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Sigma-2 Receptor Agonists as Possible Antitumor Agents in Resistant Tumors: Hints for Collateral Sensitivity

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With the aim of contributing to the development of novel antitumor agents, high-affinity σ_2 receptor agonists were developed, with 6,7-dimethoxy-2-[4-[1-(4-fluorophenyl)-1*H*-indol-3-yl]butyl]-1,2,3,4-tetrahydroisoquinoline (**15**) and 9-[4-(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-2-yl)butyl]-9*H*-carbazole (**25**) showing exceptional selectivity for the σ_2 subtype. Most of the compounds displayed notable antiproliferative activity in human MCF7 breast adenocarcinoma cells, with similar activity in the corresponding doxorubicin-resistant MCF7adr cell line. Surprisingly, a few compounds, including **25**, displayed enhanced activity in MCF7adr cells over parent cells, recalling the phenomenon of collateral sensitivity, which is under study for the treatment of drug-resistant tumors. All of the compounds showed interaction with P-glycoprotein (P-gp), and **15** and **25**, with the greatest activity, were able to revert P-gp-mediated resistance and reestablish the antitumor effect of doxorubicin in MCF7adr cells. We therefore identified a series of σ_2 receptor agonists endowed with intriguing antitumor properties; these compounds deserve further investigation for the development of alternate strategies against multidrug-resistant cancers.

Introduction

In the early 1990s, two distinct sigma (σ) receptor subtypes, namely σ_1 and σ_2, were identified after the existence of σ proteins was first proposed in 1976.[1,2] Initial research was focused on σ receptors within the central nervous system (CNS), where the most studied σ_1 receptors modulate the release of a number of neurotransmitters. Involvement in pathologies such as anxiety, depression, schizophrenia, drug addiction, and Parkinson's and Alzheimer's diseases has been demonstrated for this subtype, which seems to play a role in neuroprotection and neuroplasticity.[3–5] σ_1 Proteins have also been shown to take part in intracellular signaling through modulation of intracellular Ca^{2+} levels via inositol 1,4,5-triphosphate (IP_3) receptors.[6] A role in lipid compartmentalization and modulation of K^+ channels has also been suggested for this subtype, the signaling mechanism for which has yet to be fully understood.[6,7] Less is known about σ_2 receptors, which have yet to be cloned. The various attempts[8] to characterize this subtype recently led to the identification of the σ_2 protein as the progesterone receptor membrane component 1 (PGRMC1).[9] A significant boost in σ_2 receptor research has been given by evidence that σ proteins—mostly the σ_2 subtype—are overexpressed in a variety of human peripheral and brain tumors. In particular, σ_2 receptor density has been shown to be higher in proliferating than in quiescent tumor cells; therefore, this subtype has been proposed as an endogenous biomarker for the proliferative status of tumors.[10] Moreover, activation of σ_2 receptors with σ_2 agonists exerts antiproliferative and cytotoxic effects in tumor cells in vitro as well as in tumor xenografts in vivo.[11] Therefore, σ_2 receptors are an intriguing target for tumor diagnosis and treatment. The apoptotic mechanisms activated by σ_2 proteins are currently under investigation. There are multiple pathways activated by σ_2 receptor agonists to induce cell death; these include caspase-dependent and -independent mechanisms,[12] generation of reactive oxygen species (ROS), and autophagy.[13] Apparently, activation of the pathways depends on the tumor cell type and on the structure of the σ_2 ligand used. 1′-[4-[1-(4-Fluorophenyl)-1*H*-indol-3-yl]butan-1-yl]-spiro[isobenzofuran-1(3*H*)],4′-piperidine, (**1**, siramesine)[11] and 1-cyclohexyl-4-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)propyl]piperazine (**2**, PB28)[11] are among the most potent σ_2 agonists known (Figure 1), with the former inducing cell death by lysosomal leakage and oxidative stress,[14] and the latter modulating Ca^{2+} release from intracellular stores.[15] These two σ_2 agonists are important reference compounds in the study of σ_2 receptor ligands, and have often been used as lead compounds for the development of new generations of σ_2 receptor ligands.[11,16–21] The corresponding 4-(4-fluorophenyl)piperidine derivative of siramesine (**1**), compound **3** (Figure 1), has emerged for its sub-nanomolar σ_2 receptor affinity and appreciable selectivity.[22] Among the ligands developed from PB28 (**2**), 4-cyclohexyl-1-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-

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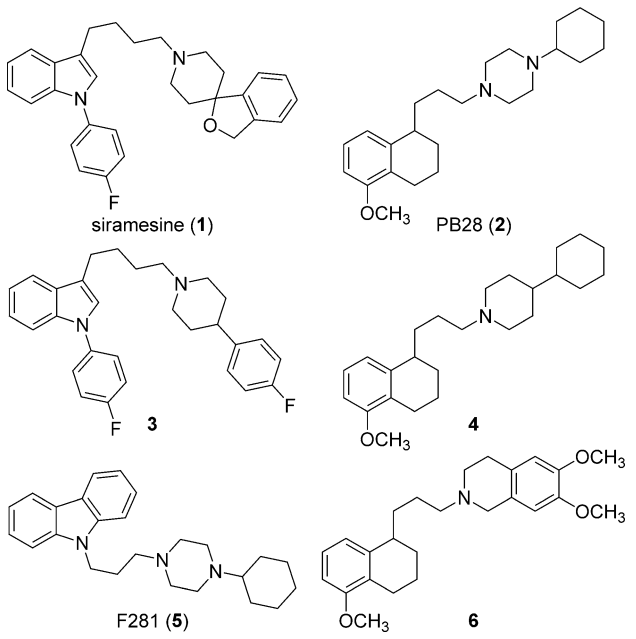


Figure 1. Reference σ_2 receptor ligands **1**–**6**.

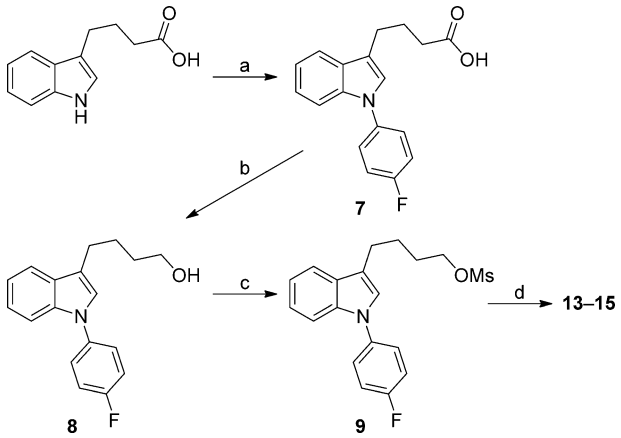
1-yl-propyl)]piperidine (**4**)[19] and 1-cyclohexyl-4-[3-(9*H*-carbazol-9-yl)propyl]piperazine (**5**, F281)[23,24] are the most promising in terms of antiproliferative activity and appreciable σ_2 receptor affinity. 6,7-Dimethoxy-2-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)propyl]-1,2,3,4-tetrahydroisoquinoline (**6**, Figure 1)[16] has emerged for its σ_2 receptor selectivity and inspired the synthesis of novel classes of 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline derivatives.[16] In our continued efforts to produce high-affinity σ_2 receptor ligands with agonist (antiproliferative) activity, we gathered our inspiration from the aforementioned compounds **1**–**6**.

The basic moieties (a–e, Table 1 below) contained in these compounds were alternatively connected to the hydrophobic portions of the same compounds (R, Table 1) in order to combine the structural features that are likely responsible for high σ_2 receptor affinity and/or activity. Binding at σ receptors and antiproliferative effects of novel and reference σ_2 receptor ligands were studied in human MCF7 breast adenocarcinoma cells and in corresponding adriamycin- (or doxorubicin)-resistant cells (MCF7adr). Data from assays with these cell lines provide an indication of the efficacy of the σ_2 receptor ligands in resistant tumors when resistance is due to overexpression of the P-glycoprotein efflux pump (P-gp, also known as multidrug-resistance protein 1, MDR1), which has been found in 50% of human cancers.[25] With this same purpose, we evaluated the interaction of our σ_2 compounds with P-gp, also based on previous results showing that σ_2 ligands are often accompanied by P-gp activity.[16,18] As we have already showed, interaction with P-gp by our σ_2 agonists may be exploited in two directions in resistant tumors: by co-administration with a classic antineoplastic drug whose activity is hampered by P-gp overexpression, or as single antitumor agents that are able to overcome P-gp-mediated resistance. This second hypothesis would also solve the pharmacokinetic problems generated by the co-administration approach (administration of a P-gp inhibitor with a classic antineoplastic drug), which is one of the strategies to overcome multidrug resistance (MDR),[26–31] but which has shown little success so far.

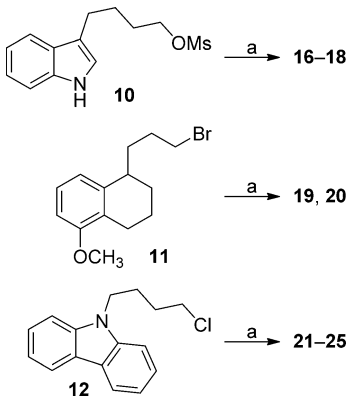
Results and Discussion

Chemistry

The synthesis of intermediate and final compounds reported herein is depicted in Schemes 1 and 2. [(4-Fluorophenyl)-1*H*-indol-3-yl]butanoic acid (**7**) was obtained from 4-(1*H*-indol-3-yl)butanoic acid by addition of 1-fluoro-4-iodobenzene, upon microwave-assisted reaction in 2-ethoxyethanol, in the presence of copper(I) iodide and potassium carbonate (Scheme 1). Reduction of **7** with lithium aluminum hydride provided the butanol derivative **8**,[22] which underwent reaction with methanesulfonyl chloride to afford the known key intermediate 4-[1-



Scheme 1. Synthesis of final compounds **13**–**15**. *Reagents and conditions:* a) CuI, K_2CO_3, 1-fluoro-4-iodobenzene, ethoxyethanol, $h\nu$, 200 °C, 40 min; b) $LiAlH_4$, Et_2O, reflux, 2 h, then RT overnight; c) MsCl, Et_3N, CH_2Cl_2, 0 °C, 1 h; d) CH_3CN or DMF and K_2CO_3 and either 1-cyclohexylpiperazine, 4-cyclohexylpiperidine, or 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline.



Scheme 2. Synthesis of final compounds **16**–**25**. *Reagents and conditions:* a) CH_3CN or DMF and K_2CO_3 and one amine among spiro[isobenzofuran-1(3*H*),4′piperidine], 4-(4-fluorophenyl)piperidine, 1-cyclohexylpiperazine, 4-cyclohexylpiperidine, or 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline.

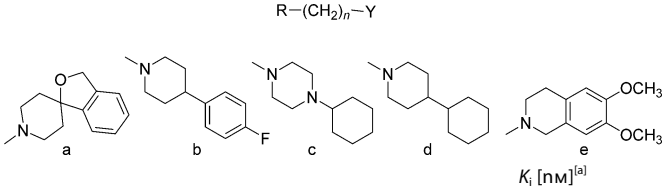
(4-fluorophenyl)-1*H*-indol-3-yl)butyl methanesulfonate (**9**).[22] Nucleophilic substitution of **9** with 1-cyclohexylpiperazine, 4-cyclohexylpiperidine,[19] or 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (basic moieties c, d, or e, respectively, Table 1), in the presence of potassium carbonate afforded final compounds **13**–**15**, respectively. Similarly, reaction of 4-(1*H*-indol-3-yl)butyl methanesulfonate (**10**) with the same basic moieties gave final compounds **16**–**18**. Reaction of (3-bromopropyl)tetralin **11**[32] with either spiro[isobenzofuran-1-(3*H*),4′piperidine][22] (a) or 4-(4-fluorophenyl)piperidine[22] (b), in the same way, afforded final compounds **19** and **20**, respectively. Each of the basic moieties a–e was allowed to react with 9-(4-chlorobutyl)carbazole **12**[33] to afford final compounds **21**–**25**. Siramesine was prepared according to published methods[22] in order to compare our results with this lead compound. All final amine compounds were converted into their hydrochloride or oxalate salts, and their physical properties are listed in table 1 of the Supporting Information.

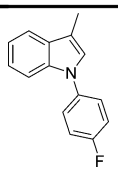
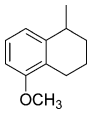
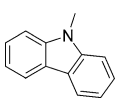
Biology

σ Receptor binding

Affinity values at the σ receptor subtypes for reference compounds **1**–**6** and novel compounds **13**–**25** are listed in Table 1 and expressed as K_i values. As for affinity at the σ_1 receptor, 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (e) was confirmed as a detrimental basic moiety, with **25** displaying the lowest affinity ($K_i = 2190$ nM). In the indole series, the presence of the 1-(4-fluorophenyl) moiety at the indole nitrogen atom led to a dramatic decrease in affinity for the σ_1 receptor, in accordance with a previous report.[22] Curiously, cyclohexylpiperidine (d) and 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (e) moieties provided appreciable σ_1 affinity in the N-unsubstituted indoles ($K_i = 1.07$ nM for **17**, and $K_i = 46.9$ nM for **18**), in contrast to the low σ_1 receptor binding observed for the tetralin-, carbazole-, and 1-(4-fluorophenyl)-substituted indole series. These differences between the N-unsubstituted and N-substituted indoles suggest that a hydrogen bond may occur between the indole NH group and the σ_1 receptor (compare **17** with **4**, **14**, **24**, and **18** with **6**, **15**, and **25**). A few compounds displayed non-competitive binding with (+)-[^{3}H]pentazocine: two were from the tetralin series (**19** and **20**), one from the N-unsubstituted indoles (**16**), and one from the carbazole series (**23**). As for σ_2 receptor binding, in the tetralin series replacement of the cyclohexylpiperazine moiety (which is present in compound **2**) with a piperidine-type group (**19** and **20**) led to a 30–60-fold decrease in σ_2 affinity, as previously shown with compound **4**.[19] The only exception was **6**, which displaced [^{3}H]-1,3-di-*O*-tolylguanidine ([^{3}H]DTG) in a very strong and non-competitive manner. Nevertheless, the presence of a cyclohexylpiperazine group in the indole series was not as convenient as in the tetralin series for σ_2 receptor affinity, with N-substituted (**13**) and N-unsubstituted (**16**) indoles showing K_i values of 28.9 and 6.50 nM respectively, in agreement with carbazole **5** ($K_i = 12.6$ nM). Notable is the σ_2 receptor affinity we observed with our protocols for siramesine (**1**, $K_i = 12.6$ nM), which is 100-fold lower than the reported value ($IC_{50} = 0.12$ nM).[22] In accordance, all siramesine *N*-(4-fluorophenyl)indole analogues displayed similar K_i values, with the four basic moieties (a, c–e) conferring similar affinity (compounds **1**, **13**, **14**, and **15**). All carbazole derivatives displayed σ_2 affinities generally similar to that of **5** (compounds **22** and **24**), with compounds **21** and **25** showing the highest σ_2 affinities (K_i values of 3.24 and 0.04 nM, respectively) among the novel compounds. The exceptional K_i value shown by **25** was counteracted by a low Hill slope ($n_H = 0.46$). Nevertheless, compound **25** emerged for its exceptional σ_2 selectivity (54750-fold) due to very low affinity for the σ_1 receptor. N-Unsubstituted indoles **17** and **18**, together with carbazole **23**, displayed non-competitive binding with [^{3}H]DTG, with the radioligand being displaced by very low test compound concentrations

Table 1. σ Receptor affinities of reference compounds **1**–**6** and new compounds **13**–**25**.



Compound	R	*n*	Y	K_i [nM][a] σ_1	σ_2
siramesine (**1**)[22]		4	a	10.5 ± 2.6	12.6 ± 0.1
3[22]		4	b	16[b]	0.27[b]
13		4	c	25.7 ± 7.3	28.9 ± 6.6
14		4	d	98.9 ± 7.1	7.28 ± 1.11
15		4	e	1390 ± 20	5.34 ± 1.22
16		4	c	70%	6.50 ± 1.45
17		4	d	1.07 ± 0.28	90%
18		4	e	46.9 ± 3.8	63%
PB28 (**2**)[34]		3	c	0.38 ± 0.10	0.68 ± 0.20
4[19]		3	d	143 ± 18	18.8 ± 5.9
6[16]		3	e	151 ± 20	96%
19		3	a	76%	40.6 ± 6.4
20		3	b	80%	22.6 ± 3.9
F281 (**5**)[23]		3	c	3450 ± 1660	12.6 ± 3.2
21		4	a	27.3 ± 5.7	3.24 ± 1.34
22		4	b	29.0 ± 1.2	11.7 ± 1.8
23		4	c	91%	60%
24		4	d	139 ± 18	7.65 ± 2.5
25		4	e	2190 ± 230	0.04 ± 0.01[c]
DTG					31.5 ± 3.3
(+)-pentazocine				3.38 ± 0.31	

[a] Values represent the mean ± SEM of $n \geq 2$ separate experiments carried out in duplicate; percent displacement at a concentration of 10^{-11} M is reported if a complete displacement curve was not obtained. [b] IC_{50} values as reported in Ref. [22]. [c] Hill slope $n_H = 0.46$.

(10^{-11} M). Therefore, the importance of the basic moiety types for conferring σ_2 selectivity was confirmed. As previously shown with a diverse panel of tetralin analogues,[19,17] the cyclohexylpiperazine ring is not optimal for conferring σ_2 selectivity, likely for the presence of the two N-atoms. By contrast, and as already shown in the tetralin series, 4-cyclohexylpiperidine,[19,34] and in particular 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline,[16] were found to have high σ_2 selectivity. Indoles and carbazoles bearing these two moieties were accompanied by a significant decrease in σ_1 affinity (**14**, **15**, **24** and **25**). Compounds **15** and **25** emerged for their notable σ_2 selectivity (260- and 54750-fold, respectively).

Calcein-AM experiments

Activity at P-gp was determined for all of the compounds except **3** by using the P-gp-overexpressing cell line MDCK-MDR1, and data are expressed as EC_{50} values (Table 2). Activity was in the micromolar range for all compounds (0.21 μM–5.38 μM), with **1** showing a considerable interaction with the efflux pump (EC_{50} = 1.41 μM). All tetralins (**2**, **4**, **6**, **19**, and **20**) displayed similar activity at P-gp, independent of the basic moiety type (EC_{50} values from 1.15 to 2.68 μM). Similar behavior was shown by carbazole derivatives **5** and **21**–**24** (EC_{50} values ranged from 1.52 to 5.38 μM), with the exception of 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline derivative **25**, which displayed a very potent interaction with P-gp (EC_{50} = 0.42 μM). The strongest interaction with P-gp was recorded by another 6,7-dimethoxytetrahydroisoquinoline derivative: the N-substituted indole derivative **15** (EC_{50} = 0.21 μM). Curiously, the presence of the 4-fluorophenyl substituent at the indole nitrogen atom led to a 10-fold increase in P-gp activity, only with 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline as the basic moiety (compare **15** with **18**). Therefore, the combination of the 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline moiety and N-substituted indoles appeared as the optimal approach to confer excellent interaction with P-gp, with **15** and **25** emerging as novel P-gp modulators worthy of further investigation.

Table 2. Activities of reference compounds **1**–**6** and new compounds **13**–**25**.

Compound	P-gp[b]	EC_{50} [μM][a] MCF7[c]	MCF7adr[d]
siramesine (**1**)	1.41 ± 0.31	12.3 ± 0.6	5.90 ± 1.21
PB28 (**2**)	3.0 ± 0.2[35]	28.4 ± 6.1	77.5 ± 10.1
4	1.92 ± 0.32	16.0 ± 2.0	17.9 ± 1.5
F281 (**5**)	2.90 ± 0.21	24.0 ± 3.1	35.2 ± 4.1
6	1.15 ± 0.2[16]	> 100	83.9 ± 13.2
13	3.44 ± 0.21	12.7 ± 0.1	13.6 ± 1.1
14	3.70 ± 0.23	14.2 ± 2.3	12.9 ± 0.8
15	0.21 ± 0.02	17.8 ± 0.4	21.8 ± 1.5
16	5.03 ± 0.61	> 100	> 100
17	4.60 ± 0.43	8.86 ± 1.81	16.4 ± 2.1
18	2.80 ± 0.41	> 100	> 100
19	2.68 ± 0.43	14.4 ± 0.6	17.1 ± 1.2
20	1.98 ± 0.32	31.4 ± 3.3	28.1 ± 2.4
21	1.58 ± 0.11	12.6 ± 2.5	17.8 ± 2.1
22	3.40 ± 0.61	25.0 ± 0.2	19.0 ± 0.9
23	1.52 ± 0.23	23.9 ± 0.9	18.6 ± 1.1
24	5.38 ± 0.50	19.9 ± 3.1	20.1 ± 2.5
25	0.42 ± 0.02	28.2 ± 5.0	17.1 ± 1.2

[a] Values represent the mean ± SEM of $n \geq 2$ separate experiments carried out in duplicate. [b] Transport inhibition in MDCK-MDR1 cells using calcein-AM (2.5 μM) as a probe. [c] Antiproliferative effect in the MCF7 cell line. [d] Antiproliferative effect in MCF7adr cells.

Antiproliferative activity in MCF7 and MCF7adr cell lines

With the exception of compound **3**, the antiproliferative activities of novel and reference compounds were evaluated in MCF7 and MCF7adr cells, expressed as EC_{50} values (Table 2). Compounds such as the N-unsubstituted indoles **16** and **18** displayed antiproliferative activity in neither of the two cell lines (EC_{50} > 100 μM). In contrast, the corresponding N-substituted indoles **13** and **15** displayed notable and similar antiproliferative activities in both cell lines (EC_{50} values: 12.7 and 13.6 μM for **13**, and 17.8 and 21.8 μM for **15**, in MCF7 and MCF7adr cells, respectively). This same difference in the antiproliferative activity between N-unsubstituted and N-substituted indoles was not shown by the couple **17**/**14**, with both compounds exerting notable antiproliferative effects in both cell lines (EC_{50} values: 8.86 and 16.4 μM for **17**, and 14.2 and 12.9 μM for **14**, in MCF7 and MCF7adr, respectively). Furthermore, N-unsubstituted indole **17** displayed the most potent activity of all the compounds studied in MCF7 cells. All the novel carbazole derivatives (**21**–**25**) displayed antiproliferative activity in both cell lines (EC_{50} from 12.6 to 28.2 μM), with spiro-isobenzofurane derivative **21** giving the best results. As for the tetralin series, all of the derivatives displayed antiproliferative activity except for the reference compound **6**. This compound, bearing a 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline moiety, curiously displayed an EC_{50} value in MCF7adr (EC_{50} = 83.9 μM) lower than that in the parental MCF7 line (EC_{50} > 100 μM). Basic moieties such as spiro-isobenzofurane- (a) and cyclohexyl- (d) piperidines led to the highest activity among tetralins in both cell lines (EC_{50}: 14.4 and 17.1 μM for **19**, and 16.0 and 17.9 μM for **4**, in MCF7 and MCF7adr cells, respectively). Moderate activity was displayed by 4-fluorophenyl derivative **20** (EC_{50}: 31.4 and 28.1 μM in MCF7 and MCF7adr, respectively), and unexpectedly disappointing activity was shown by cyclohexylpiperazine derivative **2** (EC_{50}: 28.4 and 77.5 μM in MCF7 and MCF7adr, respectively).

Collateral sensitivity

Surprisingly, some of the tested compounds displayed higher antiproliferative activity in resistant cells than in parent cells, recalling a phenomenon that has was recently termed *collateral sensitivity* (CS).[36,37] According to this phenomenon, activity is potentiated rather than decreased by the overexpression of P-gp; therefore, the MDR1-selective ratio (SR)—the compound's EC_{50} value in parental cells divided by its EC_{50} value in P-gp-overexpressing cells—should be > 1. There are different hypotheses that explain CS, and based on the evidence that

several CS agents are P-gp substrates, one of the suggested mechanisms is the activation of a futile ATP cycle, followed by increased production of ROS. In accordance with this hypothesis, **1**, which is known to activate ROS production and which was shown in this work to interact with P-gp, displayed an interesting SR, with an EC_{50} value in MCF7adr cells 2.1-fold lower than in parental cells. Lower values, but still >1, were the SR ratios observed for **22**, **23**, and **25**, all of which belong to the carbazole series. On the other hand, the differences in EC_{50} values were too small between parental and resistant cells for compounds **6**, **14**, and **20** to recall the CS phenomenon. Because the increase in ROS production generated by a futile ATP cycle may be responsible for CS, ATP consumption and ROS generation were studied for compounds **1** and **25**. For all of the compounds with antiproliferative activity in MCF7 ($EC_{50} < 100$ μM), involvement of ROS was indirectly demonstrated by the administration of the lipid antioxidant α-tocopherol: 100 μM α-tocopherol administered before the drug was able to completely rescue MCF7 cells from death (Table 3). To determine whether more ROS generation in MCF7adr than in MCF7 cells was responsible for CS, both cell lines were pretreated with increasing concentrations of α-tocopherol (1–50 μM) before the administration of **1** (25 μM) or **25** (25 μM), i.e., compounds with higher CS (Figure 2). At 1 μM, α-tocopherol was able to rescue neither MCF7 nor MCF7adr cells treated with **1**, whereas a small rescue of viability (13%) was observed in MCF7 cells treated with **25**. The differences in viability between MCF7 and MCF7adr pretreated with 10 μM α-tocopherol before administration of **1** or **25** were more significant, with viability reaching 70% only in MCF7 cells. On the other hand, MCF7adr viability was only slightly increased by α-tocopherol at 10 μM, with 40% of cells surviving after treatment with **25**, and 20% of cells surviving after administration of **1**. Pretreatment with α-tocopherol at 50 μM led to an almost complete rescue of viability in MCF7 cells treated with **1** or **25**, whereas a lower percentage of MCF7adr cells (~80%) survived. α-Tocopherol at 100 μM rescued the viability of MCF7adr as for MCF7 cells, treated with **1** or **25**. These results are in agreement with the hypothesis that the enhanced antiproliferative activity shown in resistant cells (CS) may be due to higher ROS production, likely induced by the futile ATP cycle activated by P-gp substrates. Therefore, consumption of ATP induced by compounds endowed with CS properties was evaluated, and higher ATP hydrolysis in MCF7adr than in MCF7 cells was demonstrated (Figure 3). In accordance with such a hypothesis, compound **15** did not cause enhanced ATP hydrolysis, and did not display CS, despite its very potent activity toward P-gp. Therefore, it appeared as a P-gp inhibitor that does not activate the futile ATP cycle as substrates do (Figure 3).

Table 3. Antiproliferative effect of 100 μM α-tocopherol on MCF7 cell viability.

Compound[b]	Cell viability [%][a]	
α-tocopherol[c]	−	+
siramesine (**1**)	26 ± 2.4	100
PB28 (**2**)	80 ± 5.2	100
4	60 ± 4.3	100
F281 (**5**)	60 ± 3.6	100
6	–	–
13	20 ± 0.9	100
14	20 ± 1.6	100
15	31 ± 3.2	85 ± 6.2
16	–	–
17	15 ± 0.8	80 ± 7.3
18	–	–
19	43 ± 2.4	90 ± 8.9
20	70 ± 6.1	100
21	20 ± 1.2	100
22	25 ± 1.1	100
23	43 ± 3.2	100
24	20 ± 2.1	100
25	60 ± 7.1	100

[a] Compounds tested at 25 μM. [b] Values represent the mean ± SEM of $n \geq 2$ separate experiments carried out in duplicate. [c] α-Tocopherol was used at 100 μM.

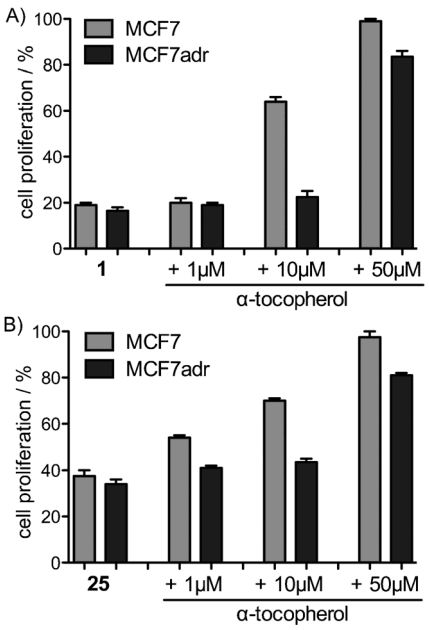


Figure 2. α-Tocopherol effect on MCF7 and MCF7adr cell viability. Antiproliferative effect of compounds A) **1** and B) **25** at 24 h in MCF7 and MCF7adr cells as indicated. Compounds **1** or **25** (25 μM) were administered alone or in combination with α-tocopherol at the indicated concentrations.

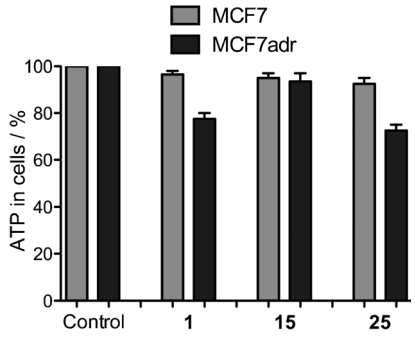


Figure 3. ATP consumption in MCF7 and MCF7adr cells as affected by compounds **1**, **15**, and **25** at a final concentration of 25 μM. Control: untreated cells.

Co-administration experiments

Compounds showing the strongest interaction with P-gp, **15** and **25**, were selected for co-administration experiments in MCF7adr cells with doxorubicin, which is a widely used chemotherapeutic drug and a P-gp substrate. As expected from a cell line in which overexpression of P-gp had been previously induced by treatment with doxorubicin, doxorubicin administered alone (up to 100 μM) did not exert any antiproliferative effect. Administration of **15** alone at 1, 10, and 25 μM left respectively 96, 89, and 12% of the cells surviving. When the compound was co-administered with 1 μM doxorubicin at the same concentrations above, a small synergistic effect was observed (cell survival rates of 76, 67, and 9%). This synergistic effect became much more pronounced with co-administration of doxorubicin at 10 μM (cell survival rates of 55, 15, and 9%). These data show that **15** inhibits P-gp, allowing doxorubicin to enter cells and to exert its cytotoxic effects. Better synergistic results were obtained when both **15** and doxorubicin were administered at 10 μM, although **15** at 1 μM also led to moderate doxorubicin activity. At 25 μM, **15** was administered above its EC_{50} value (21.8 μM, Table 2), and demonstrated its efficacy as a single agent in an MDR cell line (Figure 4). Similar behavior was shown by **25**, with better results obtained with co-administration of doxorubicin and **25** both at 10 μM (28% cell survival). Compound **25** at 25 μM also displayed its single-agent antitumor property with 15% cell survival, as already proven by its EC_{50} value (Table 2). Because compound **1** also appeared to undergo interaction with P-gp, we evaluated the properties of **1** in co-administration with doxorubicin. At 10 μM, **1** determined an 80% cell mortality as a single agent because of its EC_{50} value (5.9 μM in MCF7adr), and co-administration with doxorubicin at either 1 or 10 μM only slightly decreased viability. Compound **1** co-administered at 1 μM with 10 μM doxorubicin led to a lower synergistic effect (64% cell survival) than compound **15** (55% survival).

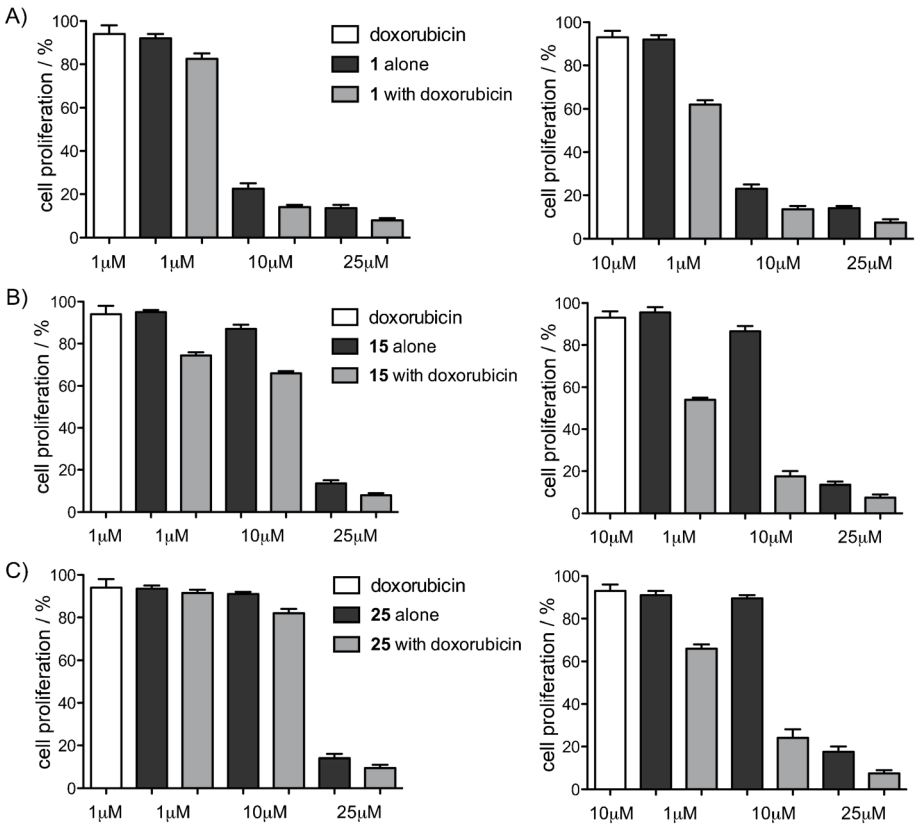


Figure 4. Co-administration of doxorubicin with σ_2/P-gp-active compounds A) **1**, B) **15**, and C) **25**. Antiproliferative effect of doxorubicin (white bars) at 1 μM (left side) and 10 μM (right side) at 24 h in the MCF7adr cell line. In comparison, σ_2/P-gp-active compounds (1, 10, and 25 μM) were administered for 24 h (black bars). After washing, compounds were co-administered with doxorubicin (1 or 10 μM) at the same concentration for 24 h (grey bars).

Conclusions

Most of the compounds synthesized displayed high σ_2 affinity, and a few displayed excellent σ_2 over σ_1 receptor selectivity. The best results in terms of σ_2 selectivity were obtained when 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline was connected to an N-substituted indole ring, with **15** displaying a 260-fold selectivity for σ_2 over σ_1. Despite the low Hill slope from the σ_2 receptor binding assay, carbazole **25** displayed exceptional σ_2 versus σ_1 selectivity (54750-fold). Therefore, novel scaffolds for high-affinity and highly σ_2-selective ligands were identified. Although no linear correlation between σ_2 receptor affinity and activity was found, most of the novel compounds exerted appreciable antiproliferative activity in breast tumor cell lines, and generally no decrease in activity was shown in MCF7adr cells, demonstrating the efficacy of these compounds in cells with P-gp-induced resistance. All compounds showed a certain degree of interaction with P-gp, with **15** and **25** displaying an unexpectedly potent activity. In co-administration with doxorubicin, both of these compounds at 10 μM were able to revert P-gp-mediated resistance, and to re-establish the antitumor activity of 10 μM doxorubicin, with some improvement also observed with doxorubicin at 1 μM. Therefore, **15** and 2**5** appear to be promising agents in MDR tumors, for use as single agents able to elude P-gp activity and kill tumor cells, or in co-administration with classic chemotherapeutic substrates of P-gp. However, the most surprising results given by some of the studied compounds (**1**, **22**, **23** and **25**) were the enhanced activities recorded in resistant MCF7adr cells over those observed in parent (non-resistant cells), recalling a phenomenon known

as collateral sensitivity. Exploitation of this phenomenon is under study as a potential alternative approach to treating drug-resistant tumors. We examined some of the possible mechanisms suggested for CS and found that **1** and **25** generate more ROS in the MCF7adr than in the MCF7 cell line, likely through a higher consumption of ATP due to interaction with P-gp. Overall, with this work we developed a few interesting and potent σ_2 receptor ligands endowed with unexpected antitumor properties that are surely worthy of further investigation for the development of alternate strategies against multidrug-resistant cancers.

Experimental Section

Chemistry

Microwave reactions were conducted in a Biotage Initiator Microwave Synthesizer. 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, ICN; 1:15 *w/w*, 15–40 μm particle size, Merck, respectively). Melting points were determined in open capillaries on a Gallenkamp electrothermal apparatus. The purity of tested compounds (in all cases $\geq$95%) was established by combustion analysis. Elemental analyses (C, H, N) were performed on a Eurovector Euro EA 3000 analyzer, and results were within $\pm$0.4% of the theoretical values unless otherwise indicated. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Mercury Varian instrument; $CDCl_3$ was used as solvent to record ^{1}H NMR on intermediate and final compounds as free basis, and the following data are reported: chemical shifts (δ) in ppm, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), integration and coupling constant(s) in Hertz. CD_3OD was used as solvent to record ^{13}C NMR on hydrochloride salts of final compounds where reported, and chemical shifts (δ) in ppm were reported. Recording of mass spectra was done on an Agilent 6890-5973 MSD GC/MS instrument and on an Agilent 1100 series LC–MSD trap system VL mass spectrometer; only significant *m/z* peaks, with percent relative intensities in parentheses, are reported. Chemicals were obtained from Aldrich and Alfa Aesar and were used without further purification.

4-[1-(4-Fluorophenyl)-1*H*-indol-3-yl)butanoic acid (7). A mixture of 4-(1*H*-indol-3-yl)butanoic acid (0.98 mmol, 0.20 g), K_2CO_3 (1.30 mmol, 0.18 g), CuI (0.25 mmol, 0.047 g), and 1-fluoro-4-iodobenzene (1.49 mmol, 0.33 g) in ethoxyethanol (3.5 mL) was warmed at 200 °C under stirring in a microwave oven for 40 min. After cooling to room temperature, H_2O (5 mL) was added to the reaction mixture, and the solvent was removed under reduced pressure. The residue was taken up with water, acidified with 3 N HCl (5 mL) and extracted with Et_2O (3×10 mL). The collected organic layers were dried (Na_2SO_4) and evaporated under reduced pressure to afford a crude residue as a brown oil, which was purified by crystallization from $CHCl_3$/*n*-hexane to provide the target compound as light-brown crystals (0.145 g, 50%): mp: 123–125 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.00–2.15 (t, 2H, *J* = 7.4 Hz, $ArCH_2CH_2$), 2.47 (t, 2H, *J* = 7.4 Hz, CH_2COOH), 2.87 (t, 2H, *J* = 7.4 Hz, $ArCH_2$), 3.80 (br s, 1H, COOH, D_2O exchanged), 7.10–7.66 ppm (m, 9H, aromatic); LC–MS (ESI^-) *m/z*: 296 $[M-H]^-$; LC–MS–MS 296: 252.

4-[1-(4-Fluorophenyl)-1*H*-indol-3-yl)-1-butanol (8). To a suspension of $LiAlH_4$ (0.66 mmol, 0.025 g) in dry THF (5 mL) kept at 0 °C and under a stream of N_2, a solution of 4-[1-(4-fluorophenyl)-1*H*-indol-3-yl)butanoic acid (**7**) (0.47 mmol, 0.14 g) in the same solvent (5 mL) was added in a dropwise manner. After holding at reflux for 2 h, the mixture was stirred at room temperature overnight. The reaction mixture was then cooled, quenched with H_2O (10 mL), and the aqueous layer was extracted with Et_2O (3×10 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated under reduced pressure to give a crude residue, which was purified by column chromatography with CH_2Cl_2/EtOAc (8:2) as eluent to afford the title compound as a yellow semisolid (0.130 g, 98%); GC–MS *m/z* 284 $[M+1]^+$ (7), 283 $[M+1]^+$ (34), 224 (100); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.60–1.90 (m, 5H, $CH_2CH_2CH_2OH$), 2.78–2.83 (m, 2H, $ArCH_2$), 3.78 (t, 2H, CH_2O), 7.09–7.70 ppm (m, 9H, aromatic).

General procedure for the synthesis of amines 13–15, 21, 22, and 25

One intermediate between **9** or **12** (0.95 mmol, 0.24 g) with one among the appropriate amines **a**–**e** (1.1 mmol) and K_2CO_3 (1.16 mmol, 0.16 g) in DMF (5 mL) was stirred at 100 °C for 20 min. The solvent was then removed under reduced pressure, and H_2O (10 mL) was added to the crude residue, and the mixture was extracted with CH_2Cl_2 (3×10 mL). The organic layers collected were dried (Na_2SO_4) and concentrated under reduced pressure to afford a crude residue, which was purified by column chromatography with CH_2Cl_2/MeOH (95:5) as eluent.

3-[4-(4-Cyclohexyl-1-piperazinyl)butyl]-1-(4-fluorophenyl)-1*H*-indole (13) was obtained as a yellow oil (0.27 g, 66% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.06–1.30 [m, 5H, cyclohexyl, $(CHH)_5$], 1.58–1.97 [m, 9H, cyclohexyl (CHH)$_5$ and $ArCH_2CH_2CH_2$], 2.17–2.31 (m, 1H, CHN), 2.40 (t, 2H, *J* = 7.6 Hz, CH_2N), 2.51–2.74 (m, 8H, piperazine), 2.81 (t, 2H, *J* = 7.3 Hz, $ArCH_2$), 7.13 (s, 1H, aromatic), 7.13–7.25 (m, 4H, aromatic), 7.40–7.46 (m, 3H, aromatic), 7.64 ppm (dd, 1H, J_{HH} = 7 Hz, J_{HF} = 1.5 Hz, aromatic); LC–MS (ESI^+) *m/z* 434 $[M+H]^+$; LC–MS–MS 434: 352, 266, 224; Anal. ($C_{28}H_{36}FN_3 \cdot 2C_2H_2O_4 \cdot {}^1/_4 H_2O$) C, H, N.

3-[4-(4-Cyclohexyl-1-piperidino)butyl]-1-(4-fluorophenyl)-1*H*-indole (14) was obtained as a yellow oil (0.29 g, 70%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.85–1.86 [m, 22H, cyclohexyl, piperidine CH_2CHCH_2, and $ArCH_2CH_2CH_2CH_2N$], 2.32–2.37 (m, 2H, piperidine CH*H*NCH*H*), 2.81 (t, 2H, *J* = 7.1 Hz, $ArCH_2$), 2.93–2.97 (m, 2H, piperidine C*H*HNC*H*H), 7.07 (s, 1H, aromatic), 7.13–7.26 (m, 4H, aromatic), 7.41–7.46 (m, 3H, aromatic), 7.64 ppm (dd, 1H, J_{HH} = 7 Hz, J_{HF} = 1.5 Hz, aromatic); LC–MS (ESI^+) *m/z* 433 $[M+H]^+$; LC–MS–MS 433: 266, 224; Anal. ($C_{29}H_{37}FN_2 \cdot HCl \cdot H_2O$) C, H, N.

6,7-Dimethoxy-2-[4-[1-(4-fluorophenyl)-1*H*-indol-3-yl]butyl]-1,2,3,4-tetrahydroisoquinoline (15) was obtained as a yellow oil (0.28 g, 65%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.70–1.88 (m, 4H, $ArCH_2CH_2CH_2$), 2.56 (t, 2H, *J* = 7.1 Hz, CH_2N), 2.7 (t, 2H, *J* = 5.8 Hz, CH_2N), 2.80–2.87 (m, 4H, 2 $ArCH_2$), 3.55 (s, 2H, $ArCH_2N$), 3.83 (s, 6H, 2 OCH_3), 6.50 (s, 1H, aromatic), 6.58 (s, 1H, aromatic), 7.16 (s, 1H, aromatic), 7.17–7.25 (m, 3H, aromatic), 7.41–7.47 ppm (m, 3H, aromatic), 7.65 (d, 1H, *J* = 8.2 Hz, aromatic); ^{13}C NMR (75 MHz, CD_3OD): δ = 22.55, 23.27, 25.34, 48.53, 51.18, 53.70, 53.80, 54.50, 107.98, 108.45, 109.85, 114.53, 114.84, 115.10, 117.40, 118.00 (d, $J_{2\text{-}CF}$ = 23 Hz), 120.93, 121.58, 123.95, 124.17, 124.20 (d, $J_{3\text{-}CF}$ = 8 Hz), 127.44, 134.73, 135.00, 147.14, 147.95, 159.52 ppm (d, $J_{1\text{-}CF}$ = 243 Hz); GC–MS *m/z*: 458 $[M]^+$ (17), 246 (42), 206 (60), 192 (100); Anal. ($C_{29}H_{31}FN_2O_2 \cdot HCl \cdot {}^1/_4 H_2O$) C, H, N.

1′-[4-(9*H*-Carbazol-9-yl)-butyl]spiro[isobenzofuran-1(3*H*),4′-piperidine] (21) was obtained as a light-yellow oil (0.22 g, 50%);

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.60–2.00 (m, 8H, CH_2CCH_2, $NCH_2CH_2CH_2$), 2.25–2.50 [m, 4H, $CH_2N(CHH)_2$], 2.78–2.90 [s, 2H, $N(CHH)_2$], 4.38 (t, 2H, J = 7.2 Hz, carbazole NCH_2), 5.05 (s, 2H, CH_2O), 7.10–8.15 (m, 12H, aromatic); GC–MS m/z: 410 $[M]^+$ (8), 202 (100); Anal. ($C_{28}H_{30}N_2O$·HCl·$^1/_4$ H_2O) C, H, N.

9-[4-(4-Fluorophenyl)piperidin-1-yl)butyl]-9*H*-carbazole (22) was obtained as a light-brown solid, which was recrystallized from EtOH to provide an ivory solid (0.17 g, 40%): mp: 121–123 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.70–2.05 [m, 10H, $CH(CH_2)_2$, $NCH_2CH_2CH_2CH_2N(CHH)_2$], 2.30–2.50 (m, 3H, ArCH, NCH_2), 2.98–3.02 [m, 2H, $N(CHH)_2$], 4.38 (t, 2H, J = 7.2 Hz, carbazole NCH_2), 6.90–8.10 ppm (m, 12H, aromatic); GC–MS m/z: 400 $[M]^+$ (12), 192 (100); LC–MS (ESI$^+$) m/z 401 $[M+H]^+$; LC–MS–MS 401: 222, 180; Anal. ($C_{27}H_{29}N_2F$·H_2O) C, H, N (H calcd: 7.47, found: 7.01).

9-[4-(6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinolin-2-yl)butyl]-9*H*-carbazole (25) was obtained as a white solid (0.32 g, 83%): mp: 104–106 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.60–2.05 (m, 4H, $NCH_2CH_2CH_2$), 2.45–2.85 (m, 6H, $ArCH_2CH_2NCH_2$), 3.45 (s, 2H, $ArCH_2N$), 3.82 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 4.38 (t, 2H, J = 7.2 Hz, carbazole NCH_2), 6.45 (s, 1H, aromatic), 6.60 (s, 1H, aromatic), 7.18–8.15 ppm (m, 8H, aromatic); ^{13}C NMR (75 MHz, CD_3OD): δ = 20.33, 23.10, 24.31, 40.19, 48.45, 51.03, 53.70, 53.80, 54.11, 107.28, 107.91, 109.82, 117.41, 117.67, 118.52, 121.42, 124.19, 138.96, 147.11, 147.92 ppm; GC–MS m/z: 414 $[M]^+$ (66), 206 (44), 192 (100); LC–MS (ESI$^+$) m/z: 415 $[M+H]^+$; LC–MS–MS 390: 222, 180; Anal. ($C_{27}H_{30}N_2O_2$·HCl·1$^1/_2$ H_2O) C, H, N.

General procedure for the synthesis of amines 16–20, 23, and 24

To a solution of one among intermediates **10**–**12** (1.9 mmol) in CH_3CN (25 mL), one among the appropriate amines **a**–**e** (2.3 mmol) and K_2CO_3 (2.3 mmol, 0.32 g) were added. The reaction mixture was held at reflux under stirring overnight. The solvent was then removed under reduced pressure, and H_2O (15 mL) was added to the crude residue. The aqueous mixture was extracted with CH_2Cl_2 (3×10 mL), and the organic layers were dried over Na_2SO_4 and concentrated under reduced pressure to afford a crude residue, which was purified by column chromatography with CH_2Cl_2/MeOH (95:5) as eluent.

3-[4-(4-Cyclohexyl-1-piperazinyl)butyl]-1*H*-indole (16) was obtained as a yellow semisolid (0.58 g, 90%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.07–1.25 [m, 5H, cyclohexyl $(CHH)_5$], 1.55–1.92 [m, 9H, cyclohexyl $(CHH)_5$ and $ArCH_2CH_2CH_2$], 2.11–2.29 (m, 1H, CHN), 2.40 (t, 2H, J = 7.6 Hz, CH_2N), 2.54–2.66 (m, 8H, piperazine), 2.77 (t, 2H, J = 7.3 Hz, $ArCH_2$), 6.97 (s, 1H, aromatic), 7.07–7.20 (m, 2H, aromatic), 7.31–7.36 (m, 1H, aromatic), 7.58–7.61 (m, 1H, aromatic), 7.97 ppm (br s, 1H, NH D_2O exchanged); GC–MS m/z: 340 $[M+1]^+$ (25), 339 $[M]^+$ (100), 181 (95), 130 (48); Anal. ($C_{22}H_{33}N_3$·2 HCl·$^1/_2$ H_2O) C, H, N.

3-[4-(4-Cyclohexyl-1-piperidino)butyl]-1*H*-indole (17) was obtained as yellow oil (0.47 g, 73%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.07–1.75 [m, 20H, cyclohexyl, piperidine CH_2CHCH_2, and $ArCH_2CH_2CH_2$], 1.78–1.86 (m, 2H, $CH_2CH_2CH_2N$), 2.31–2.36 (m, 2H, piperidine CHNCH), 2.77 (t, 2H, J = 7.3 Hz, $ArCH_2$), 2.94–3.00 (m, 2H, piperidine CHNCH), 6.97 (s, 1H, aromatic), 7.07–7.20 (m, 2H, aromatic), 7.35 (d, 1H, J = 7.7 Hz, aromatic), 7.60 (d, 1H, J = 7.5 Hz, aromatic), 7.95 (br s, 1H, NH D_2O exchanged); GC–MS m/z: 338 $[M]^+$ (11), 180 (100), 130 (66); Anal. ($C_{23}H_{34}N_2$·HCl) C, H, N.

6,7-Dimethoxy-2-[4-(1*H*-indol-3-yl)butyl]-1,2,3,4-tetrahydroisoquinoline (18) was obtained as yellow oil (0.35 g, 50% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.67–1.82 (m, 4H, $ArCH_2CH_2CH_2$), 2.54 (t, 2H, J = 7.3 Hz, CH_2N), 2.70 (t, 2H, J = 5.6 Hz, CH_2N), 2.78–2.95 (m, 4H, 2 $ArCH_2$), 3.54 (s, 2H, $ArCH_2N$), 3.83 (s, 6H, 2 OCH_3), 6.51 (s, 1H, aromatic), 6.58 (s, 1H, aromatic), 6.97 (s, 1H, aromatic), 7.10 (t, 1H, J = 7.4 Hz, aromatic), 7.18 (t, 1H, J = 7.7 Hz, aromatic), 7.34 (d, 1H, J = 7.1 Hz, aromatic), 7.61 (d, 1H, J = 7.7 Hz, aromatic), 7.97 ppm (br s, 1H, NH D_2O exchanged); GC–MS m/z: 365 $[M+1]^+$ (12), 364 $[M]^+$ (54), 246 (46), 206 (100), 192 (96); Anal. ($C_{23}H_{28}N_2O_2$·HCl·1$^1/_4$ H_2O) C, H, N.

1′-[3-(5-Methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)propyl]spiro[isobenzofuran-1(3*H*),4′-piperidine] (19) was obtained as a yellow oil (0.58 g, 78%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.48–2.20 [m, 12H, $CH(CH_2CH_2)_2$ and piperidine $C(CH_2)_2$], 2.40–2.82 (m, 7H, $N(CH_2)_3$ and benzyl CH), 2.90–3.05 (m, 2H, benzyl CH_2), 3.80 (s, 3H, OCH_3), 5.04 (s, 2H, CH_2O), 6.65 (d, 1H, J = 7.7 Hz, aromatic), 6.80 (d, 1H, J = 7.7 Hz, aromatic), 7.10 (t, 1H, J = 7.7 Hz, aromatic), 7.15–7.30 ppm (m, 4H, aromatic); GC–MS m/z: 391 $[M]^+$ (26), 202 (100); LC–MS (ESI$^+$) m/z: 392 $[M+H]^+$; LC–MS–MS 392: 203, 161; Anal. ($C_{26}H_{33}NO_2$·HCl·$^3/_4$ H_2O) C, H, N.

4-(4-Fluorophenyl)-1-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)propyl]piperidine (20) was obtained as a white semisolid (0.43 g, 60% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.45–1.85 [m, 12H, $CH(CH_2CH_2)_2$ and piperidine $CH(CH_2)_2$], 1.98–2.08 (m, 1H, piperidine CH), 2.35–2.85 (m, 7H, $N(CH_2)_3$ and benzyl CH), 3.02–3.08 (m, 2H, benzyl CH_2), 3.80 (s, 3H, OCH_3), 6.65 (d, 1H, J = 7.7 Hz, aromatic), 6.80 (d, 1H, J = 7.7 Hz, aromatic), 6.92–7.05 (m, 2H, aromatic), 7.10 (t, 1H, J = 7.7 Hz, aromatic), 7.16–7.30 ppm (m, 2H, aromatic); GC–MS m/z 381 $[M]^+$ (26), 202 (100); Anal. ($C_{25}H_{32}NOF$·HCl·$^1/_2$ H_2O) C, H, N.

9-[4-(4-Cyclohexylpiperazin-1-yl)butyl]-9*H*-carbazole (23) was obtained as a white solid (0.44 g, 60%): mp: 84–86 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.00–1.98 [m, 14H, cyclohexyl $(CH_2)_5$ and $NCH_2CH_2CH_2$], 2.05–2.78 (m, 11H, CHN, and piperazine), 4.32 (t, 2H, J = 7.2 Hz, carbazole NCH_2), 7.15–8.10 ppm (m, 8H, aromatic); GC–MS m/z: 390 $[M+1]^+$ (17), 389 $[M]^+$ (66), 346 (27), 181 (100); LC–MS (ESI$^+$) m/z: 390 $[M+H]^+$; LC–MS–MS 390: 222, 180; Anal. ($C_{26}H_{35}N_3$·2 HCl·$^3/_4$ H_2O) C, H, N.

9-[4-(4-Cyclohexyl-1-piperidino)butyl]-9*H*-carbazole (24) was obtained as a white solid (0.46 g, 62%): mp: 87–89 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.82–1.38 (m, 8H, cyclohexyl and piperidine CH and CH_2), 1.50–1.90 (m, 12H, cyclohexyl and piperidine CH, CH_2, and $NCH_2CH_2CH_2$), 1.95–2.00 (m, 2H, CH_2N), 2.25–2.40 (m, 2H, CHN piperidine), 2.82–2.95 (m, 2H, CHN piperidine), 4.32 (t, 2H, J = 7.2 Hz, carbazole NCH_2), 7.15–8.20 ppm (m, 8H, aromatic); GC–MS m/z: 388 $[M]^+$ (8), 346 (27), 180 (100); Anal. ($C_{27}H_{36}N_2$·HCl·$^1/_4$ H_2O) C, H, N.

Biology

Materials: [^{3}H]DTG (50 Ci mmol^{-1}), (+)-[^{3}H]pentazocine (30 Ci mmol^{-1}) and ATPlite 1-step Kit were purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA, USA). 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 obtained from Harlan, Italy. Cell culture reagents were purchased from EuroClone (Milan, Italy). CulturePlate 96-well plates were purchased from PerkinElmer Life Science. Calcein-AM, 3-(4,5-dimethylthiazol-2-

yl)-2,5-diphenyltetrazoliumbromide (MTT), α-tocopherol, and doxorubicin were obtained from Sigma–Aldrich (Milan, Italy).

Competition binding assays: All procedures for binding assays were described previously; σ_1 and σ_2 receptor binding assays were carried out according to Matsumoto et al.[38] The specific radioligands and tissue sources were: 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 by 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 constant (K_i) values were determined by nonlinear curve fitting by using Prism v. 3.0 (GraphPad software).[39]

Cell cultures: Human MCF7 breast adenocarcinoma was purchased from ICLC (Genoa, Italy), human MCF7adr breast adenocarcinoma (resistant to doxorubicin), were kindly provided by Prof. G. Zupi (IRE, Rome, Italy), MDCK-MDR1 cells were a gift of Prof. P. Borst, NKI-AVL Institute, Amsterdam (Netherlands). MCF7 and MDCK-MDR1 cells were grown in high-glucose DMEM 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 under a 5% CO_2 atmosphere. MCF7adr cells were grown in RPMI 1640 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 under a 5% CO_2 atmosphere.

Cell viability: Determination of cell growth was performed by MTT assay at 48 h.[40,41] On day 1, 25 000 cells per well were seeded into 96-well plates at a volume of 100 μL. On day 2, the various drug concentrations (0.1–100 μM) were added. In all the experiments, the various drug solvents (EtOH, DMSO) were added in each control to evaluate possible cytotoxicity by solvent. 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 DMSO/EtOH (1:1), and the absorbance values at λ 570 and 630 nm were determined on a microplate reader Victor 3 (PerkinElmer Life Sciences).

Effect of α-tocopherol on cell viability: The interference of ROS in cell viability was indirectly determined by MTT assay at 24 h. On day 1, 25 000 cells per well were seeded into 96-well plates in the presence or absence of various concentrations of α-tocopherol (1–100 μM). On day 2, the drugs (25 μM) were added alone and in combination with various concentrations of α-tocopherol (1–100 μM). After incubation (24 h) 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 with 100 μL DMSO/EtOH (1:1), and the absorbance values at λ 570 and 630 nm were determined on a microplate reader Victor 3 (PerkinElmer Life Sciences).

Co-administration assays: The co-administration assay with doxorubicin was performed at 72 h.[41] On day 1, 25 000 cells per well were seeded into 96-well plates at a volume of 100 μL. On day 2, three drug concentrations (1, 10, and 25 μM) were added. On day 3, the medium was removed, and the three drug concentration were added alone and in combination with doxorubicin (1 or 10 μM). 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 with 100 μL DMSO/EtOH (1:1), and the absorbance values at λ 570 and 630 nm were determined on a microplate reader Victor 3 (PerkinElmer Life Sciences).

Calcein-AM experiments: These experiments were carried out as described by Feng et al. with minor modifications.[42] Calcein-AM is a pro-fluorescent probe and a P-gp substrate. In cells overexpressing P-gp, calcein-AM is unable to permeate the cell membrane, whereas when the efflux pump is not present or is inhibited, the probe enters living cells and is converted into fluorescent calcein by intracellular esterases. Calcein is unable to diffuse through the membrane, as it is hydrophilic and is not a P-gp substrate; therefore, calcein accumulates in cells when the pump is blocked. Therefore, the fluorescent signal is directly correlated to the amount of P-gp inhibition. The MDCK-MDR1 cell line (50 000 cells per well) was seeded into black CulturePlate 96-well plates with 100 μL medium and allowed to reach confluency overnight. Test compounds at various concentrations (0.1–100 μM) were solubilized in culture medium (100 μL) and added to monolayers; 96-well plates were incubated at 37 °C for 30 min. Calcein-AM was added in 100 μL phosphate-buffered saline (PBS) to yield a final concentration of 2.5 μM, and plates were incubated for 30 min. Each well was washed with ice-cold PBS (3×100 μL). Saline buffer (100 μL) was added to each well, and the plate was read in a Victor 3 instrument (PerkinElmer) at excitation and emission wavelengths of 485 and 535 nm, respectively. Under these experimental conditions, calcein cell accumulation in the absence and presence of test compounds was evaluated, and basal-level fluorescence was estimated by untreated cells. In treated wells, the increase in fluorescence with respect to basal levels was measured. EC_{50} values were determined by fitting the percent increase in fluorescence versus log[dose].

Bioluminescence ATP assays: These experiments were performed as reported in the technical sheet of the ATPlite 1-step Kit for luminescence ATP detection based on firefly (*Photinus pyralis*) luciferase (PerkinElmer Life Sciences).[43,44] The ATPlite assay is based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin (substrate solution), and the amount of light emitted is proportional to the ATP concentration. MCF7 and MCF7adr cells were seeded into black CulturePlate 96-well plates in 100 μL of complete medium at a density of 2×10^4 cells per well. Plates were incubated overnight in a humidified atmosphere containing 5% CO_2 at 37 °C. The medium was removed, and 100 μL of complete medium in the presence or absence of various test compound concentrations (0.1–100 μM) was added. Plates were incubated for 2 h under a humidified atmosphere 5% CO_2 at 37 °C. Mammalian cell lysis solution (50 μL) was then added to all wells, and the plates were agitated for 5 min on an orbital shaker. Substrate solution (50 μL) was added to all wells, and the plates were stirred for another 5 min on an orbital shaker. Plates were dark-adapted for 10 min, and luminescence was measured on a microplate reader Victor 3 (PerkinElmer Life Sciences).

Keywords: antitumor agents · breast cancer · collateral sensitivity · multidrug resistance · sigma receptors

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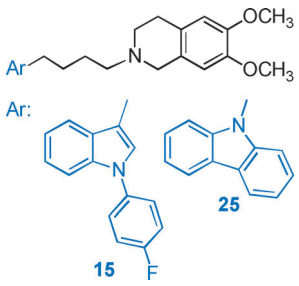
Collateral damage: We developed promising σ_2 ligands for alternative strategies against multidrug-resistant cancer. New high-affinity σ_2 agonists display antiproliferative activity in breast tumor cells; their interaction with P-gp generates higher activity in resistant than in parent cells (collateral sensitivity). Compounds co-administered with doxorubicin revert P-gp-mediated resistance.



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Sigma-2 Receptor Agonists as Possible Antitumor Agents in Resistant Tumors: Hints for Collateral Sensitivity