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Original article

From mixed sigma-2 receptor/P-glycoprotein targeting agents to selective P-glycoprotein modulators: Small structural changes address the mechanism of interaction at the efflux pump

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ABSTRACT

Generations of modulators of the efflux pump P-glycoprotein (P-gp) have been produced as tools to counteract the Multidrug Resistance (MDR) phenomenon in tumor therapy, but clinical trials were not successful so far. With the aim of contributing to the development of novel P-gp modulators, we started from recently studied high-affinity sigma-2 (σ_2) receptor ligands that showed also potent interaction with P-gp. For σ_2 receptors high-affinity binding, a basic N-atom is a strict requirement. Therefore, we reduced the basic character of the N-atom present in these ligands, and we obtained potent P-gp modulators with poor or null σ_2 receptor affinity. We also evaluated whether modulation of P-gp by these novel compounds involved consumption of ATP (as P-gp substrates do), as a source of energy to support the efflux. Surprisingly, even small structural changes resulted in opposite behavior, with amide **13** depleting ATP, in contrast to its isomer **18**. Two compounds, **15** and **25**, emerged for their potent activity at P-gp, and deserve further investigations as tools for P-gp modulation.

1. Introduction

Drug resistance is one of the major limitations in cancer treatments. This widespread phenomenon is mainly caused by overexpression of plasma membrane transporters such as P-glycoprotein (P-gp), Breast Cancer Resistant Protein (BCRP), and Multidrug Resistant associated Proteins (MRPs), all belonging to the ATP Binding Cassette (ABC) transporter proteins superfamily [1–3]. Since resistance is directed towards different drug entities, the phenomenon is named Multidrug Resistance (MDR). Overexpression of P-gp is one of the main mechanisms of MDR, and it generally leads to poor chemotherapy response and prognosis. P-gp actively pumps chemotherapeutics (e.g. vinblastine, paclitaxel, doxorubicin) out of the cancer cells leading to reduced or abolished anticancer effects. For these reasons, P-gp modulators acting as P-gp substrates (i.e. actively transported by the pump out of the cell), or as P-gp inhibitors (able to bind the pump without being effluxed) should allow chemotherapeutic agents to entering the cells and exert their anticancer properties [4]. Scientific research has been very active towards this direction with three generations of P-gp inhibitors already developed for this purpose. Nevertheless, all of these agents failed during clinical trials [5]. Therefore, more Pg-p modulators are under development. In the effort to develop sigma-2 (σ_2) receptor ligands endowed with antitumor properties, we realized that σ_2 receptors affinity and P-gp activity are often connected. σ_2 Receptors, together with the subtype-1 of σ receptors (σ_1), are endocellular proteins whose functions are not fully understood yet. Nevertheless, both these subtypes are intriguing targets for drug-related research because of their clear involvement in a number of pathologies [6,7]. In particular, interest in σ_2 receptor is due to the high densities of this subtype in several tumors. Once activated by σ_2 ligands, these proteins lead tumor cells to death through different pathways which are still under study [8–10]. With the aim of targeting σ_2 receptors, many classes of σ_2 ligands were developed and several high affinity σ_2 receptor ligands were found to interact with P-gp, either as P-gp substrates or inhibitors [10–13]. A few σ_2 receptor agonists were found to be endowed with Collateral Sensitivity (CS): their antiproliferative

Abbrevietions: ABC, ATP Binding Cassette; BCRP, Breast Cancer Resistant Protein; Calcein-AM, acetoxymethylester of Calcein; CDI, 1,1-carbonyldiimidazole; CS, Collateral Sensitivity; DTG, 1,3-di-o-tolyl-guanidine; MDCK-MDR1, Madin Darby canine kidney cells transfected with the human MDR1 gene; MDR, Multidrug Resistance; MRPs, Multidrug Resistant associated Proteins; P-gp, P-glycoprotein; ROS, reactive oxygen species.

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activity was more potent in P-gp-overexpressing cells than in P-gp-negative cells [11,13]. Further investigation led to link the CS property of these σ_2 agonists to their potent interaction as substrates at the P-gp and the consequent depletion of ATP to support P-gp-mediated active efflux of these substrates. The changes in cell energy levels and Reactive Oxygen Species (ROS) production due to ATP depletion was considered one of the causes for the higher sensitivity to mixed σ_2/P-gp agents of P-gp-overexpressing cells than P-gp-negative cells. Indeed, σ_2 agonists that displayed a potent P-gp inhibition (with no ATP cell depletion) were not endowed with CS.

In the attempt to contribute to the development of P-gp modulators, we used our previously described mixed and potent σ_2/P-gp agents as the lead compounds (Fig. 1) [11]. In the novel molecules herein reported, the hydrophobic fragments 5-methoxytetralin-1-yl, or 1-(4-fluorophenyl)indol-3-yl, or 9-carbazolyl, and the basic moieties present in the lead compounds (Fig. 1) were kept, but the basic N-atom was either inserted in an amide function, or was connected to an electron-withdrawing group. In this manner, the basic character of the N-atom, which was previously shown to be an essential requirement for σ receptor affinity [14–17], was completely abolished or reduced, in order to drastically decrease the affinity at the σ receptor, while saving the activity at the P-gp. 5-Methoxytetralin was alternatively connected through a propanoic linker to spiro[isobenzofuran-1-(3*H*),4'piperidine] (A), 4-(4-fluorophenyl)piperidine (B), 4-cyclohexylpiperidine (C), and 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (D) (Table 1). All of these amines were selected because they had been previously demonstrated to confer potent mixed P-gp/σ_2 receptor activity, when linked to 5-methoxytetralin through a propylene chain [11]. On the other hand, hydrophobic portions such as 1-(4-Fluorophenyl)indol-3-yl and 9-carbazolyl were connected through a butanoic linker only to 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (Table 2), as in compounds **5** and **6** (Fig. 1). This choice was made because this amine demonstrated a striking superiority to bind P-gp compared to other amines, when inserted on the hydrophobic moieties described above. In addition, the effect of the position of the amide function was studied through the synthesis of the corresponding 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-1-one connected through a butylene linker to the hydrophobic moieties. Another strategy for the reduction of the N-atom basic character was the exploitation of the electron-withdrawing property of the acetamide linker [18] that we used in place of the propanoic or butanoic spacer in the novel 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline derivatives. For the most potent P-gp modulators, we studied ATP consumption so that the mechanism of interaction at P-gp of these novel amides could be determined also in the CS application perspective.

2. Chemistry

The synthetic pathways for final compounds **8**–**11**, **13**, **15**, **18**, **19**, **25**, (±)-**27**, (+)-(*R*)-**27** and (−)-(*S*)-**27** are depicted in Schemes 1–4. Activation of 3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl) propanoic acid **7** with 1,1-carbonyldiimidazole (CDI) followed by addition of the appropriate amine (spiro[isobenzofuran-1-(3*H*),4'piperidine], 4-(4-fluorophenyl)piperidine, 4-cyclohexylpiperidine, or 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline) led to final amides **8**–**11** (Scheme 1). The same reaction was carried out on the already known acids 4-[1-(4-fluorophenyl)-1*H*-indol-3-yl)butanoic acid **12** [11] or 4-(9*H*-carbazol-9-yl)butanoic acid **14**, that upon activation with CDI, reacted with 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline to afford final amides **13** and **15** respectively (Scheme 1). Mesylate intermediate **16** [19] or alkyl chloride **17** [20] were used to alkylate the previously reported lactam 6,7-dimethoxy-3,4-dihydroisoquinolin-1(2*H*)-one [21] in the presence of NaH to afford final lactams **18** and **19** (Scheme 2). The synthesis for amide **25** (Scheme 3) started with reaction between indole-3-carboxyaldehyde **20** and 4-fluorophenyl-boronic acid in the presence of $Cu(AcOEt)_2$ and pyridine to afford key intermediate 1-(4-fluorophenyl)-1*H*-indole-3-

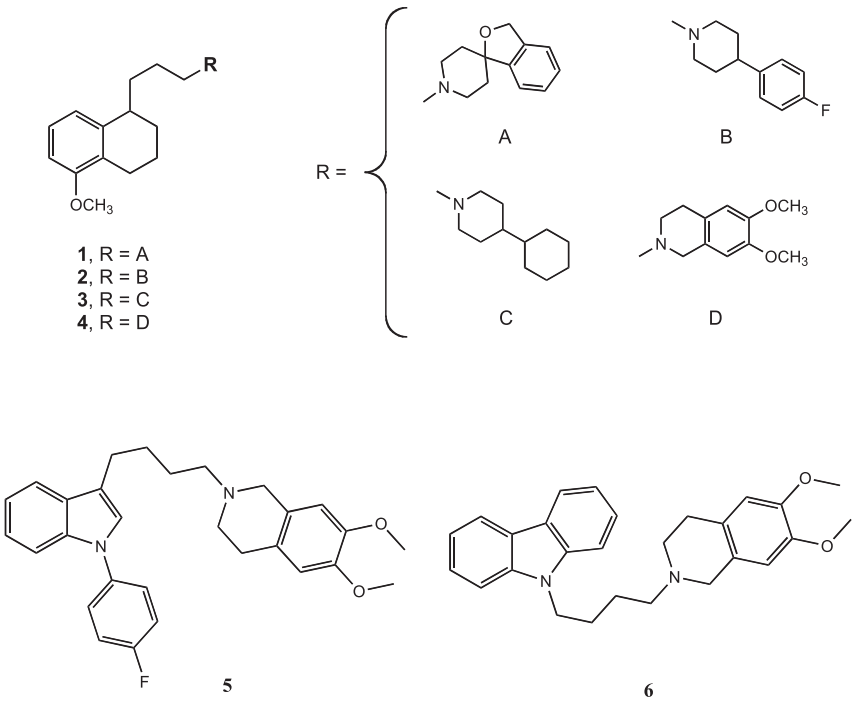
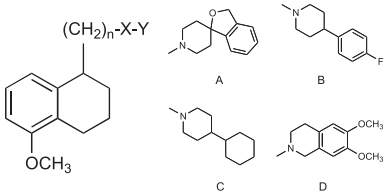


Fig. 1. Mixed σ_2-Pgp agents used as Lead Compounds.

Table 1
σ Receptor affinities, P-gp activity and ATP consumption of lead compounds **1**–**4** and novel compounds **8**–**11**, (±)-**27**, (+)-(*R*)-**27** and (−)-(*S*)-27.



Compound	*n*	X	Y	K_i (nM)[a]		EC_{50} (μM)[a]	ATP depletion	pK_a[b]
				σ_1	σ_2	P-gp		
1[c]	2	CH_2	A	76%	40.6 ± 6.4	2.68 ± 0.43	NT[d]	9.43
8	2	CO	A	260 ± 60	3210 ± 130	5.42 ± 0.90	NT[d]	−0.04
2[c]	2	CH_2	B	80%[e]	22.6 ± 3.9	1.98 ± 0.32	NT[d]	9.45
9	2	CO	B	2934 ± 323	6517 ± 265	26 ± 4.9	NT[d]	−0.87
3[c]	2	CH_2	C	143 ± 18	18.8 ± 5.9	1.92 ± 0.32	NT[d]	9.86
10	2	CO	C	>5000	>10000	30.9 ± 3.9	NT[d]	−0.46
4[c]	2	CH_2	D	151 ± 20	96%[e]	1.15 ± 0.2	Yes	8.10
11	2	CO	D	>5000	>10000	2.9 ± 0.87	Yes	−0.74
(±)-**27**	0	$NHCOCH_2$	D	>5000	1800 ± 243	10 ± 2.90	NT[d]	5.94
(+)-(*R*)-**27**	0	$NHCOCH_2$	D	>5000	1946 ± 274	9.6 ± 2.78	NT[d]	
(−)-(*S*)-**27**	0	$NHCOCH_2$	D	>5000	1761 ± 269	10 ± 2.89	NT[d]	
DTG					35.4 ± 5.8			
(+)-pentazocine				4.52 ± 0.7				
28[f]						0.82 ± 0.2		

[a] Values represent the mean ± SEM of $n \geq 2$ separate experiments, in duplicate.
[b] pK_a values were computationally calculated with ACD/Labs 7.00; version 7.07.
[c] From Ref. [5].
[d] NT = not tested.
[e] Percent displacement at a concentration of 10^{-11} M is reported since a complete displacement curve was not obtained.
[f] From Ref. [13].

carboxyaldehyde (**21**). This compound underwent reductive amination with benzylamine, $NaCNBH_3$ and $ZnCl_2$ in isopropanol, to provide intermediate amine **22**. Debenzylation of this last amine was achieved with H_2 in the presence of 10% Pd on activated carbon to provide **23**, whose acetylation with 2-bromoacetyl chloride afforded bromoacetamide **24**. This last bromide was used to alkylate 6,7-dimethoxytetrahydroisoquinoline to provide final compound **25**. Analogously, the already reported racemic (±)- and enantiomeric (+)-(*R*)- or (−)-(*S*)–*N*-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)bromoacetamide intermediates **26** [18] were reacted with 6,7-dimethoxytetrahydroisoquinoline to provide final compounds (±)-**27**, (+)-(*R*)-**27** and (−)-(*S*)-**27** (Scheme 4). Compound **25**, (±)-**27** and its enantiomers were converted to the corresponding hydrochloride salts with gaseous HCl (as reported in the Supplementary Material).

3. Results and discussion

3.1. σ receptors binding

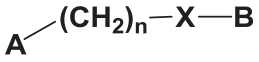
Results from radioligand binding assays are expressed as inhibition constants (K_i values) in Tables 1 and 2, together with the ionization constants (pK_a) for the N-atom in moieties A-D. As expected, the removal of the basicity of the N-atom, drastically reduced the affinity at both the σ receptor subtypes. In a way that is independent of the amine type or of the hydrophobic portion type, also the reduction of the availability of the electron pair on the N-atom determined by the presence of the acetyl electron-withdrawing group, led to the same drop in the σ affinity, so that the reduction of the basic feature of the N-atom by the acetamide linker was confirmed to be a successful strategy when σ receptors binding needs to be abolished. Most of the compounds displayed K_i values >5000 nM at the σ_1 subtype (**11**, **13**, **15**, **18**, **19**, **25** and all **27** isomers), with the only exceptions of compound **9**, whose K_i was slightly lower (2934 nM), and compound **8** which was the only one to display a certain σ_1 receptor affinity ($K_i = 260$ nM). As for the σ_2 receptor, several novel compounds displayed $K_i > 10000$ nM (**10**, **13**, **15**, **18**, **19**). Slightly lower K_i values were displayed by compounds **8** and **9** ($K_i = 3210$ nM and 6517 nM, respectively), (±)-**27** and its enantiomers (K_i values ranging from 1761 nM to 1946 nM), and by compound **25** ($K_i = 1623$ nM).

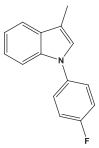
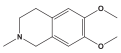
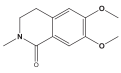
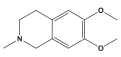
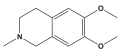
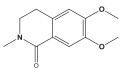
3.2. P-gp activity

3.2.1. Calcein-AM assay

Results from Calcein-AM assay are expressed as EC_{50} values in Tables 1 and 2. All of the novel compounds interacted with P-gp, with EC_{50} values ranging from 30.9 to 0.82 μM. Generally all of the amides displayed a reduced P-gp activity compared to the corresponding amines. In the tetralin-1-yl-propanamides (**8**–**11**), the 4-cyclohexylpiperidine moiety (C) and 4-(4-fluorophenyl)piperidine moiety (B) were detrimental for the interaction with the efflux pump, displaying the highest EC_{50} values ($EC_{50} = 26$ μM for **9** and $EC_{50} = 30.9$ μM for **10**, respectively), with about a 15-fold reduced P-gp activity compared to the corresponding amines [11]. On the other hand, spiropiperidine moiety (A) conferred an important interaction with P-gp (compound **8** $EC_{50} = 5.42$ μM), while 6,7-dimethoxytetrahydroisoquinoline moiety (D) led to display the most potent activity (compound **11** $EC_{50} = 2.9$ μM) among the tetralin-1-yl-propanamides. In addition, no drop in the activity was recorded with **11** compared to its amine counterpart **4** ($EC_{50} = 1.15$ μM), so that **11** was structurally modified by replacing the propanoic linker with an acetamide one. We previously showed that the presence of the acetamide between the N-tetralin structure and a piperazine basic moiety, strongly reduced the ionization pK_a of the piperazine N-atom directly attached to the linker [18].

Table 2
σ Receptor affinities, P-gp activity and ATP consumption of lead compounds **5**, **6** and novel compounds **13**, **15**, **18**, **19** and **25**.



Compound	A	*n*	X	B	K_i (nM)[a]		EC$_{50}$ (μM)[a]	ATP depletion	pK_a[b]
					σ_1	σ_2	P-gp		
5[c]		3	CH_2		1390 ± 20	5.34 ± 1.22	0.21 ± 0.02	No	8.13
13		3	CO		>5000	>10000	2.25 ± 0.4	Yes	−0.73
18		3	CH_2		>5000	>10000	2.34 ± 0.7	No	−1.06
25		1	$NHCOCH_2$		>5000	1623 ± 179	0.92 ± 0.3	No	5.97
6[c]		3	CH_2		2190 ± 230	0.04 ± 0.01	0.42 ± 0.02	Yes	7.97
15		3	CO		>5000	>10000	0.82 ± 0.2	Yes	−0.79
19		3	CH_2		>5000	>10000	1.91 ± 0.5	30%[d]	−1.08
DTG						35.4 ± 5.8			
(+)-pentazocine					4.52 ± 0.7				
28[e]							0.82 ± 0.2		

[a] Values represent the mean ± SEM of $n \geq 2$ separate experiments, in duplicate.
[b] pK_a values were computationally calculated with ACD/Labs 7.00; version 7.07.
[c] From Ref. [5].
[d] Percentage of ATP cell depletion at 100 μM.
[e] From Ref. [13].

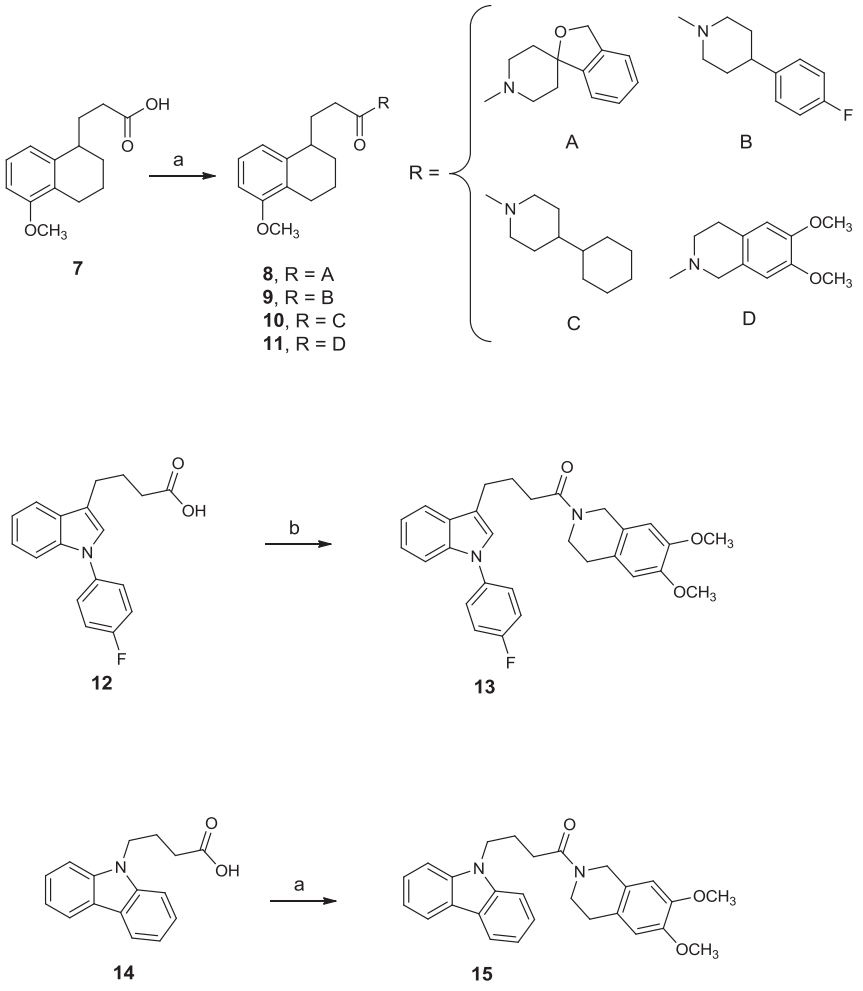
Therefore, we applied this same strategy to produce P-gp modulators, with poor affinity at the σ receptors. Although less basic, these compounds still present a protonatable N-atom (pK_a = 5.94), so that pharmacokinetic issues with which amides are often endowed, can be prevented. Thus, we prepared compound **27** in the racemic and enantiomerically pure forms. These compounds, that displayed very poor σ receptor affinity, showed a slightly weaker interaction with P-gp than propanamide **11**, and no enantioselectivity was shown by the efflux pump with enantiomers and racemate displaying similar EC$_{50}$ values (EC$_{50}$ ~ 10 μM). Amide analogues of lead compound **5** also showed a 10-fold drop in the activity at the P-gp. Butanamide **13** and its isomer **18**, that contains the amide function within the tetrahydroisoquinoline ring, presented similar EC$_{50}$ values (EC$_{50}$ = 2.25 μM and 2.34 μM, respectively). As for **11**, also for **13**, the corresponding poorly basic amine **25** was developed through the replacement of the butanoyl chain with the acetamidomethyl electron withdrawing linker. Acetamide derivative **25** (pK_a = 5.97) resulted in very poor σ receptors affinity, and in a potent interaction with P-gp (EC$_{50}$ = 0.92 μM), in line with the EC$_{50}$ value displayed by the P-gp reference compound 6,7-dimethoxy-2-{[5-methoxy-3,4-dihydro-(1*E*) (2*H*)-naphthalenylidene]propyl}-1,2,3,4-tetrahydroisoquinoline (**28**) [22]. As for the amide analogues of lead compound **6**, butanamide **15** displayed the most potent interaction with P-gp (EC$_{50}$ = 0.82 μM) among these novel compounds, with just a slight decrease compared to **6** and with a value similar to that of the reference compound **28** [22]. Lactam compound **19** also displayed a potent P-gp interaction (EC$_{50}$ = 1.90 μM) but with a 2-fold lower EC$_{50}$ value than its isomer **15**.

3.2.2. ATP depletion assay

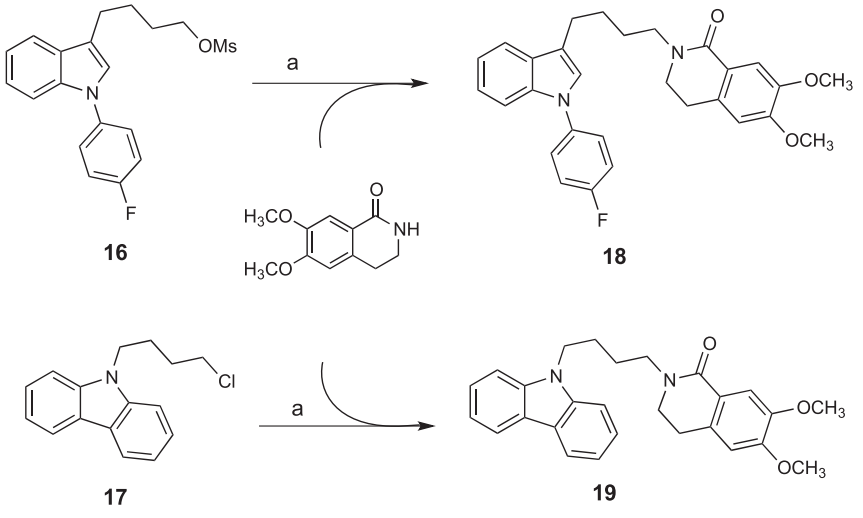
For the best P-gp modulators we evaluated also the ATP consumption in order to verify the interaction mechanism with the efflux pump. Generally, a P-gp ligand is classified as "unambiguous substrate", when it inhibits Calcein-AM transport while inducing depletion of ATP from the cell since it is actively transported. On the other hand, a P-gp inhibitor binds the transporter without being transported and therefore no ATP consumption is recorded [22,23]. We had previously verified the ATP consumption generated by the corresponding amines (lead compounds) [11], so that we could also speculate the effect of the removal/reduction of the basic feature of the N-atom on the P-gp activity. Generally, the same P-gp activity of the amines was kept in the corresponding amides. Tetralin **11** led to ATP consumption, as its corresponding amine **4** which is a deeply studied P-gp substrate [24]. Also carbazole-bearing **15** demonstrated to consume ATP upon interaction with P-gp, as its lead-compound **6**, whereas percentage of ATP cell depletion determined by lactam **19** did not allow to define unambiguously the type of P-gp modulation. On the other hand, results with the indole based derivative **13** and **18** were surprising: compound **13** improved ATP consumption in contrast with its corresponding amine **5** which was shown to behave as a P-gp inhibitor. Compound **18** (**13**-isomer), bearing an internal amide function, behaved as **5**, with no ATP consumption, as inhibitors generally do. Therefore, keeping the same basic feature of the N-atom, but changing the position of the amide function, led to opposite interaction with the P-gp. Also the acetamide **25** kept the same behavior as the lead compound **5** (with no ATP consumption) emerging as a potent P-gp inhibitor.

4. Conclusion

The results obtained from these compounds show that basicity at the N-atom in the 5,6-dimethoxy-1,2,3,4,tetrahydroisoquinoline is not determinant for the interaction with the P-gp. By contrast, it is determinant for the interaction with σ receptors, so that the strategy was successful for keeping activity at the P-gp, while strongly attenuating σ receptors affinity. More importantly, with this set of ligands we demonstrated that even small structural changes are able to modify the interaction mechanism towards P-gp, with isomeric amides such as **13** and **18**, resulting in opposite



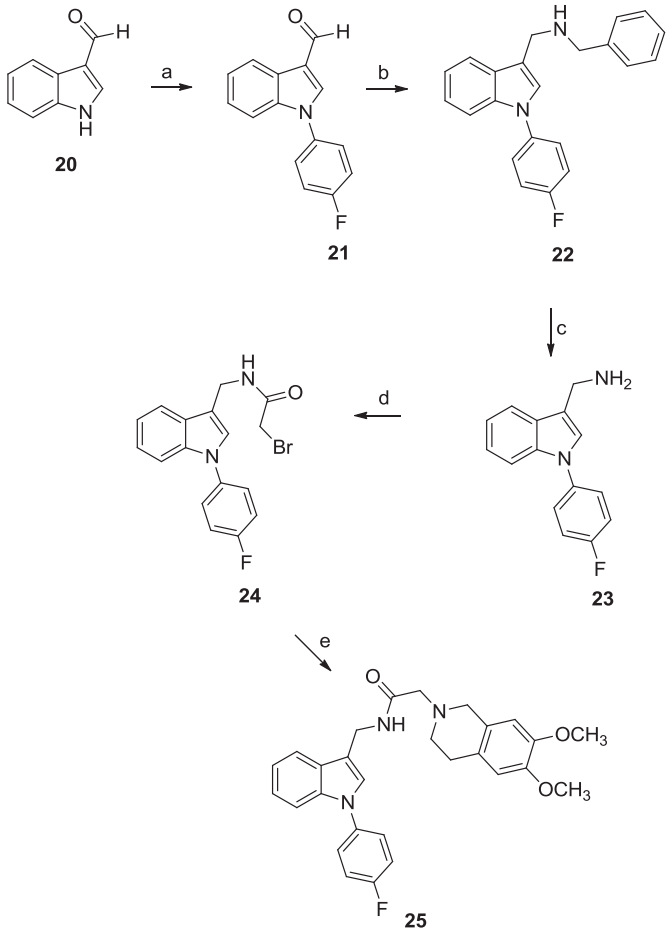
Scheme 1. Synthesis of Final Amides **8**–**11**, **13**, **15**. Reagents and conditions: (a) CDI, one amine among spiro[isobenzofuran-1-(3*H*),4'piperidine], 4-(4-fluorophenyl)piperidine, 4-cyclohexylpiperidine, or 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline, THF, room temperature, overnight; (b) CDI, 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline, THF, room temperature, overnight.



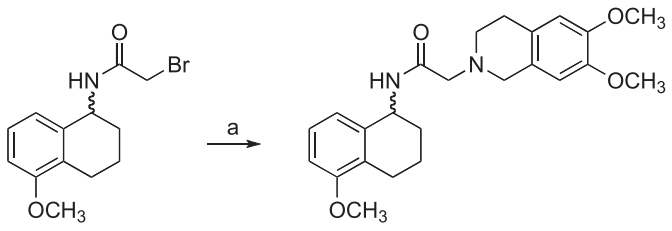
Scheme 2. Synthesis of Tetrahydroisoquinolinones derivatives. Reagents and Conditions: (a) NaH, DMF, 0 °C, 1 h, room temperature, 1 h.

ATP consumption. Also, two potent P-gp modulators, compound **15** and **25**, with different mechanisms of action at P-gp, were identified, and they deserve further investigation either as inducers of CS or as tools to circumvent P-gp activity.

All in all, this study contributed to discover novel P-gp ligands starting from small molecules that can be easily modified to address the mechanisms of interaction with P-gp while increasing potency in P-gp activity.



Scheme 3. Synthesis of Acetamide **25**. Reagents and conditions: (a) 4-Fluorophenylboronic acid, $Cu(AcOEt)_2$, pyridine, CH_2Cl_2, room temperature, overnight; (b) Benzylamine, $NaCNBH_3$, $ZnCl_2$, *i*-propanol, room temperature, 3 days (c) H_2, 10% Pd–C, MeOH, room temperature, 24 h; (d) $ClCOCH_2Br$, $NaHCO_3$, CH_2Cl_2, room temperature, 2 h; (e) 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline, K_2CO_3, CH_3CN, reflux, overnight.



Scheme 4. Synthesis of Racemate **27** and its Enantiomers. Reagents and Conditions: (a) 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline, K_2CO_3, CH_3CN, reflux, overnight.

5. Chemistry: materials and methods

5.1. Chemistry

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). Melting points were determined in open capillaries on a Gallenkamp electrothermal apparatus. Purity of tested compounds was established by combustion analysis, confirming a purity ≥95%. Elemental analyses (C, H, N) were performed on an Eurovector Euro EA 3000 analyzer; the analytical results were within ±0.4% of the theoretical values unless otherwise indicated. ^{1}H NMR (300 MHz) spectra were recorded on a Mercury Varian spectrometer. $CDCl_3$ was used as solvent to record ^{1}H NMR on intermediate and final compounds as free basis. 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. ^{13}C NMR (125 MHz) were recorded on an NMR 500 MHz, Agilent technologies using $CDCl_3$ as solvent on representative final compounds, except for compounds **25** and (±)-**27** whose spectra were recorded in CD_3OD on their hydrochloride salts: chemical shift (δ) in ppm were reported. 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. High performance liquid chromatography (HPLC) was performed on a Perkin–Elmer series 200 LC instrument using a Phenomenex Gemini RP-18 column (250 × 4.6 mm, 5 μm particle size) and equipped with a Perkin-Elmer 785A UV/VIS detector setting $\lambda = 220$ nm. Optical rotations were measured with a Perkin-Elmer 341 polarimeter at room temperature (20 °C); concentrations are expressed as grams/100 mL. Chemicals were from Aldrich and Acros and were used without any further purification.

5.2. General procedure for the synthesis of final compounds ***8*–*11*, *13*, *15***

To a solution of one of the appropriate acids among **7**, **12**, and **14** (0.67 mmol) in dry THF (10 mL), 1,1'-carbonyldiimidazole (0.87 mmol, 0.14 g) was added. The resulting mixture was stirred under argon for 2 h, and then a solution of one of the appropriate amines (0.87 mmol) in THF (10 mL) was added to it in a dropwise manner. Then few drops of DMF were added to the reaction mixture which was left overnight under stirring at room temperature. Water (10 mL) was then added and the mixture was extracted with Et_2O (3 × 10 mL). The collected organic layers were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure to afford the final amide compounds as oils which were purified by column chromatography with CH_2Cl_2/AcOEt (95:5) as eluent.

5.2.1. 1'-[3-(5-Methoxy-1,2,3,4-tetrahydronaphthalen-1-yl) propionyl]spiro[isobenzofuran-1(3H), 4'-piperidine] (8)

Was obtained as a clear yellow oil (50% yield) ^{1}H NMR δ = 0.95–1.15 (m, 4H, $CHCH_2CH_2$), 1.63–2.20 (m, 10H, $COCH_2CH_2$, $C(CH_2)_2$, benzyl CH_2), 2.42–3.00 [m, 5H, benzyl CH and $N(CH_2)_2$], 3.80 (s, 3H, OCH_3), 5.04 (s, 2H, $ArCH_2O$), 6.65–7.30 (m, 7H, aromatic); GC–MS *m*/*z* 405 (M^+, 20), 231 (95), 146 (100); LC-MS (ESI^+) *m*/*z* 428 $[M+Na]^+$. HPLC analyses using $MeOH/H_2O/CH_3CN$, 4:4:2, v/v at a flow rate 1 mL/min indicated the compound was >98% pure.

5.2.2. 4-(4-Fluorophenyl)-1-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)propionyl]piperidine (9)

Was obtained as a yellow oil (45% yield) ^{1}H NMR δ = 1.50–1.60 (m, 4H, $CHCH_2CH_2$), 1.65–2.00 [m, 7H, $CHCH_2$ and piperidine $CH(CH_2)_2$], 2.00–3.15 [m, 7H, benzyl CH and CH_2 and CH_2CO, $N(CHH)_2$], 3.80 (s, 3H, OCH_3), 3.92–4.00 (m, 1H, NC*H*H), 4.75–4.83 (m, 1H, NC*H*H), 6.60–7.20 (m, 7H, aromatic); GC–MS *m*/*z* 395 (M^+, 4), 234 (43), 221 (100). LC-MS (ESI^+) *m*/*z* 418 $[M+Na]^+$. HPLC analyses using $MeOH/H_2O/CH_3CN$, 4:4:2, v/v at a flow rate 1 mL/min indicated the compound was >98% pure.

5.2.3. 4-(4-Cyclohexyl)-1-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)propyl]piperidine (10)

Title compound was obtained as a yellow oil (45% yield) ^{1}H NMR δ = 0.80–1.38 and 1.65–2.00 [m + m, 22H, cyclohexyl CH and CH_2, piperidinyl $CH(CH_2)_2$, and $CH_2CH_2CHCH_2$], 2.30–3.00 [m, 7H, benzyl CH and CH_2 and CH_2CO, $N(CHH)_2$], 3.80 (s, 3H, OCH_3), 3.92–3.95 (m, 1H, NC*H*H), 4.60–4.70 (m, 1H, NC*H*H), 6.62 (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); GC–MS *m/z* 383 (M^+, 9), 222 (32), 209 (100). HPLC analyses using MeOH/H_2O/CH_3CN, 4:4:2, v/v at a flow rate 1 mL/min indicated the compound was >98% pure.

5.2.4. 6,7-Dimethoxy-2-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)propionyl]-1,2,3,4-tetrahydroisoquinoline (11)

Was obtained as white crystals (40% yield) ^{1}H NMR δ = 1.62–2.05 (m, 6H, $ArCH_2CH_2CH_2CHCH_2$), 2.40–2.90 (m, 7H, CH_2CO, $ArCH_2CH_2N$ and benzyl CH and CH_2), 3.62–3.75 (m, 2H, CH_2NCO), 3.84 (s, 6H, 2 OCH_3), 3.86 (s, 3H, OCH_3), 4.45 (s, 1H, ArCH*H*N), 4.65 (s, 1H, ArC*H*HN), 6.55–7.15 (m, 5H, aromatic); ^{13}C NMR: 19.11; 23.09; 26.99; 28.05; 31.23; 31.44; 37.24; 39.73; 43.36; 43.92; 47.14; 55.23; 55.98; 56.03; 106.94; 108.94; 109.51; 111.25; 111.72; 120.92; 125.57; 125,76; 125.96; 141.70; 147.70; 147.69; 147.93; 157.17; 171.92; 172.02. GC–MS *m/z* 410 (M^+ + 1, 7), 409 (M^+, 20), 235 (100). Anal. ($C_{25}H_{31}NO_4$) C, H, N.

5.2.5. 6,7-Dimethoxy-2-[4-[1-(4-fluorophenyl)-1H-indol-3-yl]butanoyl]-1,2,3,4-tetrahydroisoquinoline (13)

Was obtained as a yellow oil (50%) ^{1}H NMR δ = 2.05–2.20 (m, 2H, $NCOCH_2CH_2$), 2.45–2.58 (m, 2H, $NCOCH_2$), 2.68–2.80 (m, 2H, CH_2CH_2N), 2.85–2.95 (m, 2H, indole $ArCH_2$), 3.58–3.82 (s + m, 8H, OCH_3 and $CONCH_2$), 4.41 (s, 1H, ArC*H*HN), 4.65 (s, 1H, ArCH*H*N), 6.58–6.62 (m, 2H, aromatic), 7.16–7.65 (m, 9H, aromatic); LC-MS (ESI^+) *m/z* 495 $[M+Na]^+$; LC-MS-MS 495: 404, 376. HPLC analyses using MeOH/H_2O/CH_3CN, 4:4:2, v/v at a flow rate 1 mL/min indicated the compound was >98% pure.

5.2.6. 9-[1-(6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinolin-2-yl)butan-4-oyl]-9H-carbazole (15)

Was obtained as a white solid recrystallized from $CHCl_3$/n-Hexane (45% yield) ^{1}H NMR δ = 2.15–2.35 (m, 4H, $CH_2CH_2CONCH_2CH_2$), 2.38–2.45 (m, 1H, COCH*H*), 2.75–2.80 (m, 1H, COC*H*H), 3.22–3.35 (m, 1H, CONCH*H*), 3.78–3.85 (m + s, 7H, CONC*H*H and OCH_3), 4.15 (s, 1H, NCH*H*Ar), 4.38–4.45 (m, 2H, carbazole NCH_2), 4.65 (s, 1H, NC*H*HAr), 6.29 (s, 0.5H, aromatic), 6.53 (s, 0.5H, aromatic), 6.61 (s, 0.5H, aromatic), 6.65 (s, 0.5H, aromatic), 7.15–7.45 (m, 6H, carbazole), 8.10–8.15 (m, 2H, carbazole); ^{13}C NMR: 23.62; 23.84; 28.44; 29.22; 29.90; 39.76; 41.71; 41.87; 42.89; 43.91; 46.66; 56.01; 56.13; 108.77; 109.40; 110.70; 111.29; 113.90; 118.81; 120.26; 122.75; 122.80; 124.00; 125.26; 125.50; 125.66; 125.90; 140.50; 147.69; 147.86; 147.95; 170.63; 170.71. GC–MS *m/z*: 428 (M^+, 25), 192 (100); LC-MS (ESI^+) *m/z* 451 $[M+Na]^+$. Anal. ($C_{27}H_{28}N_2O_3 \cdot 1.6H_2O$) C, H, N.

5.3. General procedure for the synthesis of amides 18 and 19

To a suspension of NaH (2.25 mmol, 0.054 g) in dry DMF (2 mL) a solution of 6,7-dimethoxy-3,4-dihydroisoquinolin-1(2*H*)-one (0.75 mmol, 0.15 mg) in dry DMF (3 mL) was added in a dropwise manner. The mixture was stirred at 0 °C for 15 min. Then a solution of mesilate **16** or alkyl chloride **17** (0.89 mmol) in dry DMF (2 mL) was added and the resulting mixture was stirred for 1 h at 0 °C, and for 1 h at room temperature. After cooling at 0 °C, the reaction mixture was quenched with water and the solvent concentrated under reduced pressure. The residue was dissolved in H_2O and extracted with CH_2Cl_2 (3 × 10 mL). The collected organic layers were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure to afford the crude as a yellow oil. Purification through column chromatography with CH_2Cl_2/AcOEt (95:5) as eluent, followed by recrystallization from $CHCl_3$/n-hexane afforded the title compounds as white crystals (50% yield).

5.3.1. 6,7-Dimethoxy-2-[4-[1-(4-fluorophenyl)-1H-indol-3-yl]butanyl]-3,4-dihydroisoquinolin-1(2H)-one (18)

^{1}H NMR δ = 1.70–1.90 (m, 4H, $CH_2CH_2CH_2NCO$), 2.80–2.95 (m, 4H, 2 $ArCH_2$), 3.51 (t, 2H, J = 6.6 Hz, CH_2NCO), 3.58–3.64 (m, 2H, $CONCH_2$), 3.90 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 6.66 (s, 1H, aromatic), 7.20–7.60 (m, 10H, aromatic); ^{13}C NMR: 24.74; 27.38; 27.68; 27.80; 46.31; 47.19; 56.02; 56.06; 109.22; 110.14; 110.59; 116.35 ($J_{2\ C-F}$ = 22.8 Hz); 117.58; 119.34; 119.79; 122.43; 125.21; 125.83 ($J_{3\ C-F}$ = 8.5 Hz); 128.88; 129.52; 131.49; 136.03; 136.30; 147.99; 151.65; 160.78 ($J_{1\ C-F}$ = 246 Hz); 164.36. LC-MS (ESI^+) *m/z* 495 $[M+Na]^+$; LC-MS-MS 495: 266. Anal. ($C_{29}H_{29}F\ N_2O_3 \cdot 1.5H_2O$) C, H, N.

5.3.2. 9-[4-(6,7-Dimethoxy-3,4-dihydroisoquinolin-1(2H)-oxo-2-yl)butyl]-9H-carbazole (19)

^{1}H NMR δ = 1.65–1.75 (m, 2H, $CH_2CH_2CH_2CH_2$), 1.90–2.05 (m, 2H, $CH_2CH_2CH_2CH_2$), 2.79 (t, 2H, J = 6.6 Hz, $ArCH_2$), 3.37 (t, 2H, J = 6.6 Hz, CH_2NCO), 3.56 (t, 2H, J = 6.9 Hz, $CONCH_2$), 3.90 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 4.40 (t, 2H, J = 6.9 Hz, carbazole NCH_2), 6.60 (s, 1H, aromatic), 7.18–8.18 (m, 9H, aromatic); ^{13}C NMR: 25.29; 26.03; 27.67; 42.57; 46.10; 46.56; 56.03; 56.09; 108.76; 109.24; 110.57; 118.79; 120.31; 120.04; 122.85; 125.64; 131.52; 140.41; 148.03; 151.77; 164.57. LC-MS (ESI^+) *m/z* 451 $[M+Na]^+$. Anal. ($C_{27}H_{28}N_2O_3 \cdot H_2O$) C, H, N.

5.4. 1-(4-Fluorophenyl)-1H-indole-3-carboxaldehyde (21)

A solution of indole-3-carboxaldehyde **20** (0.68 mmol, 0.100 g) in CH_2Cl_2 (10 mL) was treated with pyridine (0.16 mL), 4-fluorophenyl-boronic acid (1.36 mmol, 0.19 g) and $Cu(AcOEt)_2$. The resulting mixture was stirred at room temperature overnight. HCl 2N was then added to the reaction mixture which was extracted with CH_2Cl_2 (2 × 10 mL). The collected organic layers were then washed with $NaHCO_3$ (sat solution), dried over Na_2SO_4 and concentrated under reduced pressure to afford the crude as an orange solid. Purification through column chromatography with CH_2Cl_2/AcOEt (99:1) as eluent afforded the title compound as a clear pink solid (yield 20%): mp = 151–152 °C; ^{1}H NMR δ = 7.20–7.68 (m, 7H, aromatic), 7.78 (s, 1H, aromatic), 8.35–8.42 (m, 1H, aromatic), 10.1 (s, 1H, aldehyde); LC-MS (ESI^-) *m/z* 238 $[M-H]^-$.

5.5. N-Benzyl-[1-(4-fluorophenyl)-1H-indol-3-yl]methanamine (22)

To a solution of **21** (0.84 mmol, 0.20 g) in *i*-propanol (10 mL) benzylamine (1.0 mmol, 0.11 mL), $NaCNBH_3$ (1.26 mmol, 0.079 g) and $ZnCl_2$ were added. The resulting mixture was stirred at room temperature for three days, then the solvent was removed under reduced pressure and the residue was dissolved in H_2O and extracted with CH_2Cl_2 (3 × 10 mL). The collected organic layers were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure to afford the crude as a pink oil. This was purified through column chromatography with CH_2Cl_2/MeOH (99:1) as eluent to afford the title compound as a clear yellow oil (yield 70%): GC–MS *m/z*: 330 (M^+, 25), 329 (27), 224 (100); LC-MS (ESI^+) *m/z* 331 $[M+H]^+$.

5.6. [1-(4-Fluorophenyl)-1H-indol-3-yl]methanamine (23)

A solution of benzyl derivative **22** (0.54 mmol, 0.18 g) was dissolved in MeOH (10 mL), under H_2 (4 atm) and in the presence of 10% Pd on activated carbon for 24 h. The mixture was then filtered on a Celite® pad and the filtrate was concentrated under reduced pressure to afford the title compound as a yellow oil (yield 85%): GC–MS *m/z*: 240 (M^+, 80), 224 (100).

5.7. 2-Bromo-N-[(1-(4-fluorophenyl)-1H-indol-3-yl)methyl] acetamide (24)

To a solution of **23** (0.45 mmol, 0.11 g) in dry CH_2Cl_2 (4 mL) kept at 0 °C, $BrCH_2COCl$ (0.58 mmol, 0.05 mL) and $NaHCO_3$ (0.58 mmol, 0.049 g) were added and the resulting mixture was stirred at room temperature for 2 h. After addition of H_2O, the mixture was extracted with CH_2Cl_2 (3 × 5 mL). The collected organic layers were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure to give a blue oil which was purified through column chromatography with CH_2Cl_2 as eluent to afford the title compound as a yellow oil (60% yield): GC–MS *m/z*: 362 (M^++2, 10), 360 (M^+, 10), 281 (100).

5.8. 2-(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-2-yl)-N-[1-(4-fluorophenyl)-1H-indol-3-yl)methyl]acetamide (25)

To a solution of **24** (0.18 mmol, 0.066 g) in CH_3CN (10 mL), 6,7-dimethoxytetrahydroisoquinoline (0.09 mmol, 0.017 g) and K_2CO_3 (0.09 mmol, 0.013 g) were added. The resulting mixture was stirred under reflux overnight. After cooling down, the solvent was evaporated under reduced pressure and the residue was dissolved in H_2O and extracted with CH_2Cl_2 (3 × 5 mL). The collected organic layers were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure to afford a crude residue as a yellow oil which was purified through column chromatography with CH_2Cl_2/MeOH (99:1) to afford the final compound as a white solid (yield 60%): ^{1}H NMR δ = 2.65–2.85 (m, 4H, NCH_2CH_2Ar), 3.25 (s, 2H, NCH_2Ar), 3.60 (s, 2H, $COCH_2N$), 3.78 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 4.70 (d, 2H, J = 5.5 Hz, CH_2NHCO), 6.38 (s, 1H, aromatic), 6.60 (s, 1H, aromatic), 7.05–7.70 (m, 9H, aromatic), 7.45–7.60 (br s, 1H, NH); ^{13}C NMR: 28.21; 38.09; 54.52; 57.09; 59.04; 59.12; 59.53; 113.41; 113.92; 115.35; 117.35; 120.05 ($J_{2\ C-F}$ = 23 Hz); 122.80; 122.90; 124.04; 126.57; 126.69; 129.80 ($J_{3\ C-F}$ = 8.5 Hz); 130.97; 131.67; 139.61; 140.46; 152.52; 153.35; 165.08 ($J_{1\ C-F}$ = 245 Hz); 167.60. LC-MS (ESI$^+$) *m/z* 496 $[M+Na]^+$. Anal. ($C_{28}H_{29}FN_3O_3 \cdot HCl \cdot H_2O$) C, H, N.

5.9. General procedure for the synthesis of amides (±)-27, (+)-(R)-27 and (−)-(S)-27

6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline (0.36 g, 1.58 mmol) and K_2CO_3 (0.27 g, 1.95 mmol) were added with one among the intermediate amides (±)-**26**, or (+)-(*R*)-**26** and (−)-(*S*)-**26** (0.23 g, 0.79 mmol) dissolved in CH_3CN (5 mL). The resulting reaction mixture was stirred under reflux for 24 h. The mixture was then cooled and the solvent removed under reduced pressure. The residue was dissolved in H_2O and extracted with CH_2Cl_2 (3 × 15 mL). The organic phases collected were dried over Na_2SO_4 and concentrated under vacuum to afford a crude that was purified by column chromatography using CH_2Cl_2/MeOH (98:2) as eluent. Final compounds (±)-**27**, (+)-(*R*)-**27** and (−)-(*S*)-**27** were obtained as yellow oils (60% yield).

5.9.1. 2-(6,7-dimethoxytetrahydroisoquinoline)-N-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)acetamide (±)-27

^{1}H NMR δ = 1.60–2.05 (m, 4H, CH_2CH_2), 2.52–2.80 (m, 6H, NCH_2 and benzyl CH_2), 3.20 (s, 2H, NCH_2Ar), 3.80 (s, 2H, $COCH_2N$), 3.78 (s, 3H, OCH_3), 3.80–3.82 (s + s, 6H, OCH_3), 5.15–5.25 (m, 1H, benzyl CH), 6.42 (s, 1H aromatic of tetrahydroisoquinoline), 6.52 (s, 1H aromatic of tetrahydroisoquinoline), 6.65–7.10 (m, 3H aromatic), 7.40–7.500 (m, 1H, NH); ^{13}C NMR: 22.81; 26.35; 28.28; 33.08; 51.81; 54.55; 57.16; 58.36; 59.03; 59.12; 59.59; 112.21; 113.44; 115.34; 122.98; 124.04; 126.72; 130.11; 130.18; 140.55; 152.55; 153.36; 161.16; 167.08. GC–MS *m/z* 410 (M^+, 8), 206 (62), 192 (100). Anal. ($C_{24}H_{30}N_2O_4 \cdot HCl \cdot {}^5/_4H_2O$) C, H, N.

5.9.2. 2-(6,7-dimethoxytetrahydroisoquinoline)-N-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)acetamide (+)-(R)-27

^{1}H NMR and GC–MS data are the same reported for racemic compound. $[\alpha]_D$ = −55.2° (*c* = 0.94, MeOH). Anal. ($C_{24}H_{30}N_2O_4 \cdot HCl \cdot {}^3/_2H_2O$) C, H, N.

5.9.3. 2-(6,7-dimethoxytetrahydroisoquinoline)-N-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)acetamide (−)-(S)-27

^{1}H NMR and GC–MS data are the same reported for racemic compound. $[\alpha]_D$ = +55.8° (*c* = 0.90, MeOH). Anal. ($C_{24}H_{30}N_2O_4 \cdot HCl \cdot {}^5/_4H_2O$) C, H, N.

HPLC analyses on a Daicel Chiralcel OD (*n*-hexane/*i*-propylamine/diethylamine, 9:1:0.1, flow rate 0.5 mL/min, λ = 280 nm) on both the enantiomers displayed >99% ee.

6. Biology: materials and methods

6.1. Materials

[^{3}H]-DTG (50 Ci/mmol), (+)-[^{3}H]-pentazocine (30 Ci/mmol) and ATPlite 1 step Kit were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). DTG was purchased from Tocris Cookson Ltd, UK. (+)-Pentazocine was obtained from Sigma-Aldrich-RBI srl (Milan, Italy). Male Dunkin guinea-pigs and Wistar Hannover rats (250–300 g) were from Harlan, Italy. Cell culture reagents were purchased from EuroClone (Milan, Italy). CulturePlate 96/wells plates were purchased from PerkinElmer Life Science. Calcein-AM, from Sigma–Aldrich (Milan, Italy).

6.2. Competition binding assays

All the procedures for the radioligand binding assays were previously described. σ_1 And σ_2 receptor binding were carried out according to Matsumoto et al. [25] 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 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 constants (K_i) values were determined by nonlinear curve fitting, using the Prism, version 3.0, GraphPad software [26].

6.3. Cell culture

MDCK-MDR1 cells was a gift of Prof. P. Borst, NKI-AVL Institute, Amsterdam, Nederland. MDCK-MDR1 cells were grown in DMEM

high glucose supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, in a humidified incubator at 37 °C with a 5% CO_2 atmosphere.

6.4. Calcein-AM experiment

These experiments were carried out as described by Feng et al. with minor modifications [27]. Calcein-AM is a profluorescent probe and a P-gp substrate, therefore it is not able to permeate cell membrane when P-gp is overexpressed (as in MDCK-MDR1 cell line). Upon P-gp inhibition, Calcein-AM is enabled to enter cells. Therein Calcein-AM is hydrolyzed by intracellular esterases to fluorescent Calcein, which is hydrophilic and therefore does not diffuse through the membrane. Calcein is not a P-gp substrate, and consequently it is not effluxed out of cells. Therefore, the fluorescent signal, due to Calcein accumulation within the cell, is directly correlated to the degree of P-gp inhibition mediated by the tested ligand. MDCK-MDR1 cell line (50,000 cells per well) was seeded into black CulturePlate 96/wells plate with 100 μL medium and allowed to become confluent overnight. 100 μL of different concentrations of test compounds (0.1–100 μM) were solubilized in culture medium and added to each well. The 96/wells plate was incubated at 37 °C for 30 min. 100 μL of Calcein-AM, solved in Phosphate Buffered Saline (PBS), was added to each well to yield a final concentration of 2.5 μM, and the plate was incubated for 30 min. The plate was washed 3 times with 100 μL ice cold PBS. Saline buffer (100 μL) was added to each well and the plate was read by a PerkinElmer Victor3 spectrofluorimeter at excitation and emission wavelengths of 485 nm and 535 nm, respectively. In these experimental conditions, Calcein cell accumulation in the absence and in the presence of tested compounds was evaluated and fluorescence basal level was estimated by untreated cells. In treated wells the increase of fluorescence with respect to basal level was measured. EC_{50} values were determined by fitting the fluorescence increase percentage versus log[dose].

6.5. Bioluminescence ATP assay

The ATP cell depletion was determined in cell line overexpressing P-gp (MDR1-MDCK) and therefore the effect of tested ligands on ATP consumption at different doses was ascribed to their interaction with the studied pump. Generally, a substrate or a transported ligand induces ATP cell depletion at all the studied doses ("Yes" in Tables 1 and 2) whereas an inhibitor or a not transported ligand produce no significant effect (<20%) in this assay at any tested concentration ("No" in Table 2).

This experiment was performed as reported in technical sheet of ATPlite 1 step Kit for luminescence ATP detection based on firefly (*Photinus pyralis*) luciferase (PerkinElmer Life Sciences) [28]. 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 emitted light is proportional to the ATP concentration. The plate was incubated overnight in a humidified atmosphere 5% CO_2 at 37 °C. The medium was removed and 100 μL of complete medium in the presence or absence of different concentrations of test compounds (0.1–100 μm) was added. The plate was incubated for 2 h in a humidified atmosphere 5% CO_2 at 37 °C. Then, 50 μL of mammalian cell lysis solution was added to all wells and the plate stirred for 5 min in an orbital shaker. In all wells, 50 μL of substrate solution was added and the plate stirred for 5 min in an orbital shaker. The plate was dark adapted for 10 min and the luminescence was measured on a Victor 3 microplate reader from PerkinElmer Life Sciences.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.10.082.

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