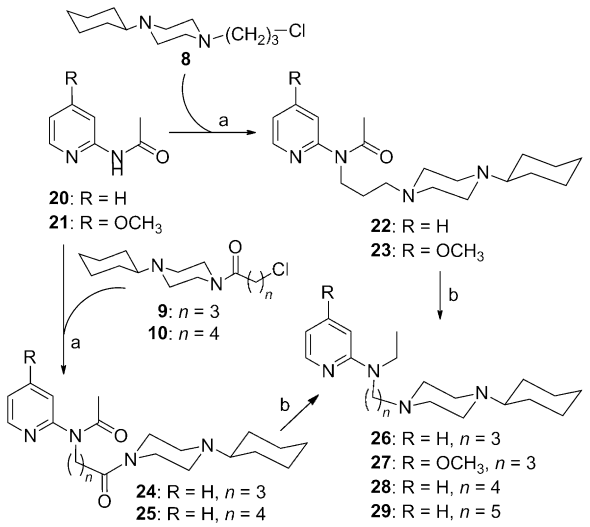
The syntheses of final compounds **12**–**15**, **18**, **19**, **22**, **23**, **26**–**29**, **35**, and **36** are depicted in Schemes 1–3. The preparation of final compounds **12**–**15**, **18**, and **19** is depicted in Scheme 1. 2-Amino-3-(bromomethyl)pyridine hydrobromide (**5**), obtained by bromination of 2-amino-3-hydroxymethylpyridine,[29] was reacted with dimethylmalonate and $NaOCH_3$ to afford intermediate **6**. Ester hydrolysis, followed by decarboxylation, provided key intermediate 3,4-dihydro-1,8-naphthyridin-2-(1*H*)-one (**7**). *N*-cyclohexylpiperazine moieties **8**–**10** were prepared by alkylation of *N*-cyclohexylpiperazine with 1-bromo-3-chloro-propane to afford intermediate **8**, and by acylation with 4-chlorobutanoyl chloride and 5-chloropentanoyl chloride to afford, respectively, intermediates **9** and **10**. Treatment of compound **7**, as well as of the commercially available 2*H*-pyrido-[3,2-*b*]-1,4-oxazin-3-(4*H*)-one **11**, with NaH and chloropropylpiperazine **8** afforded final compounds **12** and **13**, respectively. These amide compounds were reduced with borane–tetrahydrofuran (BH_3·THF) complex to afford final compounds **14** and **15**, respectively. Treatment of compound **7** with NaH and intermediates **9** or **10** furnished intermediate **16** or **17** which, upon reduction with BH_3·THF complex, provided final compounds **18** and **19** (Scheme 1).



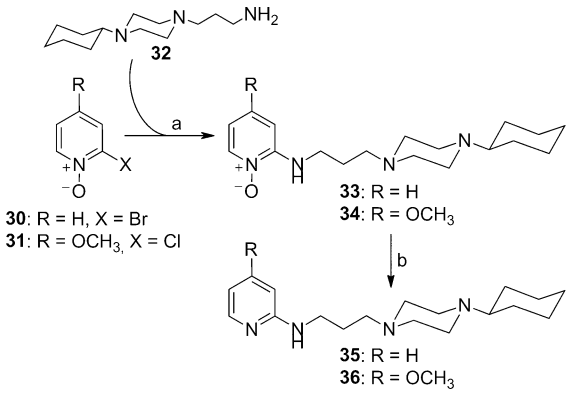
Scheme 1. Synthesis of final bicyclic 2-aminopyridine derivatives. *Reagents and conditions:* a) $H_3COOCCH_2COOCH_3$, $NaOCH_3$, CH_3OH, RT, 18 h; b) NaOH, CH_3OH, reflux, 4 h; c) HCl, CH_3OH, reflux, 18 h; d) NaH, DMF, RT, 48 h; e) BH_3·THF, THF, reflux, 3 h.

The preparation of final compounds **22**, **23**, and **26**–**29** is depicted in Scheme 2. Acetamides **20** and **21**, respectively obtained by acetylation of 2-aminopyridine and 4-methoxy-2-aminopyridine,[30] were reacted with intermediate **8** in the presence of NaH to afford compounds **22** and **23**, respectively. Intermediate **20** was reacted with chloroalkylpiperazine derivatives **9** and **10** to afford compounds **24** and **25**, respectively. Reduction with BH_3·THF complex of the amidic functionalities of compounds **22**–**25** provided final amine compounds **26**–**29**.

The synthesis of final compounds **35** and **36** is depicted in Scheme 3. Key intermediate pyridine-1-oxide derivatives **30** and **31** were commercially available. However, the latter was synthesized through a previously reported synthesis[31] starting from 2-chloro-4-nitropyridine-1-oxide via 2-chloro-4-methoxypyridine.[31] Compounds **30** and **31** underwent nucleophilic



Scheme 2. Synthesis of final monocyclic 2-aminopyridine derivatives. *Reagents and conditions:* a) NaH, DMF, RT, 48 h; b) BH_3·THF, THF, reflux, 3 h.



Scheme 3. Synthesis of final monocyclic 2-aminopyridine derivatives. *Reagents and conditions:* a) Et_3N, *n*-butanol, 120 °C, 20 h; b) PCl_3, $CHCl_3$, 80 °C, 1 h.

substitution with 3-(4-cyclohexylpiperazin-1-yl)propanamine[32] **32** to afford intermediates **33** and **34**, which were converted with PCl_3 to the final amine compounds **35** and **36**, respectively.

All of the final amine compounds were converted into their hydrochloride or oxalate salts with gaseous HCl or oxalic acid, respectively, in anhydrous diethyl ether. Physical properties of these salts are listed in the *Table of Physical Properties of Novel Compounds* in the Supporting Information, along with the values of the calculated logarithm of distribution coefficient (Clog *D*) for the corresponding free bases.[33]

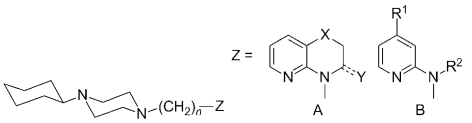
As 2-aminopyridine is endowed with fluorescence properties, the fluorescence spectra of all compounds were recorded to evaluate whether fluorescence of these new compounds could be exploited in biological assays. Fluorescence spectra of final compounds as free bases (at 10^{-5}–10^{-7} M concentrations) were recorded in organic solvents ($CHCl_3$ and EtOH) and in aqueous buffer (pH 7.4), but the quantum yields (calculated using 2-aminopyridine as the reference compound) were too low for imaging purposes, so the fluorescence properties of these molecules were no longer considered.

Radioligand binding and σ_1 and σ_2 receptor affinities

Results from binding assays are expressed as inhibition constants (K_i values) in Table 1. Although none of the newly synthesized 2-aminopyridine derivatives displayed sub-nanomolar binding affinities similar to that of **4** at both σ receptors, appreciable affinity values were reached. No selectivity between the two σ subtypes was obtained, and most of the new compounds displayed a slight preference for the σ_1 receptor (**14**, **15**, **19**, **26**, **27**, **35**, and **36**). On the other hand, compounds **12**, **18**, **22**, and **23** displayed slight σ_2 selectivity, with only **12** and **18** characterized by appreciable σ affinity.

Binding values displayed by bicyclic 1,8-naphthyridine compounds (**14**, **18**, and **19**) and by corresponding open monocyclic 2-aminopyridine analogues (**26**, **28**, and **29**) were similar (K_i: 2.06–5.66 nM for σ_1; K_i: 1.64–14.6 nM for σ_2), demonstrating that a decrease in conformational freedom does not affect the affinity at σ receptors. In addition, no significant effect of chain

Table 1. Binding data of final 2-aminopyridine derivatives.



Compd	Z	*n*	R^1	R^2	X	Y	K_i [nM][a] σ_1	σ_2
4[20]							0.38 ± 0.10	0.68 ± 0.20
12	A	3			CH_2	O	68.0 ± 7.1	16.1 ± 7.4
13	A	3			O	O	12.0 ± 3.1	16.0 ± 1.1
14	A	3			CH_2		2.06 ± 0.71	9.87 ± 0.13
15	A	3			O		4.19 ± 0.75	18.5 ± 4.1
18	A	4			CH_2		4.87 ± 1.26	1.64 ± 0.3
19	A	5			CH_2		3.44 ± 0.91	6.53 ± 1.23
22	B	3	H	$COCH_3$			353 ± 14	246 ± 53
23	B	3	OCH_3	$COCH_3$			402 ± 10	309 ± 3
26	B	3	H	CH_2CH_3			3.65 ± 1.46	14.6 ± 3.2
27	B	3	OCH_3	CH_2CH_3			7.68 ± 0.24	30.1 ± 0.7
28	B	4	H	CH_2CH_3			2.27 ± 1.09	3.54 ± 1.01
29	B	5	H	CH_2CH_3			5.66 ± 0.32	6.70 ± 1.55
35	B	3	H	H			16.4 ± 1.6	70.5 ± 13.0
36	B	3	OCH_3	H			35.5 ± 3.7	59.8 ± 7.3
(+)-pentazocine							3.31 ± 0.39	
DTG								32.0 ± 1.7

[a] Values are the means ± SEM of $n \geq 2$ separate experiments.

length (3–5 methylene groups) on σ receptor affinity was recorded. The three-methylene chain analogues were further investigated both in the bicyclic and in the monocyclic series. In the latter series, the insertion of a methoxy group in the 4-position of the pyridine (compound **27**) left affinity at both σ subtypes almost unchanged (K_i values of 7.68 and 30.1 nM for σ_1 and σ_2, respectively). The importance of the ethyl group on the 2-aminopyridine moiety was investigated with pyridine and 4-methoxypyridine analogues. Elimination of the ethyl residue resulted in a two- to fivefold decrease in affinity at both σ subtypes (comparison of **35** with **26** and **36** with **27**). Replacement of the ethyl with an acetyl group (compounds **22** and **23**) in both pyridine and 4-methoxypyridine analogues led to a dramatic decrease in affinity at the σ receptors (K_i: 353–402 nM for the σ_1 receptor; K_i: 246–309 nM for the σ_2 receptor). This evidence suggests that a certain availability of the electron lone pair in the 2-aminopyridine portion is important for a high-affinity interaction with σ receptors. In addition, the small decrease in σ binding that was recorded for secondary amines **35** and **36** suggests that, besides the lone pair availability, a certain bulk is useful for binding σ proteins.

To study the effect of decreasing lone pair availability in the bicyclic series as well, 1,8-naphthyridin-2-one derivative **12** was produced. Apparently, the electron lone pair availability in the 2-aminopyridine moiety is not a strict requirement for the interaction of bicyclic derivatives with σ_2 receptors. In fact, **12** showed an insignificant decrease in σ_2 affinity ($K_i = 16.1$ nM) relative to the non-amidic counterpart **14** ($K_i = 9.87$ nM). On the other hand, **12** showed a 30-fold lower σ_1 receptor affinity than **14**. The bicyclic series was extended with the synthesis of two isosteres in which the 1,8-naphthyridine nucleus was replaced by a pyrido-oxazine in derivative **15** and by a pyrido-oxazinone in **13**. Insertion of an oxygen atom (**15**) did not lead to pharmacodynamic changes at σ receptors relative to 1,8-naphthyridine derivative **14**. Presence of an amidic functionality (**13**) led to a decrease in σ_1 receptor affinity but not as significant as the decrease recorded for 1,8-naphthyridin-2-one **12**. Conversely, no difference was recorded at the σ_2 receptor between the amidic (**13**) and non-amidic (**15**) derivatives, as in the 1,8-naphthyridine series. Overall, the bicyclic amide derivatives (**12** and **13**) did not show the same decrease in affinity as the monocyclic derivatives (**22** and **23**), particularly at σ_2 receptors, suggesting the hypothesis that diverse binding modes for the bicyclic and monocyclic series may be possible.

Functional assays: antiproliferative activity in human neuroblastoma, human breast cancer, and mouse hippocampal cells

The antiproliferative activities of compound **4** and the newly synthesized compounds are reported as EC_{50} values in Table 2. Four cell lines, HT-22 hippocampal mouse cells, SK-N-SH human neuroblastoma cells, and MCF-7 wild-type human breast adenocarcinoma cells (MCF-7wt) and MCF-7 cells transfected with σ_1 receptor (MCF-7σ_1) were selected for activity assays. As previously reported, the SK-N-SH cell line proved to be a good model for the evaluation of σ_2 receptor-mediated antiproliferative activity, as σ_1 receptors were reported to be present in a low affinity state.[22] HT-22 cells have been used as a model for σ_1 receptor-mediated neuroprotection.[34] Scatchard analysis, which we performed on HT-22 cells to determine the content of both σ subtypes, surprisingly revealed a low σ_1 receptor density ($B_{max} = 0.169$ pmol mg^{-1} of protein), and a sevenfold higher σ_2 receptor content ($B_{max} = 1.23$ pmol mg^{-1} of protein, Figure 2). Therefore, this cell line was also mainly used for evaluation of σ_2 receptor-mediated action. To have a cell

Table 2. Antiproliferative activity of **4** and final 2-aminopyridine derivatives.

Compd	EC_{50} [μM] HT-22[a]	SK-N-SH[b]	MCF-7wt[c]	MCF-7σ_1[d]
4	30.0 ± 5.0	12.4 ± 1.2[e]	27.4 ± 4.1[f]	31.2 ± 4.2
12–**15**, **18**, **19**, **22**, **23**, **26**–**29**, **35**, **36**	> 100	> 100	> 100	> 100

Antiproliferative effect measured in: [a] mouse HT-22 hippocampal cells, [b] human SK-N-SH neuroblastoma cells, [c] human MCF-7 breast adenocarcinoma cells, and [d] human MCF-7σ_1 cells. [e] Other results for compound **4** previously reported in references [20] and [22]. [f] Other results for compound **4** previously reported in reference [23]. Values are the means ± SEM of $n \geq 2$ separate experiments.

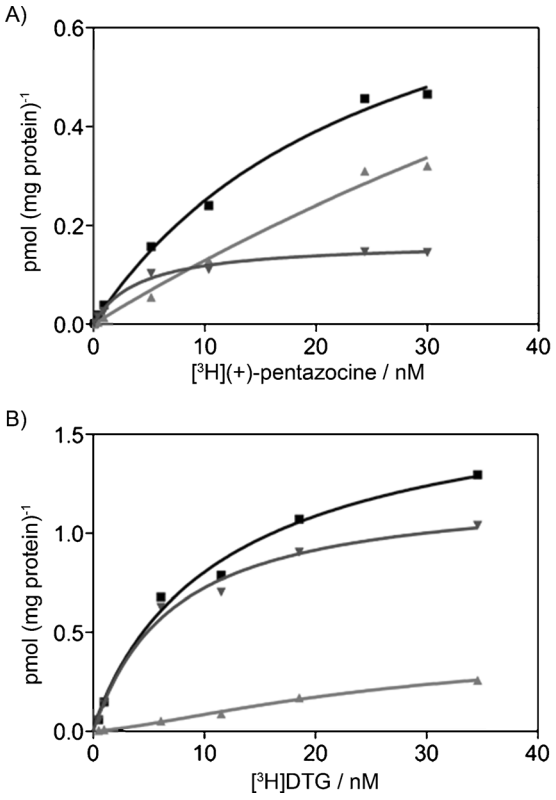


Figure 2. Saturation analysis of A) σ_1 [$K_d = 4.25$ nM, $B_{max} = 0.169$ pmol (mg protein)$^{-1}$] and B) σ_2 [$K_d = 7.032$ nM, $B_{max} = 1.23$ pmol (mg protein)$^{-1}$] receptors in membrane preparations from HT-22 cells: ■total binding; ▲nonspecific binding; ▼specific binding.

line expressing the σ_1 receptor with an appreciable density, transfection of MCF-7wt cells with the σ_1 receptor gene was conducted according to the literature.[35] The density of both subtypes was determined before transfection, and the increase in σ_1 receptor content in the newly created MCF-7σ_1 cell line was evaluated after transfection (Figure 3). Therefore, MCF-7σ_1 was mainly used for the evaluation of σ_1-mediated action, whereas MCF-7wt was used for evaluation of σ_2 receptor-mediated action. Reference compound **4**, which was previously defined as a σ_2 receptor agonist and a σ_1 receptor antagonist,[22,23] was studied in the four cell lines where it showed antiproliferative activity (Table 2), as expected for a σ_2 receptor agonist and a σ_1 receptor antagonist. On the other hand, all of the new 2-aminopyridine derivatives did not display antiproliferative activity, with EC_{50} values $>100\ \mu M$ in all of the cell lines studied. Although the involvement of other proteins in the overall action of these σ receptor ligands cannot be ruled out, these data are in agreement with previous results obtained with σ_2 ligands less lipophilic than **4**, leading to the hypothesis that lower lipophilicity values lead to lower σ_2-mediated antiproliferative activity.[27] In addition, compound **18**, which represents the highest σ_2 affinity compound among the novel 2-aminopyridines, was further investigated in an antiproliferative assay in co-administration with **4**. At the concentration used, **18** partially reversed the antiproliferative effect exerted by compound **4** in MCF-7wt cells (see the figure in the Supporting Information), showing that **18** is able to antagonize the σ_2-mediated antiproliferative action exerted by the σ_2 receptor agonist **4**.

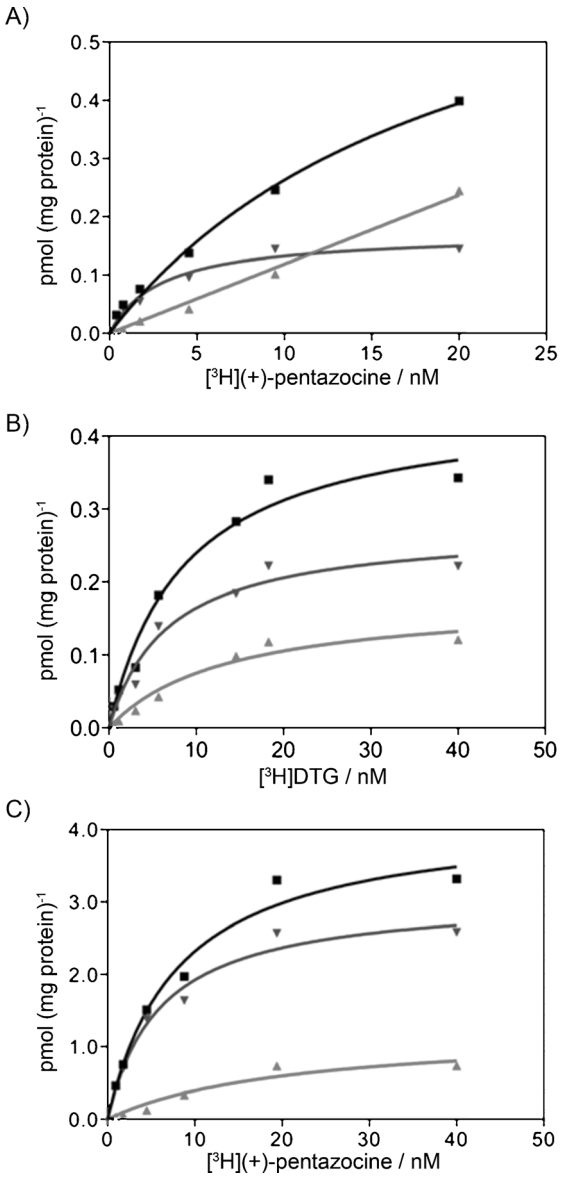


Figure 3. Saturation analysis of A) σ_1 [$K_d = 2.97$ nM, $B_{max} = 0.172$ pmol (mg protein)$^{-1}$] and B) σ_2 [$K_d = 9.3$ nM, $B_{max} = 0.323$ pmol (mg protein)$^{-1}$] receptors in membrane preparations from MCF-7wt cells, and saturation analysis of C) σ_1 [$K_d = 7.6$ nM, $B_{max} = 3.45$ pmol (mg protein)$^{-1}$] in membrane preparations from MCF-7σ_1 cells: ■total binding; ▲nonspecific binding; ▼specific binding.

σ Receptor effect on intracellular Ca^{2+} mobilization

Effects on intracellular Ca^{2+} mobilization were evaluated for representative 2-aminopyridine **18** and for reference compound **4** with the aim of understanding whether the opposite effect exerted on cell proliferation corresponds to differences in intracellular Ca^{2+} mobilization. Therefore, bradykinin-triggered Ca^{2+} response was measured for **4**, **18**, and the prototypical σ_1 agonist (+)-pentazocine ((+)-[2*S*-(2α,6α,11*R*)]-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(3-methyl-2-butenyl)-2,6-methano-3-benzazocine-8-ol) in SK-N-SH, MCF-7wt, and MCF-7σ_1 cells (Figure 4); none of the ligands affected intracellular Ca^{2+} concentration when administered alone.

Although **4** and **18** do not display σ selectivity, results obtained through experiments in the cell lines selected should allow retrieval of compound effects on each σ subtype: SK-N-SH cells express both σ receptors, with σ_1 subtype in a low affinity state; MCF-7σ_1 cells overexpress σ_1 receptors; MCF-7wt cells overexpress σ_2 receptors. As previously reported, the selective σ_1 agonist (+)-pentazocine increased bradykinin-induced Ca^{2+} mobilization in SK-N-SH and MCF-7σ_1 cells, whereas no effect was exerted in MCF-7wt cells where the σ_1 density is too low (Figure 3 A).[35,36] Compound **4**, as previously shown with carbachol in SK-N-SH cells,[37] inhibited bradykinin-triggered Ca^{2+} response in all of the cell lines studied, whereas it did not exert any effect in LoVo colon adenocarcinoma cells in which none of the σ subtypes were detected through Scatchard analysis.[36] These results support the idea that effects exerted by compound **4** in bradykinin-triggered Ca^{2+} response are σ-mediated and suggest that agonist activity at σ_2 (from SK-N-SH and MCF-7wt) and antagonist activity at σ_1 (from MCF-7σ_1) decrease such response. On the other hand, 2-aminopyridine derivative **18**, increased bradykinin-triggered Ca^{2+} response in SK-N-SH and MCF-7σ_1 cells, similar to (+)-pentazocine, implying agonist activity at the σ_1 receptor. No effect was exerted by **18** in MCF-7wt cells, where the action may be mediated by the σ_2 receptor.

Together, these data show that compounds **4** and **18** have opposite behavior and suggest that while agonist activity at

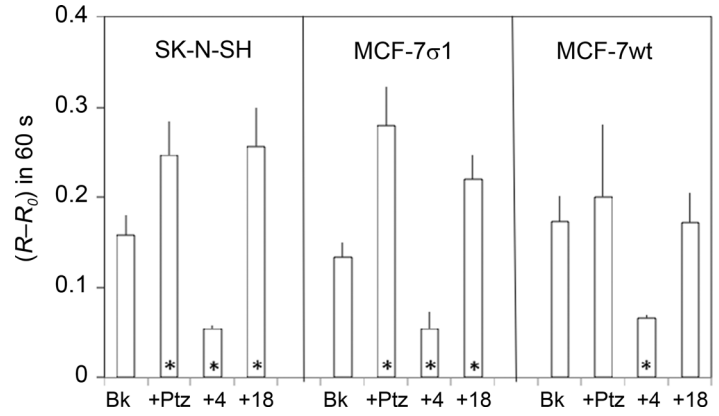


Figure 4. σ Receptor ligand effects on intracellular Ca^{2+} mobilization induced by bradykinin. Cells were pretreated with σ receptor ligands before the addition of bradykinin. Bk: bradykinin (1 μM); +Ptz: 10 min pretreatment with (+)-pentazocine (1 μM); **+4**: 3 min pretreatment with compound **4** (1 μM); **+18**: 3 min pretreatment with compound **18** (1 μM); $*p < 0.05$. In SK-N-SH and MCF-7σ_1 cells, the Ca^{2+} response induced by bradykinin was inhibited by **4** and stimulated by **18**. In MCF-7wt cells, the Ca^{2+} response induced by bradykinin was inhibited by **4** and unaffected by **18**.

the σ_2 receptor decreases bradykinin-induced Ca^{2+} response, antagonist activity may not alter such response. Therefore, compound **18** may be proposed as a σ_2 receptor antagonist and a σ_1 receptor agonist.

Conclusions

A certain number of σ_2 receptor ligands with considerable σ_2 versus σ_1 selectivities are known,[26,32,38] but no selective and high-affinity σ_2 antagonist has been reported yet, thus there is a need for σ_2 antagonists as tools for σ receptor research. Therefore, with the aim of compensating for the paucity of σ_2 antagonists, we based the development of σ_2 ligands with potential antagonist activity on previous studies. Less lipophilic analogues than σ_2 receptor agonist **4** were obtained by replacement of the tetralin nucleus with a 2-aminopyridine moiety. Compounds with high affinity for both σ receptor subtypes were obtained, and most were characterized by Clog*D* values within the optimal range for entry into cells, as demonstrated for σ_2 PET radiotracers by other authors.[38] None of the new compounds displayed antiproliferative activity in the four cell lines selected (mouse HT-22 and human SK-N-SH, MCF-7wt, and MCF-7σ_1 cells), in contrast with lead compound **4**, which consistently showed micromolar antiproliferative activity. The highest-affinity σ_2 ligand **18** was co-administered with **4** in MCF-7wt cells and partially decreased the antiproliferative effect exerted by **4**. To investigate whether the lack of antiproliferative action of the newly synthesized compounds corresponds to a different effect on Ca^{2+} mobilization, representative compounds **18** and **4** were evaluated in three tumor cell lines with diverse σ subtype content. Actually, compound **18** displayed an opposite effect than compound **4** on bradykinin-induced Ca^{2+} response. All of the results together suggest a σ_2 receptor antagonist and a σ_1 receptor agonist activity for compound **18**. In conclusion, despite the lack of σ_2 versus σ_1 selectivities, these new compounds may represent better tools for σ receptor research than the low affinity and poorly selective σ_2 receptor antagonists known.

Experimental Section

Chemistry

Both column chromatography and flash column chromatography were performed with 60 Å pore size silica gel as the stationary phase (1:30 *w/w*, 63–200 μm particle size from ICN and 1:15 *w/w*, 15–40 μm particle size from Merck, respectively). Melting points were determined in open capillaries on a Gallenkamp electrothermal apparatus. Purity of tested compounds was established by combustion analysis, confirming purity ≥98%. Elemental analyses (C, H, N) were performed on an Eurovector Euro EA 3000 analyzer; the analytical results were within ±0.4% of theoretical values. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Mercury Varian spectrometer using $CDCl_3$ and CH_3OD, respectively, as solvent. The following data were reported: chemical shift (δ) in ppm, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), integration, and coupling constant(s) in Hertz. Recording of mass spectra was done on an Agilent 6890-5973 MSD gas chromatograph/mass spectrometer and on an Agilent 1100 series LC–MSD trap system VL mass spectrometer; only significant *m/z* peaks, with their percentage of relative intensity in parentheses, are reported. Chemicals were from Sigma–Aldrich and Alfa Aesar and were used without any further purification.

General procedure for the synthesis of compounds 12, 13, 16, 17, and 22–25: NaH (1.2 mmol, 0.29 g, 60% w/w) was added to a stirred solution of the corresponding amide compound (**7**, **11**, **20**, or **21**; 1.0 mmol) in dry DMF (10 mL) under N_2. After 1 h, a solution of the appropriate chloride (1.0 mmol) in DMF was added to the suspension under N_2. The reaction mixture was stirred at room temperature for 48 h. Then, water was added to the reaction mixture, and the organic layer was separated. The aqueous phase was extracted with EtOAc (3×10 mL). The collected organic layers were dried over Na_2SO_4 and evaporated under reduced pressure to afford a crude residue, which was purified as reported below for each target compound.

1-[3-(4-Cyclohexylpiperazin-1-yl)propyl]-3,4-dihydro-1,8-naphthyridin-2(1*H*)-one (12): The yellow semi-solid was purified by column chromatography with CH_2Cl_2/CH_3OH (98:2) as eluent to afford the title compound as a yellow oil (0.125 g, 35%): ^{1}H NMR δ = 1.06–1.38 [m, 5H, cyclohexyl NCH(CH*H*)$_5$], 1.58-1.70 (m, 1H, cyclohexyl), 1.72-2.05 [m, 6H, cyclohexyl NCH(CH*H*)$_4$, CONCH$_2$C*H*$_2$CH$_2$N], 2.30–2.48 (m+t, 3H, CHN, J = 7.55 Hz, CONCH$_2$CH$_2$C*H*$_2$N), 2.54-2.80 (m, 10H, piperazine and CH$_2$C*H*$_2$CO), 2.86 (t, 2H, J = 7.9 Hz, ArC*H*$_2$CH$_2$CO), 4.17 (t, 2H, J = 7.4 Hz, CONC*H*$_2$CH$_2$CH$_2$N), 6.87-6.92 (m, 1H, aromatic), 7.42 (d, 1H, aromatic), 8.20 ppm (d, 1H, aromatic); GC–MS *m/z* 358 (M^++2, 0.4), 357 (M^++1, 4), 356 (M^+, 13), 232 (52), 218 (100), 189 (95), 181 (63), 133 (45); LC–MS (ESI$^+$) *m/z* 357 [*M*+H]$^+$, 379 [*M*+Na]$^+$; LC–MS–MS 357: 161, 188; Anal. ($C_{21}H_{32}N_4O{\cdot}2HCl{\cdot}^5/_4H_2O$) C, H, N.

4-[3-(4-Cyclohexylpiperazin-1-yl)propyl]-2*H*-pyrido[3,2-*b*]-[1,4]-oxazin-3-(4*H*)-one (13): The brown semi-solid was purified by a flash column chromatography using CH_2Cl_2/CH_3OH (95:5) as eluent to afford the title compound as a yellow oil (0.093 g, 26%): ^{1}H NMR δ = 1.06–1.36 [m, 5H, cyclohexyl, NCH(CH*H*)$_5$], 1.58-1.70 (m,

1H, cyclohexyl), 1.72–1.98 [m, 6H, cyclohexyl NCH(CH*H*)$_4$, CONCH$_2$C*H*$_2$CH$_2$N], 2.24–2.38 (m, 1H, CHN), 2.44 (t, 2H, J=7.10 Hz, CONCH$_2$CH$_2$C*H*$_2$N), 2.48–2.75 (m, 8H, piperazine), 4.17 [t, 2H, J= 7.40 Hz, CONC*H*$_2$(CH$_2$)$_2$], 4.64 (s, 2H, OCH$_2$CO), 6.87–6.92 (m, 1H, aromatic), 7.18–7.25 (m, 1H, aromatic), 7.98–8.05 ppm (m, 1H, aromatic); GC–MS m/z 360 (M^++2, 1), 359 (M^++1, 7), 358 (M^+, 31), 248 (50), 234 (59), 220 (92), 191 (100), 181 (81), 133 (42); Anal. ($C_{20}H_{30}N_4O_2$·2HCl·H_2O) C, H, N.

1-(4-Cyclohexylpiperazin-1-yl)-4-[2(1*H*)-oxo-3,4-dihydro-1,8-naphthyridin-1-yl]butan-1-one (16): The yellow solid was purified by column chromatography with CH_2Cl_2/CH_3OH (98:2) as eluent to give the target compound as a white semi-solid (0.123 g, 32%): ^{1}H NMR δ=1.06–1.32 [m, 5H, cyclohexyl NCH(CH*H*)$_5$], 1.58–1.70 (m, 1H, cyclohexyl), 1.75-1.90 [m, 4H, cyclohexyl, NCH(CH*H*)$_4$], 1.98 (m, 2H, CONCH$_2$C*H*$_2$CH$_2$CO), 2.23–2.32 (m, 1H, CHN), 2.38 [t, 2H, J= 7.4 Hz, CON(CH$_2$)$_2$C*H*$_2$CO], 2.5–2.56 [m, 4H, piperazine, CHN(C*H*$_2$)$_2$], 2.66 (t, 2H, J=8.2 Hz, ArCH$_2$C*H*$_2$CO), 2.87 (t, 2H, J=8.2 Hz, ArC*H*$_2$CH$_2$CO), 3.42 (t, 2H, piperazine, J=4.8 Hz, CONCH$_2$), 3.58 (t, 2H, piperazine, J=4.8 Hz, CONCH$_2$), 4.20 [t, 2H, J=7.1 Hz, CON-(CH$_2$)$_2$C*H*$_2$N], 6.87–6.91 (m, 1H, aromatic), 7.41–7.44 (m, 1H, aromatic), 8.20–8.22 ppm (m, 1H, aromatic); GC–MS m/z 386 (M^++2, 1), 385 (M^++1, 6), 384 (M^+, 25), 341 (65), 217 (100), 175 (58).

1-(4-Cyclohexylpiperazin-1-yl)-5-[2(1*H*)-oxo-3,4-dihydro-1,8-naphthyridin-1-yl]pentan-1-one (17): The yellow semi-solid was purified by column chromatography with CH_2Cl_2/CH_3OH (98:2) as eluent to afford the title compound as a yellow semi-solid (0.179 g, 45%): ^{1}H NMR δ=1.07–1.36 [m, 5H, cyclohexyl, NCH(CH*H*)$_5$], 1.56–1.98 [m, 9H, cyclohexyl NCH(CH*H*)$_5$ and CONCH$_2$(C*H*$_2$)$_2$CH$_2$CO], 2.28–2.43 [m, 3H, CHN and CON(CH$_2$)$_3$C*H*$_2$CO], 2.45–2.70 [m+t, 6H, J=8.2 Hz, piperazine N(CH$_2$)$_2$ and ArCH$_2$C*H*$_2$CO], 2.86 (t, 2H, J= 8.2 Hz, ArC*H*$_2$CH$_2$CO), 3.42–3.75 [m, 4H, piperazine CON(CH$_2$)$_2$], 4.16 [t, 2H, J=7.1 Hz, CONC*H*$_2$(CH$_2$)$_3$CO], 6.87–6.91 (m, 1H, aromatic), 7.41–7.43 (m, 1H, aromatic), 8.20–8.22 ppm (m, 1H, aromatic); GC–MS m/z 399 (M^++1, 3), 398 (M^+, 14), 355 (44), 231 (100), 138 (85), 126 (47).

***N*-[3-(4-Cyclohexylpiperazin-1-yl)propyl]-*N*-(2-pyridyl)acetamide (22)**: The brown residue was purified by column chromatography with $CHCl_3$ as eluent to give the title compound as pale brown oil (0.14 g, 40%): ^{1}H NMR δ=1.00-1.37 [m, 5H, cyclohexyl, NCH-(CH*H*)$_5$], 1.58–1.95 [m, 7H, cyclohexyl NCH(CH*H*)$_5$ and CONCH$_2$C*H*$_2$CH$_2$N], 1.99 (s, 3H, CH$_3$CO), 2.15–2.22 (m, 1H, NCH), 2.29–2.54 [m+t, 10H, J=7.1 Hz, CON(CH$_2$)$_2$C*H*$_2$N and piperazine], 3.87 [t, 2H, J=7.4 Hz, CONC*H*$_2$(CH$_2$)$_2$N], 7.19–7.21 (m, 2H, aromatic), 7.71–7.77 (m, 1H, aromatic), 8.50 ppm (m, 1H, aromatic); ^{13}C NMR (title compound as oxalate salt): δ=21.79, 22.32, 24.34, 24.42, 26.60, 45.12, 45.62, 54.34, 66.37, 12299, 124.74, 141.24, 149.01, 153.17, 165.84, 174.25 ppm; GC–MS m/z 345 (M^++1, 1), 344 (M^+, 1), 219 (42), 206 (100), 181 (49), 177 (39), 135 (21); LC–MS (ESI$^+$) m/z 345 [M+H]$^+$, 367 [M+Na]$^+$; LC–MS–MS 345: 135, 177; Anal. ($C_{20}H_{32}N_4O$·2$C_2H_2O_4$·H_2O) C, H, N.

***N*-[3-(4-Cyclohexylpiperazin-1-yl)propyl]-*N*-(4-methoxy-2-pyridyl)acetamide (23)**: The brown residue was purified by column chromatography with CH_2Cl_2/CH_3OH (9:1) as eluent to give the title compound as a yellow oil (0.12 g, 31%): ^{1}H NMR δ=1.04–1.27 [m, 5H, cyclohexyl, NCH(CH*H*)$_5$], 1.59–1.91 [m, 7H, cyclohexyl, NCH-(CH*H*)$_5$ and CONCH$_2$C*H*$_2$CH$_2$N], 1.99 (s, 3H, CH$_3$CO), 2.15–2.25 (m, 1H, NCH), 2.32 [t, 2H, J=7.4 Hz, CON(CH$_2$)$_2$C*H*$_2$N], 2.34–2.54 (m, 8H, piperazine), 3.81–3.86 [m, 5H, CONC*H*$_2$(CH$_2$)$_2$N and OCH$_3$], 6.71–6.76 (m, 2H, aromatic), 8.31 ppm (d, 1H, aromatic); GC–MS m/z 359 (2), 236 (88), 207 (40), 151 (55), 137 (100); LC–MS–MS (ESI$^+$) m/z 375 [M+H]$^+$, 397 [M+Na]$^+$; LC–MS–MS 375: 137, 165, 207, 333; Anal. ($C_{21}H_{34}N_4O_2$·2$C_2H_2O_4$·3/2H_2O) C, H, N.

1-(4-Cyclohexylpiperazin-1-yl)-N-acetyl-N-(2-pyridyl)-4-aminobutan-1-one (24): The brown semi-solid was purified by flash column chromatography using CH_2Cl_2/CH_3OH (95:5) as eluent, affording the title compound as a yellow oil (0.026 g, 7%): ^{1}H NMR δ=1.08–1.48 [m, 5H, cyclohexyl, NCH(CH*H*)$_5$], 1.50–2.04 [m, 10H, cyclohexyl NCH(CH*H*)$_5$, CONCH$_2$C*H*$_2$CH$_2$N and CH$_3$CO], 2.05–2.25 (m, 1H, NCH), 2.39 (t, 2H, J=7.1 Hz, CH$_2$CON), 2.65–3.10 (m, 4H, C*H*$_2$NC*H*$_2$ piperazine), 3.70–4.10 [m, 6H, CH$_3$CONC*H*$_2$ and (CH$_2$)$_2$NCO piperazine], 7.19–7.34 (m, 2H, aromatic), 7.72–7.85 (m, 1H, aromatic), 8.48–8.58 ppm (m, 1H, aromatic); LC–MS–MS (ESI$^+$) m/z 373 [M+H]$^+$, 395 [M+Na]$^+$; LC–MS–MS 373: 163, 237, 331.

1-(4-Cyclohexylpiperazin-1-yl)-*N*-acetyl-*N*-(2-pyridyl)-5-aminobutan-1-one (25): The yellow residue was purified by column chromatography with CH_2Cl_2 as eluent to afford the title compound as a yellow oil (0.12 g, 30%): ^{1}H NMR δ=1.02-1.32 [m, 5H, cyclohexyl NCH(CH*H*)$_5$], 1.52–1.68 [m, 5H, cyclohexyl NCH(CH*H*)$_5$], 1.75–1.93 [m, 4H, CONCH$_2$(C*H*$_2$)$_2$CH$_2$CO], 1.97 (s, 3H, CH$_3$CO), 2.18-2.38 [m, 3H, CHN, (CH$_2$)$_3$C*H*$_2$CO], 2.47–2.58 (m, 4H, piperazine C*H*$_2$NC*H*$_2$), 3.38–3.48 (m, 2H, piperazine CH$_2$NCO), 3.52–3.65 (m, 2H, piperazine CH$_2$NCO), 3.85 [t, 2H, J=6.8 Hz, CONC*H*$_2$(CH$_2$)$_3$CON], 7.20–7.25 (m, 2H, aromatic), 7.72–7.78 (m, 1H, aromatic), 8.50 ppm (m, 1H, aromatic); GC–MS m/z 386 (M^+, 4), 343 (32), 219 (31), 177 (85), 138 (100), 126 (58), 107 (35); LC–MS (ESI$^+$) m/z 409 [M+Na]$^+$; LC–MS–MS 409: 367.

General procedure for the synthesis of compounds 14, 15, 18, 19, and 26–29: A solution of BH_3·THF complex (1 M) in THF (2.5 mL for **12**, **13**, **22**, and **23**, and 5 mL for **16**, **17, 24**, and **25**) was added to one of the appropriate amide compounds (1.0 mmol) in anhydrous THF under N_2. The solution was stirred at reflux for 3 h. The reaction mixture was cooled, then MeOH was added to destroy the excess hydride. The solvent was removed under reduced pressure to give a white solid that was re-dissolved in MeOH. A solution of *i*PrOH saturated with $HCl_{(gas)}$ was added to this suspension, and the mixture was held at reflux for 30 min. After cooling, the solvent was evaporated under reduced pressure. The residue was re-dissolved with Na_2CO_3 (saturated solution), and the mixture was extracted with CH_2Cl_2 (3×10 mL). The collected organic layers were dried over Na_2SO_4 and evaporated under reduced pressure to give crude residues which were purified as reported below for each compound.

1-[3-(4-Cyclohexylpiperazin-1-yl)propyl]-1,2,3,4-tetrahydro-1,8-naphthyridine (14): The yellow residue was purified by column chromatography with CH_2Cl_2/CH_3OH (95:5) as eluent to give the final compound as a light yellow semi-solid (0.212 g, 62%): ^{1}H NMR δ=1.07–1.38 [m, 5H, cyclohexyl NCH(CH*H*)$_5$], 1.58–2.05 [m, 9H, cyclohexyl NCH(CH*H*)$_5$ and ArCH$_2$C*H*$_2$CH$_2$NCH$_2$C*H*$_2$CH$_2$N], 2.28–2.50 [m+t, 3H, CHN, J=7.1 Hz, N(CH$_2$)$_2$C*H*$_2$N], 2.55–2.88 (m+t, 10H, piperazine, J=6.3 Hz, ArCH$_2$), 3.36 [t, 2H, J=5.6 Hz, Ar(CH$_2$)$_2$C*H*$_2$N], 3.59 [t, 2H, J=7.1 Hz, NC*H*$_2$(CH$_2$)$_2$N], 6.38 (t, 1H, J=4.9 Hz, aromatic), 7.04 (d, 1H, J=5.5 Hz, aromatic), 7.90 ppm (d, 1H, J=4.9 Hz, aromatic); GC–MS m/z 342 (M^+, 2), 204 (100), 161 (43), 147 (86); Anal. ($C_{21}H_{34}N_4$·3HCl·5/4H_2O) C, H, N.

4-[3-(4-Cyclohexylpiperazin-1-yl)propyl]-3,4-dihydro-2*H*-pyrido-[3,2-*b*]-[1,4]-oxazine (15): The yellow residue was purified by column chromatography with CH_2Cl_2/CH_3OH (9:1) as eluent to give the title compound as a light yellow oil (0.210 g, 61%): ^{1}H NMR δ= 1.05–1.38 [m, 5H, cyclohexyl NCH(CH*H*)$_5$], 1.58–1.72 [m, 1H, cyclohexyl NCH(CH*H*)], 1.75–1.94 [m, 4H, cyclohexyl NCH(CH*H*)$_4$], 1.95–2.12 [m, 2H, NCH$_2$C*H*$_2$CH$_2$N], 2.42–2.59 (m, 3H, CHN, and

$NCH_2CH_2CH_2N$), 2.62–2.98 (m, 8H, piperazine), 3.47 (t, 2H, J= 4.54 Hz, OCH_2CH_2N), 3.62 [t, 2H, J=7.15 Hz, $NCH_2(CH_2)_2N$], 4.19 (t, 2H, J=4.54 Hz, OCH_2CH_2N), 6.44–6.50 (m, 1H, aromatic), 6.86–6.92 (m, 1H, aromatic), 7.68–7.72 ppm (m, 1H, aromatic); GC–MS m/z 346 (M^++2, 0.4), 345 (M^++1, 3), 344 (M^+, 13), 206 (100), 163 (41), 149 (59), 97 (38); Anal. ($C_{20}H_{32}N_4O{\cdot}3\,HCl{\cdot}1/2\,H_2O$) C, H, N.

1-[4-(4-Cyclohexylpiperazin-1-yl)butyl]-1,2,3,4-tetrahydro-1,8-naphthyridine (18): The yellow residue was purified by column chromatography with CH_2Cl_2/CH_3OH (98:2) as eluent, affording the title compound as a light brown semi-solid (0.257 g, 72%): ^{1}H NMR δ=1.02–1.36 [m, 5H, cyclohexyl NCH(CHH)$_5$], 1.42–1.69 [m, 5H, cyclohexyl NCH(CHH)$_5$], 1.72–2.00 [m, 6H, $ArCH_2CH_2CH_2NCH_2(CH_2)_2CH_2N$], 2.21–2.78 [m+t, 13H, CHN, J=6.8 Hz, $N(CH_2)_3CH_2N$, piperazine, and $ArCH_2(CH_2)_2N$], 3.35 [t, 2H, J=5.6 Hz, $Ar(CH_2)_2CH_2N$], 3.57 [t, 2H, J=7.1 Hz, $NCH_2(CH_2)_3N$], 6.36 (t, 1H, J=4.9 Hz, aromatic), 7.04 (d, 1H, J=5.2 Hz, aromatic), 7.90 ppm (d, 1H, J=4.9 Hz, aromatic); GC–MS m/z 358 (M^++2, 0.1), 357 (M^++1, 0.2), 356 (M^+, 1), 218 (100), 147 (39); LC–MS (ESI$^+$) m/z 357 [M+H]$^+$; LC–MS–MS 357: 147, 189, 223; Anal. ($C_{22}H_{36}N_4{\cdot}3\,HCl{\cdot}5/2\,H_2O$) C, H, N.

1-[5-(4-Cyclohexylpiperazin-1-yl)pentyl]-1,2,3,4-tetrahydro-1,8-naphthyridine (19): The yellow residue was purified by column chromatography with CH_2Cl_2/CH_3OH (98:2) as eluent to give the title compound as an orange semi-solid (0.333 g, 90%): ^{1}H NMR δ=1.06–1.42 [m, 7H, cyclohexyl NCH(CHH)$_5$, $N(CH_2)_2CH_2(CH_2)_2N$], 1.49–1.70 [m, 5H, cyclohexyl NCH (CHH)$_5$], 1.78–2.04 [m, 7H, $ArCH_2CH_2CH_2NCH_2CH_2CH_2CH_2N$, and CHN], 2.35 [t, 2H, J= 7.8 Hz, $(CH_2)_4CH_2N$], 2.55–2.71 [m+t, 10H, J=6.0 Hz, piperazine, $ArCH_2(CH_2)_2N$], 3.35 [t, 2H, J=5.6 Hz, $Ar(CH_2)_2CH_2N$], 3.55 [t, 2H, J= 7.4 Hz, $NCH_2(CH_2)_4N$], 6.37 (t, 1H, J=4.9 Hz, aromatic), 7.05 (d, 1H, J=5.5 Hz, aromatic), 7.91 ppm (d, 1H, J=4.9 Hz, aromatic); ^{13}C NMR (title compound as hydrochloride salt): δ=17.99, 21.44, 21.75, 23.12, 23.44, 24.69, 25.14, 36.89, 39.98, 42.03, 45.20, 49.55, 52.17, 64.17, 109.99, 122.51, 131.55, 138.69, 148.17 ppm; GC–MS m/z 371 (M^++1, 1), 370 (M^+, 3), 232 (100), 147 (30); Anal. ($C_{23}H_{38}N_4{\cdot}3\,HCl{\cdot}3\,H_2O$) C, H, N.

3-(4-Cyclohexylpiperazin-1-yl)-*N*-Ethyl-*N*-(2-pyridyl)propanamine (26): The yellow residue was purified by column chromatography with CH_2Cl_2/CH_3OH (95:5) as eluent to give the title compound as a light brown oil (0.248 g, 75%): ^{1}H NMR δ=1.02–1.30 [m+t, 8H, J=7.1 Hz, CH_3 and cyclohexyl NCH(CHH)$_5$,], 1.58–1.94 [m, 7H, cyclohexyl NCH(CHH)$_5$ and $NCH_2CH_2CH_2N$], 2.20–2.30 (m, 1H, CHN), 2.37 (t, 2H, J=7.1 Hz, $NCH_2CH_2CH_2N$), 2.40–2.71 (m, 8H, piperazine), 3.43-3.55 [m, 4H, $CH_3CH_2NCH_2(CH_2)_2$], 6.44-6.48 (m, 2H, aromatic), 7.35-7.41 (m, 1H, aromatic), 8.11 ppm (d, 1H, J=4.9 Hz, aromatic); ^{13}C NMR (title compound as oxalate salt): δ=10.91, 21.49, 24.43, 26.59, 44.97, 45.75, 46.28, 53.73, 66.34, 112.14, 135.89, 144.15, 151.13, 166.14 ppm; GC–MS m/z 331 (M^++1, 2), 330 (M^+, 4), 192 (100), 149 (37), 135 (73), 107 (33); Anal. ($C_{20}H_{34}N_4{\cdot}3\,C_2H_2O_4$) C, H, N.

3-(4-Cyclohexylpiperazin-1-yl)-*N*-Ethyl-*N*-(4-methoxy-2-pyridyl)-propanamine (27): The yellow oil was purified by column chromatography with $CH_2Cl_2/CH_3OH/NH_4OH$ (9:1:0.1) as eluent to give the title compound as a pale yellow oil (0.198 g, 55%): ^{1}H NMR δ= 1.07–1.29 [m+t, 8H, J=7.1 Hz, CH_3 and cyclohexyl NCH(CHH)$_5$], 1.60–1.99 [m, 7H, cyclohexyl NCH(CHH)$_5$ and $NCH_2CH_2CH_2N$], 2.30–2.33 (m, 1H, CHN), 2.40 [t, 2H, J=7.1 Hz, $(CH_2)_2CH_2N$], 2.56–2.67 (m, 8H, piperazine), 3.42–3.54 [m, 4H, $CH_3CH_2NCH_2(CH_2)_2N$], 3.78 (s, 3H, OCH_3), 5.93 (d, 1H, J=2.2 Hz, aromatic), 6.12–6.14 (dd, 1H, J= 5.7 Hz and J'=2.2 Hz, aromatic), 7.96 ppm (d, 1H, J=5.7 Hz, aromatic); GC–MS m/z 360 (M^+, 1), 222 (100), 179 (50), 165 (83), 137 (48); Anal. ($C_{21}H_{36}N_4O{\cdot}3\,C_2H_2O_4$) C, H, N.

3-(4-Cyclohexylpiperazin-1-yl)-*N*-Ethyl-*N*-(2-pyridyl)butanamine (28): The yellow solid residue was purified by column chromatography with CH_2Cl_2/CH_3OH (95:5) as eluent to give the title compound as a pale yellow semi-solid (0.248 g, 73%): ^{1}H NMR δ=0.98–1.38 [m+t, 8H, J=7.1 Hz, CH_3 and cyclohexyl NCH(CHH)$_5$], 1.45–1.64 [m, 5H, cyclohexyl NCH(CHH)$_5$], 1.70–2.05 [m, 4H, $NCH_2(CH_2)_2CH_2N$], 2.22–2.44 [m, 3H, CHN and $N(CH_2)_3CH_2N$)], 2.50–2.80 (m, 8H, piperazine), 3.35–3.56 [m, 4H, $CH_3CH_2NCH_2(CH_2)_3N$], 6.38–6.50 (m, 2H, aromatic), 7.32–7.45 (m, 1H, aromatic), 8.11 ppm (d, 1H, J=4.9 Hz, aromatic); LC–MS (ESI$^+$) m/z 345 [M+H]$^+$; LC–MS–MS 345: 149, 177, 223; Anal. ($C_{21}H_{36}N_4{\cdot}3\,C_2H_2O_4{\cdot}3/4\,H_2O$) C, H, N.

3-(4-Cyclohexylpiperazin-1-yl)-*N*-Ethyl-*N*-(2-pyridyl)pentanamine (29): The yellow solid residue was purified by column chromatography with CH_2Cl_2/CH_3OH (85:15) as eluent to give the title compound as a yellow oil (0.194 g, 54%): ^{1}H NMR δ=1.06–1.38 [m+t, 10H, J=7.1 Hz, CH_3CH_2N, cyclohexyl NCH(CHH)$_5$, and $N(CH_2)_2CH_2(CH_2)_2N$], 1.44–1.69 [m, 5H, cyclohexyl NCH(CHH)$_5$], 1.70–1.98 (m, 4H, $NCH_2CH_2CH_2CH_2CH_2N$), 2.20–2.38 [m+t, 3H, J=7.9 Hz, $N(CH_2)_4CH_2N$ and CHN], 2.43–2.78 (m, 8H, piperazine), 3.40 [t, 2H, J=7.4 Hz, $NCH_2(CH_2)_4N$], 3.49 (q, 2H, J=7.1 Hz, CH_3CH_2N), 6.40–6.47 (m, 2H, aromatic), 7.35-7.41 (m, 1H, J=6,8 Hz, aromatic), 8.11 ppm (d, 1H, J=4,9 Hz, aromatic); GC–MS m/z 359 (M^++1, 1), 358 (M^+, 5), 220 (100), 181 (34), 135 (26), 125 (43); Anal. ($C_{22}H_{38}N_4{\cdot}2\,C_2H_2O_4{\cdot}1/2\,H_2O$) C, H, N.

General procedure for the synthesis of intermediate compounds 33 and 34: Amine **32** (1.0 mmol, 0.22 g) was added to a solution of pyridine-1-oxide derivative **30** or **31** (1.0 mmol) in *n*-butanol (4 mL) in the presence of Et_3N (1.0 mmol, 0.14 mL). The reaction mixture was heated at 120 °C for 20 h. After cooling, the solvent was evaporated under reduced pressure to give a brown oil residue.

3-(4-Cyclohexylpiperazin-1-yl)-*N*-(2-pyridyl-*N*-oxide)propanamine (33): The brown oil was purified by column chromatography with CH_2Cl_2/MeOH (9:1) as eluent affording the title compound as a yellow semi-solid (0.178 g, 56%): ^{1}H NMR δ=1.10–1.74 (m, 7H, cyclohexyl), 1.84–2.03 [m, 5H, 3H of cyclohexyl and $NHCH_2CH_2$], 2.16–2.29 (m, 1H, cyclohexyl CHN), 2.69 [t, 2H, J=5.7 Hz, $(CH_2)_2CH_2N$], 2.79–2.98 (m, 4H, piperazine), 3.26–3.37 (m, 6H, 4 piperazine and $NHCH_2$), 6.49–6.54 (m, 2H, aromatic), 7.15–7.24 (m, 1H, aromatic), 8.06–8.16 (m, 1H, aromatic), 8.26 ppm (broad s, 1H, NH, D_2O exchanged); LC–MS (ESI$^+$) m/z 319 [M+H]$^+$; LC–MS–MS 319: 135, 181, 237.

3-(4-Cyclohexylpiperazin-1-yl)-*N*-(4-methoxy-2-pyridyl-*N*-oxide)-propanamine (34): The brown oil was purified by column chromatography with $CHCl_3$/MeOH (9:1) as eluent affording the title compound as an orange oil (0.30 g, 86%): ^{1}H NMR δ=1.11–1.78 (m, 7H, cyclohexyl), 1.90–2.16 (m, 5H, 3H of cyclohexyl and $NHCH_2CH_2$), 2.16–2.35 (m, 1H, cyclohexyl CHN), 2.74 [t, 2H, J= 5.2 Hz, $(CH_2)_2CH_2N$], 3.03–3.11 (m, 4H, piperazine), 3.30–3.40 [m, 6H, 4 piperazine and $NHCH_2(CH_2)_2$], 3.84 (s, 3H, OCH_3), 5.95–6.00 (m, 1H, aromatic), 6.15–6.20 (m, 1H, aromatic), 7.95–8.00 (m, 1H, aromatic), 8.06 ppm (broad s, 1H, NH, D_2O exchanged); LC–MS (ESI$^+$) m/z 349 [M+H]$^+$; LC–MS–MS 349: 137, 165, 267.

General procedure for the synthesis of Final Compounds 35 and 36: To a suspension of intermediate **33** or **34** (1.0 mmol) in $CHCl_3$ (15 mL) cooled at 0 °C, PCl_3 (3.0 mmol, 0.26 mL) was added in a dropwise manner, and the mixture was heated for 1 h at 80 °C. After cooling, water was added, and the reaction mixture was made alkaline by adding NaOH and extracted with $CHCl_3$ (3× 70 mL). The collected organic solutions were dried (Na_2SO_4) and the crude residue was purified as reported below.

3-(4-Cyclohexylpiperazin-1-yl)-*N*-(2-pyridyl)propanamine (35): The crude yellow oil was purified by column chromatography with CH_2Cl_2/CH_3OH (9:1) as eluent to give the title compound as a yellow semi-solid (0.142 g, 47%): ^{1}H NMR $\delta = 1.02$–1.36 [m, 5H, cyclohexyl NCH(CH*H*)$_5$,], 1.55–1.75 [m, 1H, cyclohexyl NCH(CH*H*)], 1.76–1.98 [m, 6H, cyclohexyl NCH(CH*H*)$_4$ and NHCH$_2$C*H*$_2$CH$_2$N], 2.17–2.30 (m, 1H, CHN), 2.37 [t, 2H, $J = 7.1$ Hz, NH(CH$_2$)$_2$C*H*$_2$N], 2.40–2.71 (m, 8H, piperazine), 3.33 [t, 3H, $J = 6.6$ Hz, NHC*H*$_2$(CH$_2$)$_2$], 5.28 (broad s, 1H, NH, D_2O exchanged), 6.35 (d, 1H, $J = 8.2$ Hz, aromatic), 6.50–6.54 (m, 1H, aromatic), 7.34–7.40 (m, 1H, aromatic), 8.04–8.07 ppm (m, 1H, aromatic); ^{13}C NMR (title compound as oxalate salt): 22.75, 24.45, 26.62, 38.69, 45.78, 49.97, 54.15, 66.33, 113.02, 135.06, 135.26, 144.09, 152.09, 166.70; GC–MS *m/z* 303 ($M^+ +1$, 0.2), 302 (M^+, 0.8), 164 (100), 121 (52); Anal. ($C_{18}H_{30}N_4$·5/2$C_2H_2O_4$·1/2H_2O) C, H, N.

3-(4-Cyclohexylpiperazin-1-yl)-*N*-(4-methoxy-2-pyridyl)propanamine (36): The crude brown semi-solid was purified by column chromatography with EtOAc and petroleum ether (8:2) as eluent to give the title compound as a yellow semi-solid (0.160 g, 48%): ^{1}H NMR $\delta = 1.06$-1.29 [m, 5H, cyclohexyl NCH(CH*H*)$_5$,], 1.59–1.63 [m, 1H, cyclohexyl NCH(CH*H*)], 1.73–1.91 [m, 6H, cyclohexyl NCH(CH*H*)$_4$ and NHCH$_2$C*H*$_2$CH$_2$N], 2.23–2.29 (m, 1H, cyclohexyl CHN), 2.42-2.65 [t+m, 10H, $J = 6.8$ Hz, (CH$_2$)$_2$C*H*$_2$N and piperazine), 3.02 (broad s, 1H, NH, D_2O exchanged), 3.26–3.32 [m, 2H, NHC*H*$_2$(CH$_2$)$_2$N], 3.77 (s, 3H, OCH$_3$), 5.82 (d, 1H, $J = 2.2$ Hz, aromatic), 6.15–6.17 (m, 1H, $J = 5.7$ Hz, aromatic), 7.88 ppm (d, 1H, $J = 5.7$ Hz, aromatic); LC–MS (ESI$^+$) *m/z* 333 [*M*+H]$^+$, 355 [*M*+Na]$^+$; LC–MS–MS 333: 137, 165; Anal. ($C_{19}H_{32}N_4O$·3$C_2H_2O_4$·1/2H_2O) C, H, N.

Biology

Competition binding assays: All of the procedures for the binding assays were previously described. σ_1 And σ_2 receptor binding were carried out according to Matsumoto et al.[39] [^{3}H]-DTG, 1,3-di-2-tolylguanidine (30 Ci mmol^{-1}) and (+)-[^{3}H]-pentazocine (34 Ci mmol^{-1}) were purchased from PerkinElmer Life Sciences (Zavantem, Belgium). DTG was purchased from Tocris Cookson Ltd. (UK). (+)-Pentazocine was obtained from Sigma–Aldrich-RBI s.r.l. (Milan, Italy). Male Dunkin guinea pigs and Wistar Hannover rats (250–300 g) were from Harlan (Italy). The specific radioligands and tissue sources were, respectively: (a) σ_1 receptor, (+)-[^{3}H]-pentazocine, guinea pig brain membranes without cerebellum; (b) σ_2 receptor,[^{3}H]-DTG in the presence of 1 μM (+)-pentazocine to mask σ_1 receptors, rat liver membranes. The following compounds were used to define the specific binding reported in parentheses: (a) (+)-pentazocine (73–87%), (b) DTG (85–96%). Concentrations required to inhibit 50% of radioligand specific binding (IC_{50}) were determined using six to nine different concentrations of the drug studied in two or three experiments with samples in duplicate. Scatchard parameters (K_d and B_{max}) and apparent inhibition constants (K_i values) were determined by nonlinear curve fitting, using Prism GraphPad software (version 3.0).[40]

Saturation binding assay: Saturation experiments were carried out as described by Vilner et al. with minor modifications.[41] Membranes of human MCF-7wt and MCF-7σ_1 breast adenocarcinoma cells and mouse HT-22 hippocampal cells were prepared according to Colabufo et al.[22] σ_1 Receptors were radiolabeled using (+)-[^{3}H]-pentazocine concentrations of 0.4–40 nM. Samples contained 200 μg membrane protein, radioligand, and 10 μM (+)-pentazocine to determine nonspecific binding. Samples were incubated in a final volume of 500 μL (50 mM Tris, pH 8.0) for 120 min at 25 °C. Incubations were stopped by addition of 1 mL ice-cold buffer (50 mM Tris, pH 7.4), then the suspension was filtered through GF/C presoaked in 0.5% polyethylenimine (PEI) for at least 30 min prior to use. The filters were washed twice with 1 mL ice-cold buffer. σ_2 Receptors were radiolabeled using [^{3}H]-DTG concentrations of 0.5–40 nM. Samples containing 200 μg membrane protein, radioligand, 10 μM DTG (to determine nonspecific binding), and 1 μM (+)-pentazocine (to mask σ_1 receptors) were equilibrated in a final volume of 500 μL (50 mM Tris, pH 8.0) for 120 min at 25 °C; the subsequent manipulations were as described above for σ_1 receptors. Scatchard parameters (K_d and B_{max}) were determined by nonlinear curve fitting, using the Prism GraphPad software (version 3.0).[40]

Cell culture: The human SK-N-SH neuroblastoma and the human MCF-7 breast adenocarcinoma were obtained from Interlab Cell Line Collection (ICLC, Genoa, Italy). The MCF-7σ_1 line was created in our laboratory. The HT-22 cell line was a gift from Dr. Alessandra Rossi, (Heinrich–Pette Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany). MCF-7wt, MCF-7σ_1, and HT-22 cells were grown in DMEM high glucose supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, in a humidified incubator at 37 °C with a 5% CO_2 atmosphere. SK-N-SH cell line was routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 1 mM sodium pyruvate, and 1% non-essential amino acids in a humidified incubator at 37 °C with a 5% CO_2 atmosphere. Cell culture reagents were purchased from EuroClone (Milan, Italy). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide), G418 (geneticin), and fura-2-acetoxymethyl ester (Fura-2-AM) were obtained from Sigma–Aldrich (Milan, Italy); FuGENE HD transfection reagent was purchased from Promega (Milan, Italy); Opti-MEM was obtained from Invitrogen.

Cell viability: Determination of cell growth was performed using the MTT assay at 48 h.[23] On day 1, 25000 cells per well were seeded into 96-well plates in a volume of 100 μL. On day 2, the various drugs at concentrations between 0.1–100 μM were added. In all of the experiments, the various drug solvents (EtOH, DMSO) were added in each control to evaluate a possible solvent cytotoxicity. After the established incubation time with drugs, MTT (0.5 mg mL^{-1}) was added to each well, and after 3-4 h incubation at 37 °C, the supernatant was removed. The formazan crystals were solubilized using 100 μL of DMSO/EtOH (1:1) and the absorbance values at 570 and 630 nm were determined on a Victor 3 microplate reader from PerkinElmer Life Sciences.

Construction of expression vector harboring σ_1 receptor complete coding sequence (CDS): Total RNA was extracted from 1×10^6 MCF-7wt cells using a GenElute Mammalian Total RNA Miniprep kit (Sigma–Aldrich) and reverse transcribed with GeneAmp RNA PCR core kit (Applied Biosystems). The full-length coding region of the human σ_1 receptor (GenBank accession number NM_005866.2) was amplified from MCF-7wt cDNA using iProof High Fidelity DNA Polymerase (Bio-Rad), 10 pmol of each primer (Table 3) and 1 μL of dNTPs (10 mM for each nucleotide) in a final volume of 50 μL of regular buffer reaction mixture. PCR was run under the following conditions: pre-incubation at 94 °C for 1 min, run for 30 cycles at 94 °C for 10 sec, 55 °C for 30 sec, and 72 °C for 1 min, extension at 72 °C for 7 min. The PCR amplification product was purified using the High Pure PCR Product Purification kit (Roche) and digested with HindIII and BamHI (Roche). The digested DNA fragment was ligated with purified HindIII- and BamHI-digested pcDNA3.1(+) vector (Invitrogen) in the sense orientation. Top10 chemically competent *Escherichia coli* cells (Invitrogen) were transformed with the

Table 3. Primers used for amplification and sequencing of σ_1 receptor CDS.

Name	Sequence 5′→3′
SIGMA1FOR	CGAAAGCTTATGCAGTGGGCCGTGGGC
SIGMA1REV	CAGGGATCCTCAAGGGTCCTGGCCAAAGAGG
pcDNAF	AATACGACTCACTATAGGGA
SIGMA1FOR300	TCCGAGTATGTGCTGCTCTT
pcDNAR	AGAAGGCACAGTCGAGGC

construct described above and the vector amplified. The plasmid DNA was isolated using a High Pure Plasmid Isolation kit (Roche).[42] The fidelity of the final human σ_1 receptor insert in pcDNA3.1(+) plasmid was verified by DNA sequencing using a BigDye Terminator kit (Applied Biosystems) and the primers shown in Table 3.

MCF-7 transfection with σ_1 receptor: To develop stable MCF-7σ_1 cell lines, MCF-7wt cells were plated at a density of 3×10^6 cells in 10 mL growth medium in 100 mm Petri dishes, and incubated at 37 °C overnight. Cells were transfected with 17 μg of pcDNA3.1(+) vector containing the target σ_1 DNA sequence as per standard protocol, using FuGENE HD transfection reagent in Opti-MEM medium without serum. Vector-expressing cells were selected using geneticin (G418). After transfection, cells were placed in normal DMEM growth medium. After 1 day, cells were detached with trypsin/EDTA and replated into DMEM growth medium containing geneticin (800 μg mL^{-1}) and cultured for 25 days. Surviving cell clones were picked out and propagated separately in 60 mm Petri dishes in the same medium with 800 μg mL^{-1} geneticin. To suppress reversion of the phenotype, all subsequent cell culture was carried out in DMEM growth medium as described above, supplemented with 800 μg mL^{-1} geneticin.[35]

Fluorescence measurements for intracellular Ca^{2+} response detection: Cells were seeded onto glass cover-slips at a density of 20,000 cm^2 and used for fluorescence measurements after 5 days. Intracellular [Ca^{2+}] was estimated using the dual-wavelength ratiometric probe Fura-2; protocols have been described elsewhere (Galiano et al., 2004).[43] A HEPES buffer was used containing 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.6 mM $MgCl_2$, 11 mM glucose, and 25 mM HEPES (pH 7.4). Agonists or other drugs were directly pipetted into the chamber without perfusion. The experiment was run at 22 °C. Each trace shown is the mean of values from at least eight representatives and at least 50 total cells from four experiments, performed with different cell batches; only one in four S.E.M. values was plotted. In Figure 3, "($R-R_0$) in 60 s" is the difference between the mean value of 12 and 10 fluorescence ratio values, measured every 5 s before and after addition of the drug under investigation. Where indicated, data were statistically analyzed with Student's *t*-test for unpaired values.[36]

Supporting Information

Elemental analyses of the novel end products; formulas of hydrochloride and oxalate salts, crystallization solvents, melting points and Clog *D* values, antiproliferative effects in MCF-7wt cells of compound **4** alone and in co-administration with **18**, description of the preparation and spectroscopy data for the intermediate compounds **6**–**10**, **20**, and **21**.

Acknowledgements

The authors thank Dr. Alessandra Rossi for kindly providing HT-22 cells and Prof. Giuseppe Cassano and Giuseppe Gasparre (Dipartimento di Bioscienze, Biotecnologie e Scienze Farmacologiche, Università degli Studi di Bari ALDO MORO, Bari, Italy) for support with experiments on Ca^{2+} response.

Keywords: 2-aminopyridines · calcium · *N*-cyclohexylpiperazines · σ receptors

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Received: May 11, 2012
Revised: July 20, 2012
Published online on ■■ ■■, 0000

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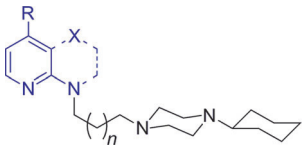
2-Aminopyridine Derivatives as Potential σ_2 Receptor Antagonists



A reduced fat option: *N*-Cyclohexylpiperazine derivatives linked to a 2-aminopyridine moiety were generated as less lipophilic analogues of the σ_2 agonist PB28. The new *N*-cyclohexylpiperazines, which display high affinity for σ subtypes, are devoid of antiproliferative activity and may be proposed as σ_2 receptor antagonists.

