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Small P-gp modulating molecules: SAR studies on tetrahydroisoquinoline derivatives

Nicola Antonio Colabufo,[a,*] Francesco Berardi,[a] Mariangela Cantore,[a] Maria Grazia Perrone,[a] Marialessandra Contino,[a] Carmela Inglese,[a] Mauro Niso,[a] Roberto Perrone,[a] Amalia Azzariti,[b] Grazia Maria Simone,[b] Letizia Porcelli[b] and Angelo Paradiso[b]

[a]*Dipartimento Farmacochimico, Università degli Studi di Bari, via Orabona, 4, 70125 Bari, Italy*
[b]*National Cancer Institute Giovanni Paolo II, Via Hahnemann, 10, 70126 Bari, Italy*

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Abstract—The development of small molecules as P-gp modulating agents and SAR studies on these ligands represented the aim of the present work. A series of 6,7-dimethoxytetrahydroisoquinoline derivatives was prepared and their ability to inhibit P-gp activity has been evaluated. The basic nucleus of these compounds, common to the best P-gp inhibitors such as Tariquidar and Elacridar, has been functionalized with no-basic moiety from our studied sigma receptor ligands displaying potent P-gp inhibition. The best results were obtained for compounds **3c** and **3a** (EC_{50} = 1.64 and 4.86 μM, respectively) and these results were remarkable because Elacridar showed in the same biological evaluation similar inhibitory activity (EC_{50} = 2 μM). SAR studies displayed that the removal of double bond on the spacer or its shifting into tetraline ring decreased the P-gp inhibiting activity. Moreover, the P-gp inhibition mechanism of tested compounds was investigated by three selected biological experiments. The results displayed that only compound **3c** was P-gp inhibitor as Elacridar, while compound **3a** and reference compounds Cyclosporin A and Verapamil modulated P-gp activity saturating the efflux pump as substrates. Flow cytometry studies carried out in Doxorubicin resistant breast cancer cell line (MCF7/Adr) confirmed that compound **3c** increased Doxorubicin cell accumulation 5.7-fold. In addition, in MCF7/Adr, antiproliferative effect of 5 μM Doxorubicin shifted from 5% to 95% when co-administered with compound **3c** (20 μM). The present study suggested a new class of small molecules displaying P-gp inhibitor activity differing from reference compounds Elacridar and Tariquidar for a simplified, and in the meantime, efficacious no-basic moiety.
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1. Introduction

The ATP-binding cassette (ABC) transporters represent the largest family of transmembrane (TM) proteins.[1,2] These proteins bind ATP and use the hydrolysis energy to extrude various molecules across all cell membranes. In bacteria, these transporters are predominantly involved in the import of essential compounds that cannot be obtained by diffusion (sugars, vitamins, metal ions, etc.) into the cell.[3,4] In eukaryotes, most ABC pumps transport compounds from the cytoplasm to the outside of the cell or into an intracellular compartment such as endoplasmic reticulum (ER), mitochondria, and peroxisome.[4] In humans, 49 ABC genes that are organized into seven subfamilies (A–G) have been described.[5] Many ABC genes are involved in human genetic diseases[6–9] and other ABC genes display an important role in multidrug resistance (MDR).[10–13] The most supported mechanism of MDR is the overexpression of ABC transporters localized in the cell membrane, which determines this pharmacological effect by effluxing a variety of chemotherapeutic drugs from tumor cells.[14,15] The mostly involved ABC transporter in MDR is *ABCB1* (*ABCB* subfamily) well known as P-glycoprotein (P-gp).[16] P-gp is localized in several biological compartments such as on the apical membrane of hepatocytes, on the mucosal cells in the gastrointestinal tract, on the apical lumen of gastrointestinal enterocytes, on the canalicular membrane of hepatocytes, the proximal tubular cells of the kidney, and the luminal membrane of brain capillary endothelial cells.[17–20] P-gp both modulates the efflux of many structurally different drugs and

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* Corresponding author. Tel.: +39 080 5442727; fax: +39 080 5442231; e-mail: colabufo@farmchim.uniba.it

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controls their intestinal absorption.[21] Other ABC transporters related to MDR are ABCC1-7 proteins (*ABCC* subfamily) well known as MRP1-7 (multidrug resistance protein) and ABCG2 (*ABCG* subfamily) known as BCRP (Breast Cancer Resistance Protein).[16,22] During the treatment of human cancer with chemotherapeutic agents, MDR is frequently observed and P-gp has been the first ABC transporter investigated for resistance induction.[23,24] Traditional P-gp substrates include, e.g., cytotoxic agents, anthracyclines (Doxorubicin, Daunorubicin), vinca alkaloids (Vinblastine and Vincristine), taxanes (Paclitaxel, Docetaxel), colchicines, epipodophyllotoxins (Etoposide, Teniposide), and also some novel antitumor drugs, such as Imatinib, ET-743, and Gentuzumab are effluxed out of tumor cells by P-gp activity.[25–28]

Many drugs have been investigated in the last years for modulating the P-gp transport and among them a calcium channel blocker such as Verapamil and the antisteroid Tamoxifen (Fig. 1).[29–31] These compound, were not specifically developed to block P-gp since many of them were substrates for other transporters and enzyme system, resulting in unpredictable pharmacokinetic interaction.[24,31] Furthermore, most of these inhibitors presented low affinity for P-gp and were administered in high doses which displayed unacceptably high level of toxicity that, in turn, caused severe side effects.[32]

The second generation of P-gp modulators has been developed and the mostly studied compounds are Biricodar, Valspodar (PSC-833)[32–34] (Fig. 1), dexverapamil, dexniguldipine and indolizilsulfone derivative (SR33557), a triazinoaminopiperidine derivative (S9788). These compounds are more potent and less toxic than first generation compounds but some limitations have been evidenced.[35] For example, Biricodar and PSC-833 inhibited cytochrome P450A4-mediated metabolism of paclitaxel, while vinblastine has made it difficult to establish a safe but efficient dose of coadministered chemotherapy agents, which has led to the restricted use of this second generation of modulators in the treatment of MDR cancers.[36,37]

The third generation of P-gp modulators includes Zosuquidar[38,39] (Fig. 1) and other compounds bearing dimethoxytetrahydroisoquinoline moiety such as Elacridar (Phase I)[40,41] and Tariquidar (Phase III).[42–45] These inhibitors have displayed no pharmacokinetic interaction with chemotherapeutic drugs and showed high potency and specificity for P-gp. Nevertheless, the results of clinical trials on Tariquidar showed that it displayed limited clinical activity to restore sensitivity to anthracycline or taxane chemotherapy.[44] Moreover, in these complex molecules, structure–activity relationship (SAR) studies are not so easy to carry out and to date the contribution of each portion to reverse MDR is not clarified.[46]

Obviously, these studies can be easily performed starting from small molecules. Indeed, in our previous work, we carried out SAR studies on a new class of arylmethyloxyphenyl derivatives (Fig. 2, formula **I**) as small molecules displaying P-gp inhibition activity.[47]

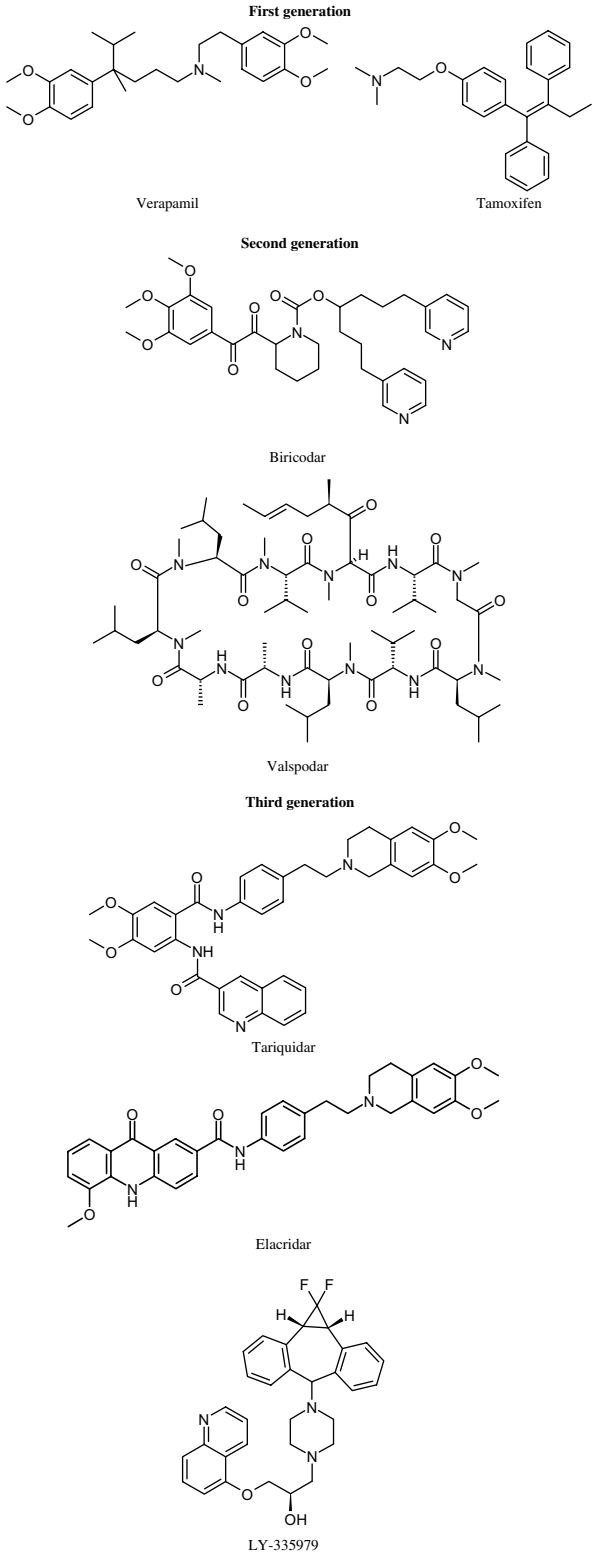


Figure 1. P-gp modulators.

The development of small molecules as P-gp modulating agents and SAR studies on these ligands similarly represented our target of the present work. Recently, we studied PB28 (Fig. 2), a cyclohexylpiperazine derivative displaying a potent P-gp modulating activity in MCF7/Adr cells.[48,49] In order to improve its P-gp modulating activity, we replaced cyclohexylpiperazine with a

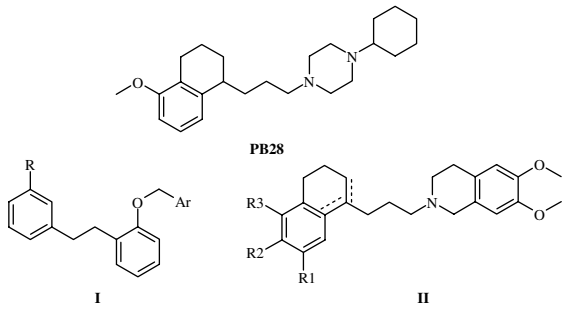
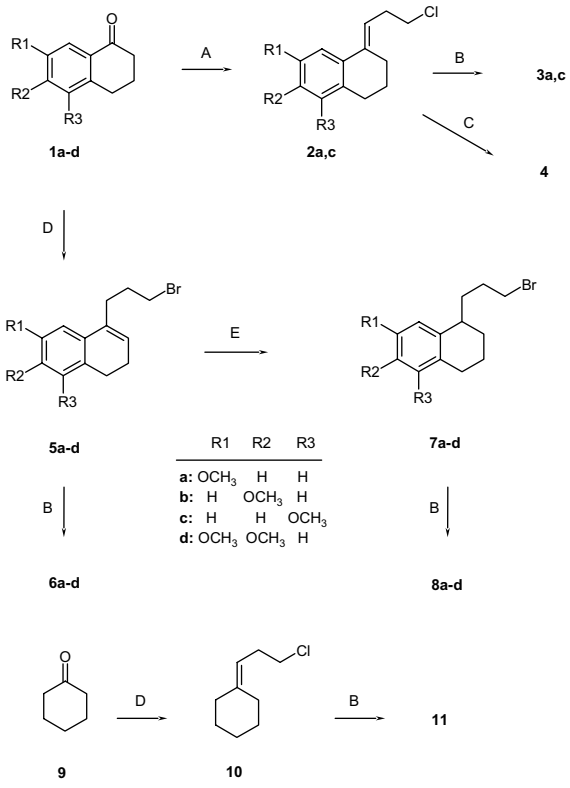


Figure 2. PB28 and general formula of synthesized compounds.

dimethoxytetrahydroisoquinoline moiety (Fig. 2, formula **II**) such as in Tariquidar and Elacridar where it is functionalized with a bulking and planar substituent.

The set of compounds presented in this work was characterized by 6,7-dimethoxytetrahydroisoquinoline nucleus bearing as substituent the same no-basic portion of PB28.

Since this portion can be easily modified, we investigated the conformational restriction by inserting a double bond in the spacer and into tetraline ring. Aims of the present work were to investigate the effect of the replacement of basic nucleus of PB28 with 6,7-dimethoxytetrahydroisoquinoline on MDR activity and also the influence of the modifications on the no-basic portion of these compounds.



Scheme 1. Reagents: (A) cyclopropylMgBr, 3 N HCl; (B) 6,7-dimethoxytetrahydroisoquinoline; (C) piperidine; (D) cyclopropylMgBr, 48% HBr; (E) H_2/Pd (5%).

2. Results and discussion

2.1. Chemistry

The preparation of the final compounds **3a**, **3c**, **4**, **6a–d**, **8a–d**, and **11** is depicted in Scheme 1. The key intermediates *E*-**2a**, *E*-**2c**, **5a–d**, and **10** were prepared by Grignard's reaction starting from the appropriate ketone (**1a–d** for intermediates *E*-**2a**, *E*-**2c**, and **5a–d**, while **9** for intermediate **10**). Key intermediates **2a**, **2c**, and **10** have been obtained by kinetic control that failed starting from tetralones **1b**, **1d**. The preparation of intermediate **5a–c**, *E*-**2c** has been previously reported.[50–52]

The intermediates **7a–d** have been obtained by catalytically reducing double bond of corresponding unsaturated derivatives **5a–d** under hydrogen atmosphere.[50,52]

Final compounds **3a**, **3c**, **6a–d**, **8a–d**, and **11** were obtained by alkylating commercially available amine 6,7-dimethoxytetrahydroisoquinoline. Final compound **4** was prepared by reaction between intermediate *E*-**2c** and piperidine.

2.2. Structure–activity relationships studies

For all compounds the ability to inhibit [^{3}H]vinblastine transport in Caco-2 cell monolayer has been determined. With respect to PB28 displaying EC_{50} = 0.55 μM, all tested compounds **3a, 3c**, **4**, **6a–d**, **8a–d**, and **11** were less potent. The best results were found, as listed in Table 2, for compounds **3c** and **3a** bearing a (*E*)-double bond on the spacer (EC_{50} = 1.64 and 4.86 μM, respectively). The corresponding saturated derivatives **8c** and **8a** displayed EC_{50} = 6.35 and 10.2 μM, respectively. Compounds **6c** and **6a** having a double bond in tetraline ring showed with respect to compounds **3c** and **3a** high decrease of P-gp inhibition activity (EC_{50} = 15 and 35.5 μM, respectively).

The position of methoxy substituent influenced the activity. In fact, the best results were obtained for compounds **3c**, **8c**, and **6c** for each studied series where $R_1 = R_2 = H$ and $R_3 = OCH_3$. The shifting of methoxy in other positions of tetraline ring decreased the activity in all series (**3c** vs **3a**, **6c** vs **6a**, and **6b**, **8c** vs **8a** and **8b**). The dimethoxy derivatives **6d** and **8d** displayed moderate P-gp inhibition activity (EC_{50} = 31.6 and 17.9 μM, respectively). These findings led to consider compound **3c** as lead compound and starting from it we prepared compound **4** where the aromatic portion on the basic tetrahydroisoquinoline moiety has been removed. The results displayed that this modification moderately decreased P-gp inhibition activity with respect to compound **3c** (EC_{50} = 8.2 vs 1.64 μM). Moreover, from **3c**, compound **11** has been prepared where the aromatic portion of the tetraline ring was removed. The results displayed that this modification decreased the inhibition activity ten fold with respect to lead compound **3c** (EC_{50} = 15.2 vs 1.64 μM).

Nevertheless lead compound **3c** was found to be two fold less potent than PB28, it displayed higher P-gp inhi-

bition activity than reference compounds Cyclosporin A and Verapamil (EC_{50} = 80 and 20 μM, respectively). In addition, compound **3c** showed the same P-gp inhibition value as that of Elacridar (EC_{50} = 2.0 μM) one of the most studied P-gp inhibitors.

2.3. Pharmacology

The target compounds **3a**, **3c**, **4**, **6a–d**, **8a–d**, and **11** as hydrochloride salts were evaluated for determining the Apparent Permeability (P_{app}) both in basolateral–apical (B–A) and apical–basolateral (A–B) transports.[47,53] Monolayer Caco-2 cells grown on permeable filters express human P-gp and are commonly employed to characterize P-gp substrate. In this assay, substrates generally displayed BA/AB ratio > 2 while nonsubstrate or inhibitors displayed BA/AB < 2.[54] Moreover, the ATPase activation monitored by ATP cell depletion due to each compound has been evaluated. In this assay, only substrates caused ATP cell depletion. The cell ATP was unchanged for treating in the presence of inhibitor and nonsubstrate.[55] Furthermore, specific radiolabeled P-gp substrate [^{3}H]vinblastine has been employed and in this assay both substrate and inhibitor displayed interacting effect with the radioligand transport, while nonsubstrates are unable to compete with radiolabeled substrate.[56,57] By comparing the results of these three assays, two categories of P-gp ligands are elicited (Table 1). In category I, unambiguous nonsubstrates, unambiguous substrates, and inhibitors are listed. A compound is defined as nonsubstrate when it is non transported, ATPase is not activated by it, and it is unable to modulate [^{3}H]vinblastine transport. A substrate is transported, it activates ATPase, and displays competition toward [^{3}H]vinblastine transport. Finally, an inhibitor differs from a nonsubstrate because it competes with radioligand substrate transport. This experimental subclassification is an efficacy characterization for an inhibitor and a nonsubstrate but it is limitative for substrate identification. Actually, the substrate characterization presents several deviations by typical substrate definition as reported in category I. In fact, a substrate could be a non transported substrate (category IIA) such as Verapamil, Ketoconazole, and Nifedipine, if it is non transported. Moreover, it could be classified as a substrate of category IIB_1, such as Daunorubicin, if it is unable both to activate ATPase and to compete with [^{3}H]vinblastine. In addition, a substrate could be transported, it could activate ATPase but it could be unable to compete with [^{3}H]vinblastine (category IIB_2) such as Taxol. Cyclosporin A is a substrate example of category IIB_3, where the substrate is transported and competes with [^{3}H]vinblastine but it is unable to activate ATPase.

2.4. P-gp inhibition mechanism investigation

In monolayer efflux assay the Apparent Permeability (P_{app}) both in basolateral–apical (P_{app}, B–A) and apical–basolateral (P_{app}, A–B) directions was determined for each compound. The BA/AB ratio is <2 for compounds **3c** and **6d** and reference compound Verapamil (BA/AB = 1.6, 1.5, and 1.2, respectively) while the other compounds displayed BA/AB > 3.1.

As regards ATPase activation, several compounds were unable to deplete ATP (**3a**, **3c**, **4**, **6b**, **11**). All other compounds activated ATPase pump at 100 μM with different percentage as displayed in Table 2. Moreover, all compounds inhibited [^{3}H]vinblastine transport as reported in Section 2.2. Combining the results of these three biological assays, only compound **3c** and reference compound Elacridar are "claimed" P-gp inhibitors. On the contrary, tetraline derivatives **8a–d** were unambiguous substrates. All the other compounds were substrates but differently characterized: **3a**, **4**, **6b**, **11** were transported substrates Cyclosporin A–like, while **6d** was a nontransported substrate as Verapamil.

As data standing, we better characterized, by flow cytometry, the compounds **3c**, **3a** and **8c** and reference compounds Cyclosporin A and Verapamil displaying the best P-gp modulating activity although with a different mechanism.

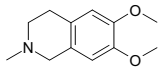
As depicted in Figure 3a–e, in MCF7/Adr cells, overexpressing P-gp, the simultaneous treatment with Doxorubicin and each compound increased Doxorubicin accumulation, as shown by 20 μM compound **3c** (Fig. 3b) which increased Doxorubicin accumulation 5.7-fold, in the cells (Table 3). In the same flow cytometry experiment, compounds **3a** (Fig. 3a) and **8c** (Fig. 3c) displayed an increased Doxorubicin cell accumulation of 4.1- and 4.8-fold, respectively (Table 3).

Table 1. Evaluation of drug interaction with P-glycoprotein by three specific biological assays[54]

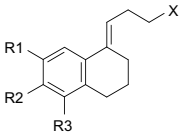
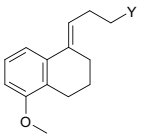
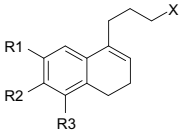
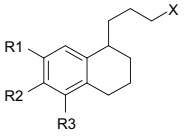
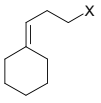
Categories of P-gp ligands		Methods of measurements in Caco-2 cell line		
		Monolayer efflux	ATPase activation	[^{3}H]-Substrate transport inhibition
Category I				
	Unambiguous nonsubstrates	No	No	No
	Unambiguous substrates	Yes	Yes	Yes
	Inhibitors	No	No	Yes
Category II				
IIA	Nontransported substrates	No	Yes	Yes
IIB_1	Transported substrates	Yes	No	No
IIB_2	Transported substrates	Yes	Yes	No
IIB_3	Transported substrates	Yes	No	Yes

Table 2. Biological in vitro assays

	R1	R2	R3
a:	OCH_3	H	H
b:	H	OCH_3	H
c:	H	H	OCH_3
d:	OCH_3	OCH_3	H

X: 

Y: 

Compound		P-gp inhibition	ATPase activation	P_{app}[c] (B–A) nm/s	P_{app}[c] (A–B) nm/s	P_{app}[c] (BA/AB)
		EC_{50} ± SEM (μM)[a]				
3a		4.86 ± 1.10	No	6567	1663	3.9
3c		1.64 ± 0.20	No	386	237	1.6
4		8.2 ± 0.5	No	2564	584	4.4
6a		35.5 ± 2.5	Yes (20)[b]	3316	610	5.4
6b		83.7 ± 5.2	No	1506	407	3.7
6c		15 ± 2.5	Yes (30)	1140	160	7.1
6d		31.6 ± 2.7	Yes (15)	1030	672	1.5
8a		10.2 ± 0.80	Yes (15)	1703	460	3.7
8b		8.15 ± 0.30	Yes (27)	3510	828	4.2
8c		6.35 ± 0.25	Yes (70)	1038	56	18
8d		17.9 ± 1.5	Yes (62)	4399	868	5.1
11		15.2 ± 1.5	No	2403	774	3.1
PB28		0.55 ± 0.02	No	4358	2496	1.7
Cyclosporin A		80 ± 7.5	No	153[d]	15.9[d]	9.6[d]
Verapamil		20 ± 1.0	Yes (52)	687[d]	591[d]	1.2[d]
Elacridar		2.0	No[d]			<2[d]

[a] Data ± SEM are the mean of three independent determinations (samples in triplicate).
[b] Percentage activation at 100 μM shown given in parentheses. Data are the mean of three independent determinations with samples in triplicate.
[c] Data are the mean of three independent determinations (samples in triplicate) each with SEM <10%.
[d] See Ref. 54.

At 20 μM the reference compounds Cyclosporin A (Fig. 3d) and Verapamil (Fig. 3e) displayed an increase of Doxorubicin cell accumulation of 5- and 3.3-fold, respectively.

In graphs (Fig. 3a–e) this accumulation can be appreciated by the shift to right of Doxorubicin curve. The following step was to verify if Doxorubicin cell accumulation, induced by our compounds, improved the antiproliferative effect of the chemotherapeutic. In fact, at 5 μM, Doxorubicin itself displayed a low antiproliferative effect (5%) as listed in Table 3.

The results of Doxorubicin antiproliferative effect as drugs combination with studied compounds are shown in Table 3. The results are well correlate with the increased Doxorubicin accumulation; i.e. 20 μM compound **3c** together with Doxorubicin inhibited cell growth of about 95% . Moreover, high antiproliferative effect was induced by Doxorubicin in the presence of compounds **3a** and **8c** and Cyclosporin A (77%, 85% and 83%, respectively). In the presence of Verapamil, Doxorubicin displayed moderate increase of antiproliferative effect (50%).

Moreover, compounds **3a**, **3c**, and **8c** were studied in the absence of Doxorubicin for the cytotoxicity and antiproliferative effects at 24 and 48 h, respectively. The cytotoxicity of tested compounds at 100 μM, determined by LDH measurement in cell medium after treatment,[48] was negligible (< 20% for each studied compound). Analogously, in the antiproliferative assay[48] at 48 h

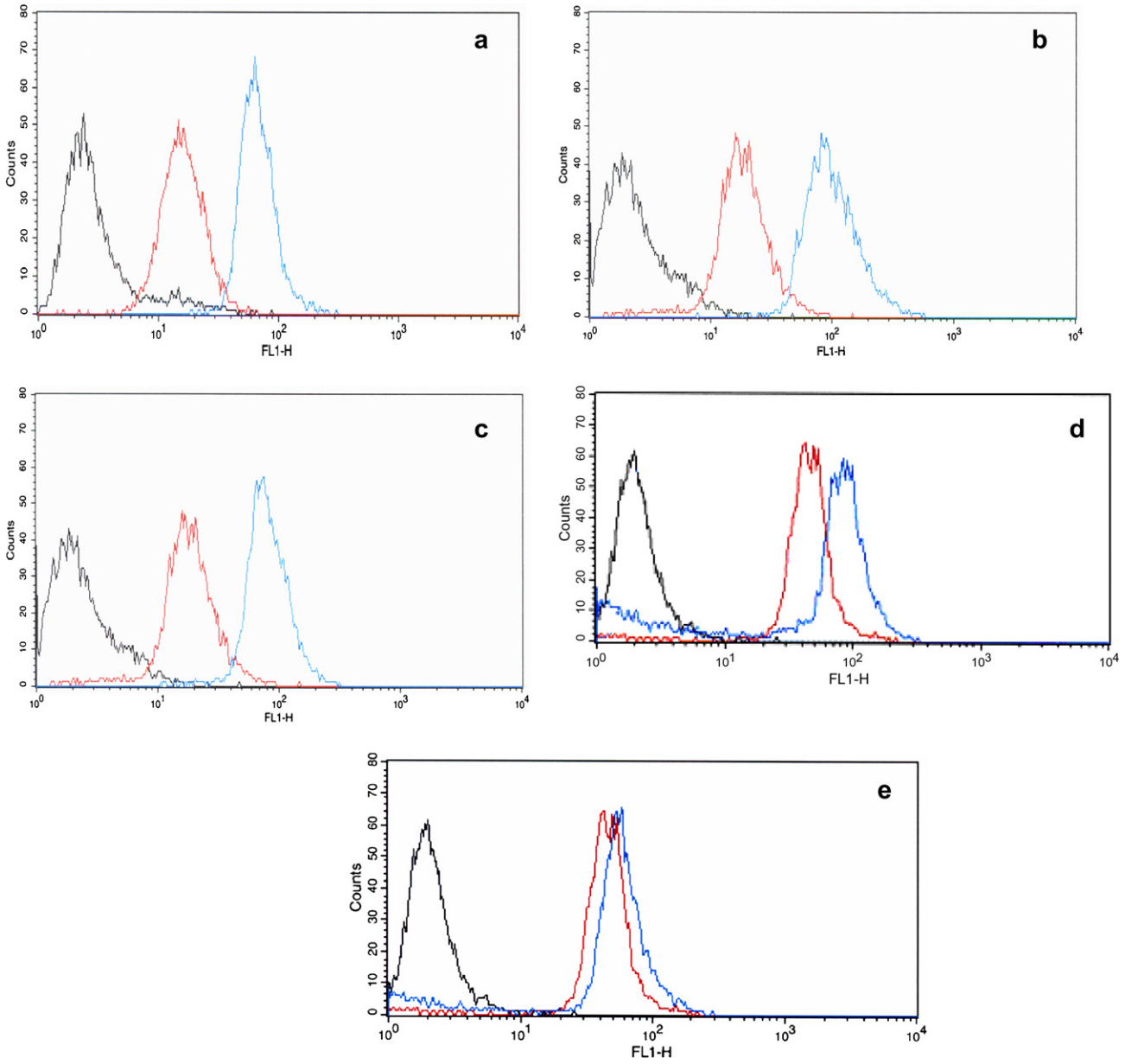


Figure 3. Flow cytometry analysis with MCF7/Adr cells. Autofluorescence (black), 50 μM Doxorubicin (red), 20 μM of tested compounds in the presence of 50 μM Doxorubicin (blue). (a) Compound **3a**. (b) Compound **3c**. (c) Compound **8c**. (d) Cyclosporin A. (e) Verapamil.

Table 3. Capability of the newly synthesized P-gp modulating agents to increase Doxorubicin intracellular accumulation in MCF7/Adr cell line (left column)

Compound	-Folds of increased 50 μM Doxorubicin intracellular accumulation[a]	Antiproliferative effect[b] (%) (5 μM Doxorubicin)
Doxorubicin	1	5 ± 1.0
Inhibitor		
20 μM compound **3a** + Doxorubicin	4.1 ± 1.2	77 ± 2.3
20 μM compound **3 c** + Doxorubicin	5.7 ± 1.0	95 ± 1.8
20 μM compound **8c** + Doxorubicin	4.8 ± 0.5	85 ± 2.1
20 μM Cyclosporin A + Doxorubicin	5.0 ± 0.7	83 ± 2.0
20 μM Verapamil + Doxorubicin	3.3 ± 0.8	50 ± 2.5

Doxorubicin antiproliferative effect of drugs combination (right column).
[a] Data ± SEM are the mean of three independent determinations samples in triplicate.
[b] Data are the mean of three independent determinations samples in triplicate.

the tested compounds, at 100 μM, displayed low effect. Only compound **3c** showed at this concentration 25% of antiproliferative effect while compounds **3a** and **8c** displayed an effect < 20%.

These findings demonstrated that the drugs combination, Doxorubicin with newly synthesized P-gp modulating agents, improved Doxorubicin antiproliferative effect in MCF7/Adr tumor cells.

3. Conclusions

In this paper, we identified among several newly synthesized compounds derivative **3c** as a P-gp modulating agent. It inhibited the drug efflux pump and strongly enhanced anthracyclines' effectiveness. This finding encouraged us to patent this pharmaceutical class as new small molecules with P-gp modulating activity.[58]

Another aspect of the evaluation of P-gp modulating agents is the mechanism involved in the pump efflux inhibition and it has been investigated combining three biological assays. The mechanism of P-gp inhibition is a critical pharmacokinetic step. In fact at high concentrations both substrate and inhibitor efficaciously blocked pump efflux but at low concentrations only inhibitor displayed this effect.[59] In the future we will perform the full biological characterization of these compounds, determining their selectivity toward other ABC transporters involved in MDR and identifying other hypothetic targets with which they could interfere, by expression microarray technologies. Moreover, on the basis of compound **3c**, other semirigid congeners might be prepared in order to investigate the requirements for obtaining compounds acting as P-gp inhibitors.

4. Experimental

4.1. Chemistry

Column chromatography was performed with 1:30 Merck silica gel 60Å (63–200 μm) as the stationary phase. Melting points were determined in open capillaries on a Gallenkamp electrothermal apparatus. Elemental analyses (C, H, N) were performed on Eurovector Euro EA 3000 analyzer; the analytical results were within ±0.4% of the theoretical values for the formula given. ^{1}H NMR spectra were recorded in $CDCl_3$ at 300 MHz on a Varian Mercury-VX spectrometer. All spectra were recorded on the free bases. All chemical shift values are reported in ppm (δ). Recording of mass spectra was done on an HP6890-5973 MSD gas chromatograph/mass spectrometer; only significant *m*/*z* peaks, with their percentage of relative intensity in parentheses, are reported. All spectra were in accordance with the assigned structures. ESI-MS analyses were performed on an Agilent 1100 LC/MSD trap system VL. The UV/vis spectra were recorded with LAMBDA BIO20 spectrophotometer PerkinElmer. The spectroscopic characterization of compounds ***E*-2c**, **5a**, **5c**, **7a–c** has been reported in our previous works.[50–52]

4.2. General procedure for the synthesis of amines 3a, 3c, 4, 6a–d, 8a–d, and 11

A mixture of haloalkylderivatives *E*-**2a**, **2c** or **5a–d** or **7a–d** or **10** (0.50 mmol) and amine, 6,7-dimethoxytetrahydroisoquinoline or piperidine (1.2 mmol), and anhydrous Na_2CO_3 (0.50 mmol) in 20 mL DMF was stirred overnight. The solvent was evaporated and the residue was partitioned between H_2O (20 mL) and $CHCl_3$ (30 mL). The organic layer was dried (Na_2SO_4) and concentrated in vacuo. The crude residue was chromatographed as following reported for each final compound.

4.3. 6,7-Dimethoxy-2-{3-[7-methoxy-3,4-dihydro-2*H*-naphthalen-(1*E*)-ylidene]-propyl}-1,2,3,4-tetrahydro-isoquinoline (3a)

Yellow oil, 90% yield from column chromatography (eluent $CHCl_3$/MeOH 19:1). GC–MS *m*/*z* 394 (M^+ + 1, 4), 393 (M^+ 11), 206 (100), 165 (6). ^{1}H NMR δ 1.78–1.85 (m, 2H, $CH_2CH_2CH_2$), 2.50–2.89 (mm, 12H, $CH_2CH_2CH_2$, NCH_2CH_2 tetrahydroisoquinoline $CHCH_2CH_2N$), 3.49–3.88 (mm, 11H, NCH_2 tetrahydroisoquinoline, 9H, CH_3), 6.00 (t, 1H, J = 7 Hz, C=CH), 6.50–7.09 (m, 5H aromatic). UV–vis: λ_{max} = 260 nm; ε = 2235 M^{-1} cm^{-1} (PBS). Anal. ($C_{25}H_{31}NO_3$·HCl) C, H, N (hydrochloride salt melted at 232–235 °C).

4.4. 6,7-Dimethoxy-2-{3-[4-methoxy-3,4-dihydro-2*H*-naphthalen-(1*E*)-ylidene]-propyl}-1,2,3,4-tetrahydro-isoquinoline (3c)

Yellow oil, 58% yield from column chromatography (eluent $CHCl_3$/MeOH 19:1). GC–MS *m*/*z* 395 (M^+ + 2, 1), 394 (M^+ + 1, 3), 393 (M^+ 9), 207 (16), 206 (100). ^{1}H NMR δ 1.79–1.87 (m, 2H, $CH_2CH_2CH_2CH$), 2.47–2.93 (mm, 12H, $CH_2CH_2CH_2CH$, NCH_2CH_2 tetrahydroisoquinoline, $CHCH_2CH_2N$), 3.63 (s, 2H, NCH_2 tetrahydroisoquinoline), 3.82–3.87 (3s, 9H, CH_3), 6.03 (t, 1H, J = 7 Hz, C=CH), 6.60–7.26 (m, 5H, aromatic). UV–vis: λ_{max} = 260 nm; ε = 8300 M^{-1} cm^{-1} (PBS). Anal. ($C_{25}H_{31}NO_3$·HCl) C, H, N (hydrochloride salt melted at 237–240 °C).

4.5. 1-{3-[4-Methoxy-3,4-dihydro-2*H*-naphthalen-(1*E*)-ylidene]-propyl}-piperidine (4)

White solid, 88% yield from column chromatography (eluent CH_2Cl_2/MeOH 19:1). GC–MS *m*/*z* 286 (M^+ + 1, 1), 285 (M^+ 1), 98 (100). ^{1}H NMR δ 1.25–1.85 (m, 10H, 3 of CH_2 piperidine, $CH_2CH_2CH_2C$ tetraline), 2.43–2.47 (m, 8H, 2 of CH_2 piperidine, $CHCH_2CH_2N$, $CH_2CH_2CH_2C$ tetraline), 2.70 (t, 2H, J = 7.0 $CH_2CH_2CH_2C$ tetraline), 3.81 (s, 3H, CH_3), 6.67–7.19 (m, 3H, aromatic). UV–vis: λ_{max} = 258 nm; ε = 12440 M^{-1} cm^{-1} (PBS). Anal. ($C_{19}H_{27}NO$·HCl) C, H, N (hydrochloride salt melted at 203–205 °C).

4.6. 6,7-Dimethoxy-2-[3-(7-methoxy-3,4-dihydro-naphthalen-1-yl)-propyl]-1,2,3,4-tetrahydro-isoquinoline (6a)

Yellow oil, 53% yield from column chromatography (eluent CH_2Cl_2/EtAc 7:3). ESI^+/MS *m/z* 394 (MH^+). GC–MS *m/z* 395 ($M^+ + 2$, 4), 394 ($M^+ + 1$, 22), 393 (M^+ 79), 206 (100), 191 (60), 164 (32). ^1H NMR δ 1.82–1.90 (m, 2H, $CH_2CH_2CH_2N$), 2.19–2.25 (m, 2H, $CH_2CH_2CH{=}C$), 2.46–2.85 (m, 10H, $CH_2CH_2CH{=}C$, $CH_2CH_2CH_2N$, NCH_2CH_2 tetrahydroisoquinoline), 3.57 (s, 2H, NCH_2 tetrahydroisoquinoline), 3.82–3.90 (s, 9H, CH_3), 5.89 (t, 1H, C=CH, *J* = 5 Hz), 6.51–7.06 (m, 5H, aromatic). Anal. ($C_{25}H_{31}NO_3$·HCl) C, H, N (hydrochloride salt melted at 212–216 °C).

4.7. 6,7-Dimethoxy-2-[3-(6-methoxy-3,4-dihydro-naphthalen-1-yl)-propyl]-1,2,3,4-tetrahydro-isoquinoline (6b)

Yellow oil, 55% yield from column chromatography (eluent CH_2Cl_2/EtAc 7:3). GC–MS *m/z* 395 ($M^+ + 2$, 3), 394 ($M^+ + 1$, 13), 393 (M^+ 47), 218 (62), 206 (100), 191 (47). ^1H NMR δ 1.83–1.88 (m, 2H, $CH_2CH_2CH_2N$), 2.19–2.26 (m, 2H, $CH_2CH_2CH{=}C$), 2.50–2.82 (m, 10H, $CH_2CH_2CH{=}C$, $CH_2CH_2CH_2N$, NCH_2CH_2 tetrahydroisoquinoline), 3.56 (s, 2H, NCH_2 tetrahydroisoquinoline), 3.79–3.83 (s, 9H, CH_3), 5.75 (t, 1H, C=CH, *J* = 6.2 Hz), 6.50–7.21 (m, 5H, aromatic); UV–vis: λ_{max} = 280 nm; ε = 10119 $M^{-1}\,cm^{-1}$ (PBS). Anal. ($C_{25}H_{31}NO_3$·HCl) C, H, N (hydrochloride salt melted at 205–208 °C).

4.8. 6,7-Dimethoxy-2-[3-(4-methoxy-3,4-dihydro-naphthalen-1-yl)-propyl]-1,2,3,4-tetrahydro-isoquinoline (6c)

Yellow oil, 90% yield from column chromatography (eluent CH_2Cl_2/MeOH 19:1). ESI^+/MS *m/z* 392 (MH^+). GC–MS *m/z* 394 ($M^+ + 1$, 20), 393 (M^+ 70), 392 (53), 218 (86), 219 (42), 206 (100). ^1H NMR δ 1.80–1.85 (m, 2H, $CH_2CH_2CH_2N$), 2.20–2.24 (m, 2H, $CH_2CH_2CH{=}C$), 2.47–2.84 (m, 10H, $CH_2CH_2CH{=}C$, $CH_2CH_2CH_2N$, NCH_2CH_2 tetrahydroisoquinoline), 3.58 (s, 2H, NCH_2 tetrahydroisoquinoline), 3.82 (s, 9H, CH_3), 5.89 (t, 1H, C=CH, *J* = 4 Hz), 6.50–7.26 (m, 5H, aromatic). Anal. ($C_{25}H_{31}NO_3$·HCl) C, H, N (hydrochloride salt melted at 227–229 °C).

4.9. 2-[3-(6,7-Dimethoxy-3,4-dihydro-naphthalen-1-yl)-propyl]-6,7-dimethoxy-1,2,3,4-tetrahydro-isoquinoline (6d)

Yellow oil, 83% yield from column chromatography (eluent CH_2Cl_2/MeOH 19:1). GC–MS *m/z* 425 ($M^+ + 2$, 4), 424 ($M^+ + 1$, 14), 423 (M^+ 39), 218 (69), 206 (100), 191 (44), 164 (19). ^1H NMR δ 1.84–1.94 (m, 2H, $CH_2CH_2CH_2N$), 2.18–2.26 (m, 2H, $CH_2CH_2CH{=}C$), 2.46–2.84 (m, 10H, $CH_2CH_2CH{=}C$, $CH_2CH_2CH_2N$, NCH_2CH_2 tetrahydroisoquinoline), 3.60 (s, 2H, NCH_2 tetrahydroisoquinoline), 3.82–3.88 (s, 12H, CH_3), 5.78 (t, 1H, C=CH, *J* = 4 Hz), 6.50-6.83 (m, 4H, aromatic); UV–vis: λ_{max} = 280 nm; ε = 9435 $M^{-1}\,cm^{-1}$ (PBS). Anal. ($C_{26}H_{33}NO_4$·HCl) C, H, N (hydrochloride salt melted at 219–224 °C).

4.10. 6,7-Dimethoxy-2-[3-(7-methoxy-1,2,3,4-tetrahydro-naphthalen-1-yl)-propyl]-1,2,3,4-tetrahydro-isoquinoline (8a)

Yellow oil, 63% yield from column chromatography (eluent CH_2Cl_2/EtAc 7:3). ESI^+/MS *m/z* 392 (MH^+). GC–MS *m/z* 397 ($M^+ + 2$, 2), 396 ($M^+ + 1$, 10), 395 (M^+ 37), 206 (100), 193 (25), 192 (54). ^1H NMR δ 1.58–1.90 (m, 8H, $CH_2CH_2CH_2CH$, $CH_2CH_2CH_2N$), 2.56–2.84 (m, 9H, $CH_2CH_2CH_2CH$, $CH_2CH_2CH_2N$, NCH_2CH_2 tetrahydroisoquinoline), 3.60 (s, 2H, NCH_2 tetrahydroisoquinoline), 3.76–3.83 (s, 9H, CH_3), 6.52–6.99 (m, 5H, aromatic). Anal. ($C_{25}H_{33}NO_3$·HCl) C, H, N (hydrochloride salt melted at 205–207 °C).

4.11. 6,7-Dimethoxy-2-[3-(6-methoxy-1,2,3,4-tetrahydro-naphthalen-1-yl)-propyl]-1,2,3,4-tetrahydro-isoquinoline (8b)

Yellow oil, 55% yield from column chromatography (eluent CH_2Cl_2/EtAc 7:3). ESI^+/MS *m/z* 396 (MH^+). GC–MS *m/z* 397 ($M^+ + 2$, 3), 396 ($M^+ + 1$, 17), 395 (M^+ 60), 206 (100), 192 (41), 164 (30). ^1H NMR δ 1.57–1.91 (m, 8H, $CH_2CH_2CH_2CH$, $CH_2CH_2CH_2N$), 2.54–2.85 (m, 9H, $CH_2CH_2CH_2CH$, $CH_2CH_2CH_2N$, NCH_2CH_2 tetrahydroisoquinoline), 3.58 (s, 2H, NCH_2 tetrahydroisoquinoline), 3.76–3.84 (s, 9H, CH_3), 6.51–7.11 (m, 5H, aromatic). Anal. ($C_{25}H_{33}NO_3$·HCl) C, H, N (hydrochloride salt melted at 219–223 °C).

4.12. 6,7-Dimethoxy-2-[3-(5-methoxy-1,2,3,4-tetrahydro-naphthalen-1-yl)-propyl]-1,2,3,4-tetrahydro-isoquinoline (8c)

Yellow oil, 77% yield from column chromatography (eluent CH_2Cl_2/MeOH 19:1). ESI^+/MS *m/z* 396 (MH^+). GC–MS *m/z* 397 ($M^+ + 2$, 2), 396 ($M^+ + 1$, 10), 395 (M^+ 39), 206 (100). ^1H NMR δ 1.64-1.78 (m, 8H, $CH_2CH_2CH_2CH$, $CH_2CH_2CH_2N$), 2.54–2.84 (m, 9H, $CH_2CH_2CH_2CH$, $CH_2CH_2CH_2N$, NCH_2CH_2 tetrahydroisoquinoline), 3.58 (s, 2H, NCH_2 tetrahydroisoquinoline), 3.80-3.84 (s, 9H, CH_3), 6.52–7.26 (m, 7H, aromatic). Anal. ($C_{25}H_{33}NO_3$·HCl) C, H, N (hydrochloride salt melted at 207–211 °C).

4.13. 2-[3-(6,7-Dimethoxy-1,2,3,4-tetrahydro-naphthalen-1-yl)-propyl]-6,7-dimethoxy-1,2,3,4-tetrahydro-isoquinoline (8d)

Yellow oil, 55% yield from column chromatography (eluent CH_2Cl_2/MeOH 19:1). ESI^+/MS *m/z* 426 (MH^+). GC–MS *m/z* 427 ($M^+ + 2$, 4), 426 ($M^+ + 1$, 16), 425 (M^+ 47), 206 (100), 192 (58), 164 (30). ^1H NMR δ 1.53–1.89 (m, 8H, $CH_2CH_2CH_2CH$, $CH_2CH_2CH_2N$), 2.55–2.85 (m, 9H, $CH_2CH_2CH_2CH$, $CH_2CH_2CH_2N$, NCH_2CH_2 tetrahydroisoquinoline), 3.61 (s, 2H, NCH_2 tetrahydroisoquinoline), 3.82–3.90 (s, 12H, CH_3), 6.50–7.76 (m, 4H, aromatic). Anal. ($C_{26}H_{35}NO_4$·HCl) C, H, N (hydrochloride salt melted at 212–215 °C).

4.14. 2-(3-Cyclohexylidene-propyl)-6,7-dimethoxy-1,2,3,4-tetrahydro-isoquinoline (11)

Yellow oil, 17% yield from column chromatography (eluent CH_2Cl_2/MeOH 19:1). GC–MS *m*/*z* 316 ($M^+ + 1$, 2), 315 (M^+ 10), 206 (100). ^{1}H NMR δ 1.52 (m, 6H, 4 of CH_2 cyclohexen), 2.1–2.3 (m, 4H, 2 of CH_2 cyclohexen), 2.32–2.35 (m, 2H, $CHCH_2CH_2N$), 2.50–2.54 (m, 2H, NCH_2CH_2, isoquinoline), 2.75–2.83 (m, 4H, NCH_2CH_2, isoquinoline and $CHCH_2CH_2N$), 3.60 (s, 2H, NCH_2Ar, isoquinoline), 3.82 and 3.83 (2s, 6H, 2 of CH_3), 5.10 (t, 2H, J = 7.0 Hz CH), 6.51–6.58 (m, 2H, aromatic). UV–vis: λ_{max} = 280 nm; ε = 3238 M^{-1} cm^{-1} (PBS). Anal. ($C_{20}H_{29}NO_2$·HCl) C, H, N (hydrochloride salt dec. at 238–241 °C).

5. Biological methods

5.1. Cell lines

The breast cancer cell line of human origin, MCF7/Adr (resistant to Adriamycin or Doxorubicin), was routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100,000 U/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator at 37 °C with a 5 % CO_2 atmosphere. Caco-2 cells were grown in DMEM with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine.

The cells were trypsinized twice a week with trypsin/ethylenediaminetetraacetic acid (EDTA) (0.02%/0.02%) and the medium was changed twice a week.

5.2. Permeability experiments

5.2.1. Preparation of Caco-2 monolayer. This procedure has been previously reported by Leopoldo et al.[60] Briefly, Caco-2 cells were harvested with trypsin-EDTA and seeded onto MultiScreen Caco-2 assay system at a density of 10,000 cells per well. The culture medium was replaced every 48 h for the first 6 days and every 24 h thereafter, and after 21 days in culture, the Caco-2 monolayer was utilized for the permeability experiments. The Trans Epithelial Electrical Resistance (TEER) of the monolayers was measured daily before and after the experiment using an epithelial voltohommeter (Millicell®-ERS; Millipore, Billerica, MA). Generally, TEER values obtained are greater than 1000 Ω for a 21-day culture.

5.2.2. Drug transport experiment. Apical to basolateral (P_{app}, A–B) and basolateral to apical (P_{app}, B–A) permeability of drugs were measured at 120 min and at various drug concentrations (1–100 μM).[47] Drugs were dissolved in Hanks' balanced salt solution (HBSS, pH 7.4) and sterile filtered. After 21 days of cell growth, the medium was removed from filter wells and from the receiver plate. The filter wells were filled with 75 μL of fresh HBSS buffer and the receiver plate with 250 μL per well of the same buffer. This procedure was repeated twice, and the plates were incubated at 37 °C for 30 min. After incubation time, the HBSS buffer was removed and drug solutions added to the filter well (75 μL). HBSS without the drug was added to the receiver plate (250 μL). The plates were incubated at 37 °C for 120 min. After incubation time, samples were removed from the apical (filter well) and basolateral (receiver plate) side of the monolayer and then were stored in a freezer (−20 °C) pending analysis.

Compounds **6a**, **6c** and **8a**–**d** were lyophilized at −52 °C and 0.06 bars for 24 h and desalted by adding 500 μL of CH_3CN (in order to remove phosphate salts coming from HBSS), then 500 μL of 50 mM of Ammonium Acetate, pH 5, was added. The resulting solutions were analyzed by ESI-MS analyses in order to determine the concentration of drug in the samples. The concentration of compounds **3a, 3c**, **4**, **6b** and **6d**, and **11** was measured using UV spectroscopy.

The apparent permeability (P_{app}), in units of nm per second, was calculated using the following equation:

$$P_{app} = \left(\frac{V_A}{\text{Area} \times \text{time}}\right) \times \left(\frac{[\text{drug}]_{\text{acceptor}}}{[\text{drug}]_{\text{initial}}}\right)$$

where V_A is the volume (in mL) in the acceptor well; Area is the surface area of the membrane (0.11 cm^2 of the well); time is the total transport time in seconds (7200 s); $[drug]_{acceptor}$ is the concentration of the drug measured by ESI-MS analyses or UV spectroscopy; $[drug]_{initial}$ is the initial drug concentration (1×10^{-4} M) in the apical or basolateral wells.

5.3. Cell ATP availability assay

This experiment was performed as reported in technical sheet of ATPlite 1step Kit for luminescence ATP detection using Victor3, from PerkinElmer Life Sciences.[55] Caco-2 cells were seeded into 96-well microplates in 100 μL of complete medium at a density of 2×10^4 cells/well. The plate was incubated O/N in a humidified atmosphere of 5% CO_2 at 37 °C. The medium was removed and 100 μL of complete medium in the presence or absence of different concentrations ranging from 1 to 100 μM of test compounds was added. The plate was incubated for 2 h in a humidified atmosphere of 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, the plate stirred for 5 min as reported above. The plate was dark adapted for 10 min and the luminescence was measured in Victor3, from Perkin-Elmer Life Sciences.

5.4. [^{3}H]Vinblastine transport inhibition

Caco-2 cells were seeded onto MultiScreen Plates at 10,000 cells/well for 21 days measuring the integrity of the cell monolayers by Trans Epithelial Electrical Resistance (TEER, $\Omega \times cm^2$) with an epithelial voltohommeter. Mature Caco-2 cell monolayer exhibited a TEER > 800 $\Omega \times cm^2$ prior to use in transport experiments. Transport experiments for tested compounds were carried out as described by Taub et al.[56]

In each well to basolateral (BL) compartment in the absence and in the presence of P-gp inhibitors (from 200 nM to 400 μM) was added 20 nM [^{3}H]vinblastine for 120 min at 37 °C and its appearance in the apical (AP) compartment was monitored. At 120 min, 20 μL sample was taken from donor compartment to determine the concentration of radioligand remaining in the donor chamber at the end of the experiment. Samples were analyzed using LS6500 Beckman Counter. For each compound, [^{3}H]vinblastine transport inhibition was calculated as radioactivity difference between radioligand in the presence and absence of compound. These differences were expressed as inhibition percentage at single drug concentration.

5.5. Cell antiproliferative effect

The antiproliferative effect was evaluated using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay as reported by Colabufo et al. with minor modifications.[48] The cells were seeded to 96-well plates in the absence and presence of known concentrations of test compound for 48 h. In each well 10 μL of MTT solution (5 mg/mL) freshly prepared was added and the plate was incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 3–4 h. MTT solution was removed and 200 μL of EtOH/DMSO (1:1) was added to each well to dissolve the blue formazan solid crystals. The optical density was measured at 570 and 650 nm wavelenghts using Victor3, from Perkin-Elmer Life Sciences.

5.6. Cytotoxicity assay

The assay was performed using the CytoTox-One kit from the Promega Corp. (Madison, WI, USA) as reported in previous paper.[48] Cell death was determined as the release of lactate dehydrogenase (LDH) into the culture medium. The percentage of cytotoxicity was calculated relative to the LDH release from total lysis of cells in untreated control. It is assumed here that the drug-treated wells and the control wells contained the same total number of cells (dead plus alive cells) at the end of the treatment period. Therefore, the cytotoxic effect of tested compounds was unaffected by any underestimation of cytotoxicity that could occur because of decreased total number of cells in the treated samples compared to the untreated control. Cells were seeded into 96-well plates for optical performance in the fluorescent cell-based assay in 100 μL of complete medium in the presence or absence of different concentrations of test compounds. The plate was incubated for 24 h in a humidified atmosphere of 5% CO_2 at 37 °C and then 100 μL of substrate mix in assay buffer was added. Ten microliters of lysis solution was added to untreated wells in order to estimate total LDH. Plates were kept protected from light for 10 min at room temperature and 50 μL of stop solution was added to all wells. The fluorescence was recorded using a LS55 Luminescence Spectrometer PerkinElmer with a 560 nm excitation wavelength and a 590 nm emission wavelength. The cytotoxicity percentage was estimated as follows: 100 × (LDH in medium of treated cells—culture medium background)/(total LDH in untreated cells—culture medium background).

5.7. Intracellular Doxorubicin accumulation

The modulation of intracellular Doxorubicin accumulation by **3a**, **3c**, and **8c**, Cyclosporin A, and Verapamil was evaluated by flow cytometry, utilizing Verapamil as standard of P-gp inhibition.[49] In all experiments, the various drug-solvents (EtOH, DMSO) were added in each control to evaluate the solvent influence. In MCF7/Adr cells, the standard and the newly synthesized P-gp compounds were utilized at 20 μM for 2 days exposure, and during the second day Doxorubicin was given at 50 μM (3 days-drug IC_{50} concentration). After incubation, the cell media were removed and trypsin–EDTA was used to detach the cells from the plates. Cells were harvested, washed twice in ice-cold PBS (pH 7.4), and were placed on ice (less than 1 h) until analysis. Fluorescence measurements of individual cells were performed with a Becton–Dickinson FACScan equipped with an ultraviolet argon laser. Analysis was gated to include single cells on the basis of forward and side light scatter and was based on the acquisition of data from 5000 cells. Log fluorescence was collected and displayed as single parameter histograms. The mean fluorescence intensity of Doxorubicin in the Doxorubicin-treated cells, arbitrarily established as 100%, represented the positive control and the auto-fluorescence of untreated cells, arbitrarily established as 0%, was the negative control.

5.8. Effect of antiproliferative drug combination[48]

In MCF7/Adr, each newly synthesized compound was utilized at 20 μM; Doxorubicin at 5 μM (IC_{50} after 3 days drug exposure when the P-gp was not overexpressed); Verapamil and Cyclosporin A were utilized as reference compounds. The schedule of drug administration was the P-gp inhibitors plus Doxorubicin for 2 days followed, after two wash steps with complete medium, by Doxorubicin for 1 day. The analysis of cell growth inhibition was performed using the MTT assay. On day one, 10,000 cells/well were plated in 96-well plates in a volume of 200 μL and on day two, the various drugs alone or in combination were added. Six control wells (untreated cells) and six wells for each treatment were used in each experiment. In all experiments, the various drug-solvents (EtOH, DMSO) were added in each control to evaluate a possible solvent cytotoxicity. Then 0.5 mg/mL MTT was added to each well and, after 1-h incubation at 37 °C, the supernatant was removed. To solubilize the formazan crystals, 100 μL of DMSO was added and the absorbance values at 570 and 630 nm were determined on the microplate reader SpectraCount (Packard—USA). The cell growth inhibitory activity of drugs was expressed as percentage of control (untreated cells).

6. Statistical analysis

The EC_{50} values of the compounds reported in Table 2 were determined by nonlinear curve fitting utilizing the GraphPad Prism program.[61]

Supplementary materials

Elemental Analysis and experimental section for key intermediates **2a**, **5b**, **5d**, **7d**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.09.039.

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