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New structure–activity relationship studies in a series of *N,N*-bis(cyclohexanol)amine aryl esters as potent reversers of P-glycoprotein-mediated multidrug resistance (MDR)

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ABSTRACT

As a continuation of previous research on a new series of potent and efficacious P-gp-dependent multidrug resistant (MDR) reversers with a *N,N*-bis(cyclohexanol)amine scaffold, we have designed and synthesized several analogs by modulation of the two aromatic moieties linked through ester functions to the *N,N*-bis(cyclohexanol)amine, aiming to optimize activity and to extend structure–activity relationships (SAR) within the series. This scaffold, when esterified with two different aromatic carboxylic acids, gives origin to four geometric isomers (*cis*/*trans*, *trans*/*trans*, *cis*/*cis* and *trans*/*cis*).

The new compounds were tested on doxorubicin-resistant erythroleukemia K562 cells (K562/DOX) in the pirarubicin uptake assay. Most of them resulted in being potent modulators of the extrusion pump P-gp, showing potency values ($[I]_{0.5}$) in the submicromolar and nanomolar range. Of these, compounds **2b**, **2c**, **3d**, **5a–d** and **6d**, showed excellent efficacy with a α_{max} close to 1. Selected compounds (**2d**, **3a**, **3b**, **5a–d**) were further studied to evaluate their doxorubicin cytotoxicity potentiation (RF) on doxorubicin-resistant erythroleukemia K562 cells and were found able to enhance significantly doxorubicin cytotoxicity on K562/DOX cells.

The results of both pirarubicin uptake and the cytotoxicity assay, indicate that the new compounds of the series are potent P-gp-mediated MDR reversers. They present a structure with a mix of flexible and rigid moieties, a property that seems critical to allow the molecules to choose the most productive of the several binding modes possible in the transporter recognition site.

In particular, compounds **5c** and **5d**, similar to the already reported analogous isomers **1c** and **1d**,[29] are potent and efficacious modulators of P-gp-dependent MDR and may be promising leads for the development of MDR-reversal drugs.

1. Introduction

The development of resistance in cancer cells and microorganisms is the main obstacle to achieving success with chemotherapy. Frequently, following drug treatment, cells become resistant to a variety of chemotherapeutic drugs that are even structurally and mechanistically unrelated, thus resulting in so-called multidrug resistance (MDR).[1] In humans, MDR is associated with the overexpression of transporter proteins acting as extrusion pumps that perform an ATP-dependent active outward transport of chemotherapeutic drugs.[2,3] These proteins such as ABCB1, ABCC subfamily and ABCG2, belong to the ABC super family of multidrug transporters and, in normal conditions, are expressed in cells of several tissues where they have an important physiological role regulating the permeability of biological membranes,[3] the secretion of physiologically important lipophilic molecules[4] and the extrusion of xenobiotics that enter the organism.[5]

Among the MDR transporter proteins, ABCB1 (P-gp) is the most extensively studied and in recent years several biochemical, biophysical and bioinformatic tools have been used to study its mechanism of action and to explore its structural characteristics.[6–8] Even if there is no high-resolution crystal structure of human P-gp, several homology models have been proposed, based on

Abbreviations: EDCl, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimmide hydrochloride; DMAP, 4-(*N,N*-dimethylamino)pyridine; DOX, doxorubicin.

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the resolved structure of related bacterial proteins[9–15] which have provided useful information on the P-gp mechanism of action and its recognition site. An important advancement in the field was the recently described structure of murine P-gp[16] which has 87% sequence identity to human ABCB1 transporter and obviously is a better template for homology models.[17,18]

Although an atomic structure of human P-gp is still missing, several important features of its structure are known, in particular its recognition site that results characterized as a large, polymorphous drug binding domain where a variety of molecules can be accommodated in a plurality of binding modes, including π–π, ion–π, hydrogen bonds and hydrophobic interactions. This means that, unlike receptors and enzymes, P-gp and sister proteins lack pre-existent and specific binding sites for the large variety of chemicals that they are able to transport. This characteristic makes it difficult to develop useful general pharmacophores but at the same time appears fruitful in terms of drug research since it implies that efficient modulators can be found in very different chemical classes, as indeed is the case.[19–21]

In the past 30 years, P-gp overexpression in cancer cells has been considered a possible therapeutic target for circumventing MDR.[22] Many P-gp-dependent MDR reversers, that should act by being co-administrated with chemotherapeutic drugs, have been identified[20,23] and a few have reached clinical trials.[24] Nevertheless, no drug of this kind has been cleared for the market so far. Like substrates, even ABC transporter-targeting drugs present a variety of chemical structures, suggesting that most of them bind to the large substrate-recognition sites with the same plurality of binding modes proposed for substrates.

The lack of a high-resolution crystal structure of human P-gp and sister proteins, as well as their ability to interact with a very large variety of chemical species, makes it nearly impossible to use the two classic approaches of medicinal chemistry: structure-based (including fragment-based drug design, FBDD) and ligand-based drug design, to discover new drugs. As a consequence, search for ABC protein inhibitors is, at present, mostly done by extensive screening of synthetic or natural compounds and by chemical modulation of those found active.

A general pharmacophore for P-gp interaction could be very useful to direct the synthesis of more potent and selective reversers. However, structure–activity relationship studies performed until now have proved to be of limited value in attaining this goal, because they have identified only some general characteristics such as the importance of hydrogen bond acceptor groups, aromatic or hydrophobic features and the presence of a protonable nitrogen atom.[25–27] As expected from the broad substrate selectivity of ABC transporters like P-gp, more detailed SARs seem valid only within a specific chemical class as the case of the model proposed by Pajeva and Wiese on verapamil-related MDR reversers.[28]

In an ongoing search for potent P-gp-dependent MDR reversers started a few years ago, we used some of the information available about the recognition site of P-gp, reasoning that in a large recognition site like that proposed, flexible basic molecules carrying suitably positioned aryl moieties could easily find the most productive interaction, compensating for the entropy toll with enthalpy gain. The discovery of potent P-gp dependent MDR reversers among the compounds synthesized showed that this was indeed the case.[29] Then, considering that an excess of rotatable bonds is usually detrimental to proper pharmacokinetics, we decided to reduce the very high flexibility of linear structures and designed and synthesized a new series of compounds characterized by a *N,N*-bis(cyclohexanol)amine scaffold linked to two aromatic moieties by ester functions.

When esterified with different acids the *N,N*-bis(cyclohexanol)amine moiety gives origin to four geometric isomers (*cis*/*trans*, *trans*/*trans*, *cis*/*cis* and *trans*/*cis*) with varying geometry and possibly different biological activity. Some of these novel derivatives had low nanomolar potency and very high efficacy in inhibiting P-gp-dependent nuclear pirarubicin efflux in doxorubicin-resistant erythroleukemia K562 cells (K562/DOX) and rat intestinal mucosa ATPase activity, and on increasing the cytotoxicity of doxorubicin towards the K562/DOX cell line.[30,31] Among the several sets of isomers synthesized, those containing the *trans*-3-(3,4,5-trimethoxyphenyl)acrylic acid moiety combined with the 3,4,5-trimethoxybenzoic acid one (compounds **1a–d**, Table 1) showed the best MDR-reversing activity both in terms of potency and efficacy.

Taking into consideration the outstanding potency and efficacy of compounds **1a–d**, we decided to extend the structure–activity relationships studies in this series of molecules by studying new analogues characterized by the *N,N*-bis(cyclohexanol)amine scaffold with suitably modulated aromatic ester portions (Chart 1).

First of all, the *trans*-3-(3,4,5-trimethoxyphenyl)vinyl moiety (A) was combined with 4-cyano-4-(3,4-dimethoxyphenyl)-5-methylpentyl and 9*H*-fluorene moieties (C and D structures of Chart 1) that are present in compounds active at nanomolar doses reported in our previous work on verapamil-derived MDR reversers[32,33] to give sets **2** and **3**.

Then, to investigate the consequence of geometric modulation of the *trans*-3-(3,4,5-trimethoxyphenyl)acrylic acid moiety (A), present in set **1**, analogous compounds where this residue was substituted by 3-(3,4,5-trimethoxyphenyl)propanoic acid (E) (set **4**) or 3-(3,4,5-trimethoxyphenyl)propiolic acid (F) (set **5**) were synthesized. Preliminary results of this research have been anticipated in a letter.[34]

The good activity of compounds containing the 3-(3,4,5-trimethoxyphenyl)propiolic acid moiety (**5a–d**) prompted us to synthesize other compounds containing this aromatic residue combined with the 2,2-bis(4-methoxyphenyl)acetic acid residue and with the 9H-fluorene-9-carboxylic acid one (sets **6a–d** and **7a–d**).

The reversal activity of the studied compounds was evaluated by the pirarubicin doxorubicin-resistant erythroleukemia K562 cell uptake assay that was used as preliminary screening. The compounds that showed the best results in this test (**2d**, **3a**, **3b**, **5a–d**) were further studied by evaluating their doxorubicin cytotoxicity potentiation (RF) on the K562/DOX cell line.

2. Chemistry

Compounds **2a–d**, **3a–d**, **6a–d** and **7a–d** were obtained following the reaction pathways described in Schemes 1 and 2 and their chemical and physical characteristics are reported in Table S1 (Supplementary data). The synthesis of compounds **4a–d** and **5a–d** has been previously reported.[34]

Key intermediates **11a–d** (Scheme 1) were synthesized starting from the 4-oxocyclohexyl ester **8** obtained by esterification of 4-hydroxycyclohexanone[35] with 3-(3,4,5-trimethoxyphenyl)propiolic acid[36] in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCl) and 4-dimethylaminopyridine (DMAP). Reductive amination of compound **8** with commercially available *trans*-4-aminocyclohexanol gave an approximately 1:1 mixture of *cis*/*trans* and *trans*/*trans* isomers (a/b) of alcohol **9** (**9a/b**). The same reaction with *cis*-4-aminocyclohexanol[37] gave an approximately 1:1 mixture of *cis*/*cis* and *trans*/*cis* isomers (c/d) of **9** (**9c/d**), as it results from ^{1}H NMR spectra. In both cases titanium (IV) isopropoxide as Lewis acid catalyst and $NaBH_3CN$ as reducing agent were used, according to the Mattson procedure.[38]

A chromatographic separation was performed on the mixtures **9a/b** and **9c/d**, and the pure isomers **9a–d** were obtained. Their

configuration was attributed on the basis of the ^{1}H NMR characteristics of the cyclohexane protons.[31] ^{1}H NMR spectra indicate that the substituents on the two cyclohexane rings are in a *cis*/*trans* (series a), *trans*/*trans* (series b), *cis*/*cis* (series c) and *trans*/*cis* (series d) configuration.

The pure secondary aminoalcohols **9a–d** were alkylated by reductive methylation with HCOOH/HCHO to give the corresponding tertiary aminoalcohols **11a–d**.

In the same manner were also obtained aminoalcohols **10a–d**, whose synthesis has been previously described.[31]

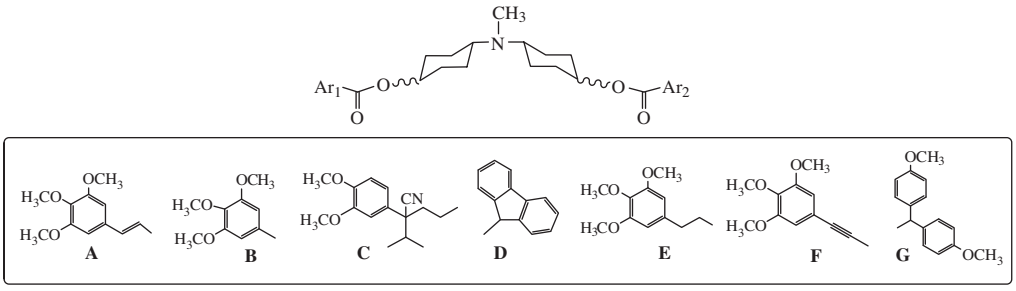
Final compounds **2a–d**, **3a–d**, **6a–d** and **7a–d** (Scheme 2) were obtained by reaction of **10a–d** (for **2** and **3** sets) and **11a–d** (for **6** and **7** sets) with 4-cyano-4-(3,4-dimethoxyphenyl)-5-methylhexanoic acid,[39] 9*H*-fluorene-9-carboxylic acid or 2,2-bis-(4-methoxyphenyl)acetic acid[40] in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCl) and 4-dimethylamino-pyridine (DMAP).

3. Biological studies

3.1. Modulation of pirarubicin uptake

The ability of compounds of sets **2–7** to modulate P-gp action was evaluated on the K562/DOX cell line. K562 is a human leukemia cell line established from a patient with chronic myelogeneous leukemia in blast transformation.[41] K562/DOX cells resistant to doxorubicin overexpress a unique membrane glycoprotein, P-gp.[42,43] The uptake of THP-adriamycin (pirarubicin) was measured by a continuous spectrofluorometric signal of anthracycline at 590 nm (λ_{ex} = 480 nm) after incubation of the cells, following the protocols reported in previous papers.[32,44] In brief, P-gp-blocking activity is described by: (i) α, which represents the fold increase in the nuclear concentration of pirarubicin in the presence of the P-gp modulator and varies between 0 (in the absence of the modulator) and 1 (when the amount of pirarubicin in resistant cells is

Table 1
MDR-reversing activity of compounds **1**–7



N	Ar_1	Ar_2	$[I]_{0.5}$ μM[a]	α_{max}[b]
1a *cis*/*trans*	B	A	0.092 ± 0.015[c]	0.85 ± 0.03
1b *trans*/*trans*	B	A	0.32 ± 0.10[c]	0.81 ± 0.03
1c *cis*/*cis*	B	A	0.03 ± 0.01[c]	0.80 ± 0.02
1d *trans*/*cis*	B	A	0.012 ± 0.001[c]	0.98 ± 0.03
2a *cis*/*trans*	A	C	0.24 ± 0.04	0.88 ± 0.03
2b *trans*/*trans*	A	C	0.14 ± 0.02	0.99 ± 0.01
2c *cis*/*cis*	A	C	0.15 ± 0.03	0.99 ± 0.01
2d *trans*/*cis*	A	C	0.08 ± 0.03	0.89 ± 0.05
3a *cis*/*trans*	A	D	0.08 ± 0.01	0.81 ± 0.03
3b *trans*/*trans*	A	D	0.075 ± 0.01	0.94 ± 0.04
3c *cis*/*cis*	A	D	0.18 ± 0.04	0.90 ± 0.03
3d *trans*/*cis*	A	D	0.15 ± 0.03	0.99 ± 0.01
4a *cis*/*trans*	B	E	0.63 ± 0.2[d]	0.75 ± 0.03
4b *trans*/*trans*	B	E	0.61 ± 0.18[d]	0.76 ± 0.03
4c *cis*/*cis*	B	E	1.10 ± 0.3[d]	0.76 ± 0.05
4d *trans*/*cis*	B	E	0.35 ± 0.1[d]	0.77 ± 0.02
5a *cis*/*trans*	B	F	0.08 ± 0.01[d]	0.98 ± 0.02
5b *trans*/*trans*	B	F	0.07 ± 0.01[d]	0.99 ± 0.01
5c *cis*/*cis*	B	F	0.07 ± 0.01[d]	0.99 ± 0.01
5d *trans*/*cis*	B	F	0.02 ± 0.01[d]	0.99 ± 0.01
6a *cis*/*trans*	F	G	0.13 ± 0.03	0.76 ± 0.03
6b *trans*/*trans*	F	G	0.14 ± 0.04	0.74 ± 0.02
6c *cis*/*cis*	F	G	0.12 ± 0.04	0.94 ± 0.04
6d *trans*/*cis*	F	G	0.02 ± 0.001	0.99 ± 0.01
7a *cis*/*trans*	F	D	0.12 ± 0.04	0.96 ± 0.04
7b *trans*/*trans*	F	D	0.50 ± 0.07	0.77 ± 0.02
7c *cis*/*cis*	F	D	0.05 ± 0.01	0.89 ± 0.05
7d *trans*/*cis*	F	D	0.29 ± 0.08	0.91 ± 0.06
Verapamil			1.60 ± 0.3	0.70 ± 0.07
MM36			0.05 ± 0.01[e]	0.70 ± 0.06

[a] Concentration of the inhibitor that causes a 50% increase in nuclear concentration of pirarubicin (α = 0.5).
[b] Efficacy of MDR-modulator and maximum increase that can be obtained in the nuclear concentration of pirarubicin in resistant cells.
[c] See Ref. 31.
[d] See Ref. 34.
[e] See Ref. 32.

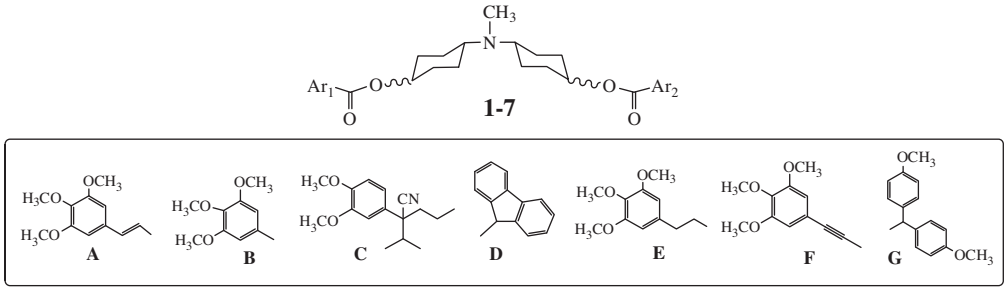
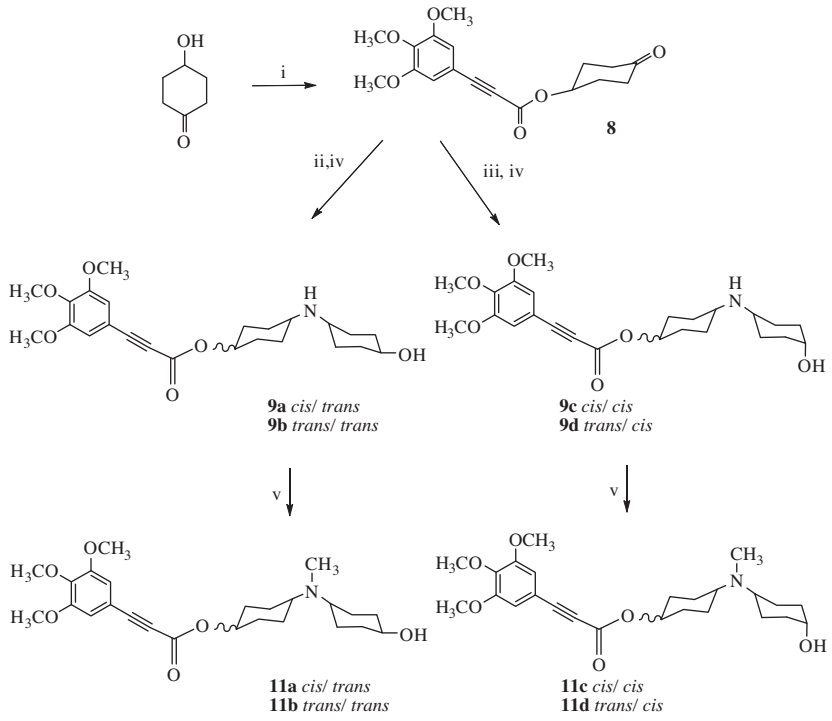


Chart 1. General structure of designed compounds.



Scheme 1. Synthesis of key intermediates **11a–d**. Reagents and conditions: (i) anhydrous CH_2Cl_2, 3-(3,4,5-trimetoxyphenyl)propiolic acid,[36] 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCl), 4-dimethylaminopyridine (DMAP); (ii) *trans*-4-aminocyclohexanol,[37] $(i\text{-PrO})_4Ti$, $NaBH_3CN$; (iii) *cis*-4-aminocyclohexanol, $(i\text{-PrO})_4Ti$, $NaBH_3CN$; (iv) chromatographic separation; (v) HCOOH/HCHO.

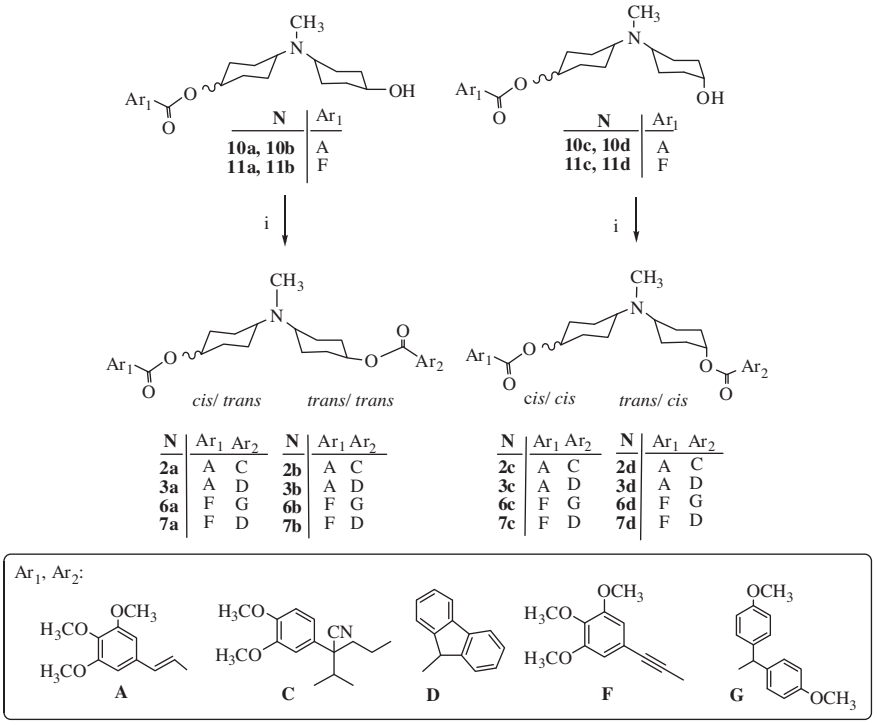
the same as in sensitive cells); (ii) α_{max}, which expresses the efficacy of the P-gp modulator and is the maximum increase that can be obtained in the nuclear concentration of pirarubicin in resistant cells with a given compound; and (iii) $[I]_{0.5}$, which measures the potency of the modulator and represents the concentration that causes a half-maximal increase ($\alpha = 0.5$) in the nuclear concentration of pirarubicin (see Table 1).

This test indicates that the compounds inhibit the P-gp-operated extrusion of the reporter molecule pirarubicin, as does the reference molecule verapamil.

3.2. Cytotoxicity test and MDR reversal

The ability of a compound to enhance the growth inhibitory effects of doxorubicin in cancer cell lines showing MDR due to P-gp overexpression, has also been proved useful in the quantification and characterization of MDR reversal by modulators of the MDR phenotype. The reversal effects on the MDR phenotype of selected compounds (**2d**, **3a**, **3b**, **5a–d**) were investigated on K562/DOX cells. Preliminarily, the compounds were evaluated for their intrinsic cytotoxicity on both K562 and K562/DOX cell lines by the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay,[45] then the MDR-reversal activity of studied compounds was tested using the same method in the presence of doxorubicin. Based on the values of $[I]_{0.5}$, two concentrations were chosen, one very close to the $[I]_{0.5}$ value (0.1 μM) and the other ten times higher (1 μM). Verapamil was used as reference compound.

The IC_{50} doxorubicin values resulting in 50% inhibition of cell growth compared to untreated control were calculated from plotted results. The reversal-fold (RF) values, a measure of MDR reversal, were calculated by dividing the doxorubicin IC_{50} value on K562/DOX cells in the absence of modulators by the doxorubicin IC_{50} values in the same cells in the presence of modulators (see Table 2).



Scheme 2. Synthesis of final compounds **2a–d**, **3a–d**, **6a–d**, and **7a–d**. The synthesis of compounds **10a–d** is similar to that of compounds **11a–d** and described in Ref. 31. Reagents and conditions: (i) anhydrous CH_2Cl_2, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), 4-dimethylaminopyridine (DMAP), Ar_2COOH (4-cyano-4-(3,4-dimethoxyphenyl)-5-methylhexanoic acid,[39] 9*H*-fluorene-9-carboxylic acid or 2,2-bis(4-methoxyphenyl)acetic acid.[40]

4. Results

4.1. Modulation of pirarubicin uptake.

The results obtained in doxorubicin-resistant erythroleukemia K562 cells are reported in Table 1, together with those of MM36 (Fig. 1, the most potent compound that we have discovered in previous studies[32]) and verapamil (the gold standard of P-gp inhibition) used as reference compounds.

The new compounds, except compound **4c**, show potency values ($[I]_{0.5}$) in the submicromolar and nanomolar range; among them, compounds **2b–c**, **3d**, **5a–d** and **6d** show excellent efficacy with an α_{max} close to 1. They are therefore potent and efficacious modulators of the P-gp extrusion pump.

4.2. Cytotoxicity test and MDR reversal

The reversal effects (RF) of compounds on the MDR phenotype have been investigated in K562/DOX cells at 0.1 and 1.0 μM-concentrations. The results are reported in Table 2 together with those of the reference compound verapamil tested at 2.0 and 20 μM and of compounds **1a–d**.[31] Compounds **2d**, **3a**, **3b**, **5a–d** and verapamil tested at 1.0 μM without doxorubicin had no cytotoxic effects on K562 and K562/DOX cells (data not shown), therefore, we tested all compounds at 0.1 and 1.0 μM concentrations in the presence of doxorubicin. The lowest concentration (0.1 μM) of compounds moderately reversed the resistance of K562/DOX cells with RF values ranging between 1.6 and 3.0; at this concentration, compound **5b** had no reversal effect (RF = 0.6). Comparing the RF value obtained at both concentrations, the compounds **2d**, **3a, 5b**, **5c** and **5d** at a higher tested concentration (1.0 μM) caused a 2- to 4-fold increase in RF (RF = 10.7, 5.0, 1.9, 4.5, 8.5, respectively) compared to the lower concentration (0.1 μM) (RF = 3.0, 2.3, 0.6, 2.3, 2.0, respectively). Compounds **3b** and **5a** instead showed the same RF values at both concentrations.

Table 2
Effects of MDR-reversing agents **2d**, **3a**, **3b**, **5a–d** in comparison to **1a–d** on doxorubicin cytotoxicity in K562 and K562/DOX cells

Compounds	K562 cells	K562/DOX cells	
	IC_{50} (μM)[a]	IC_{50} (μM)[a]	RF[b]
DOXO[c]	0.03 ± 0.002	2.84 ± 0.047	
DOXO + **1a** (1 μM)[c]	0.05 ± 0.011	0.37 ± 0.130	7.7
DOXO + **1b** (1 μM)[c]	0.05 ± 0.010	0.31 ± 0.030	9.2
DOXO + **1c** (1 μM)[c]	0.04 ± 0.015	0.29 ± 0.060	9.8
DOXO + **1d** (1 μM)[c]	0.05 ± 0.009	0.14 ± 0.030	20.3
DOXO	0.05 ± 0.007	2.99 ± 0.200	
DOXO + **2d** (0.1 μM)	0.04 ± 0.010	0.99 ± 0.200	3.0
DOXO + **2d** (1 μM)	0.013	0.28 ± 0.080	10.7
DOXO + **3a** (0.1 μM)	0.04 ± 0.020	1.3 ± 0.060	2.3
DOXO + **3a** (1 μM)	0.03 ± 0.017	0.6 ± 0.100	5.0
DOXO + **3b** (0.1 μM)	0.02 ± 0.004	1.56 ± 0.410	2.0
DOXO + **3b** (1 μM)	0.02 ± 0.004	1.06 ± 0.270	2.9
DOXO + **5a** (0.1 μM)	0.14 ± 0.020	1.85 ± 0.300	1.6
DOXO + **5a** (1 μM)	0.07 ± 0.016	1.98 ± 0.200	1.5
DOXO + **5b** (0.1 μM)	0.09 ± 0.020	4.8 ± 0.600	0.6
DOXO + **5b** (1 μM)	0.07 ± 0.020	1.54 ± 0.070	1.9
DOXO + **5c** (0.1 μM)	0.04 ± 0.040	1.3 ± 0.100	2.3
DOXO + **5c** (1 μM)	0.04 ± 0.010	0.67 ± 0.030[d]	4.5
DOXO + **5d** (0.1 μM)	0.06 ± 0.010	1.47 ± 0.070	2.0
DOXO + **5d** (1 μM)	0.06 ± 0.040	0.35 ± 0.053[d]	8.5
DOXO + verapamil (2 μM)	0.09 ± 0.030	2.51 ± 0.160	1.2
DOXO + verapamil (20 μM)	0.05 ± 0.006	0.63 ± 0.060	4.7

[a] Mean ± SE of at least three determinations or mean of two determinations performed with quadruplicate cultures at each drug concentration tested and measured as described in Section 6.

[b] Reversal fold of MDR was determined by dividing the doxorubicin IC_{50} values on K562/DOX cells in the absence of modulators by those in the presence of modulators.

[c] From Ref. 31. Note that in that paper the DOXO IC_{50} value on K562 and K562/DOX cells was slightly different from that obtained in the present tests.

[d] p <0.001 versus control doxorubicin-treated. Where not specified the IC_{50} value is not significant compared to control cells (K562 and K562/DOX cells treated with doxorubicin only).

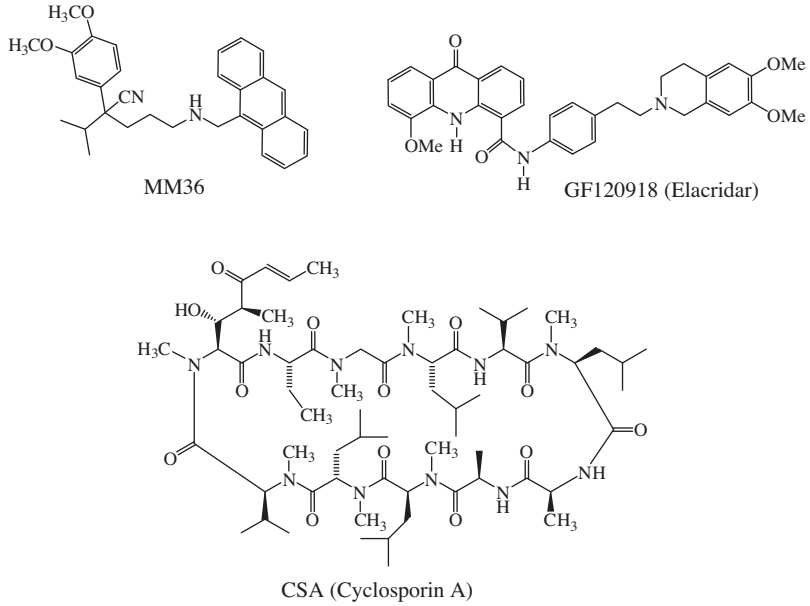


Figure 1. MM36[32], GF120918[20] (Elacridar) and CSA[19] (Cyclosporin A) structures.

The RF data indicate that all the *N,N*-bis(cyclohexanol)amine aryl esters tested were moderately active at a 0.1 μM concentration with the exception of compound **5b**. Their activity was substantially increased at 1.0 μM with the exception of compounds **3b** and **5a.** All compounds were more potent than the reference drug verapamil at 2.0 μM. Compounds **2d** and **5d** were the most effective, exhibiting a similar behavior to that of isomers **1a**–**c** (RF = 7.7, 9.2 and 9.8, respectively). They were, however, less effective than the **1d** isomer (RF = 20.3).

4.3. Chemical stability

In order to evaluate the chemical stability of our compounds during the biological tests, a selection of derivatives were maintained under the same conditions as the cytotoxicity test (37 °C for 3 days in the cell culture medium described in the Section 6) in four different concentrations (10^{-3} to 10^{-6} M). The stability of the compounds was verified by TLC and by LC–ESI-MS (Supplementary data); both analytical methods showed that the tested compounds are stable under these conditions. however, at the moment, no information is available on their metabolic stability. Studies are in progress to evaluate the metabolic profile of these compounds.

5. Discussion

In the search for potent and efficient reversers of P-gp-dependent multidrug resistance, we synthesized a series of compounds characterized by an *N,N*-bis(cyclohexanol)amine scaffold linked to two different aromatic moieties through ester functions. This scaffold gives origin to four geometric isomers (*cis*/*trans*, *trans*/*trans*, *cis*/*cis* and *trans*/*cis*), resulting in a library of 24 pure isomers (sets **2**–**7**). All compounds were screened in the preliminary test through the evaluation of pirarubicin uptake on doxorubicin-resistant erythroleukemia K562 cells (K562/DOX) as a measure of their ability to reverse P-gp extruding action. In Table 1, where the results of this assay are reported, we have included the data of the four isomers of the previously studied set **1**.[30,31] Accordingly we will take into consideration the isomers of this set, that are the most interesting compounds of the class, in the following discussion.

We will discuss the results obtained in this ranking test in three ways: (i) by analyzing the behavior of the isomers in each set; (ii) by comparing each set with the others; (iii) by examining the whole library of compounds.

(i) In five out of the seven sets the most active isomer is the *trans*/*cis* one (**1d**, **2d**, **4d**, **5d**, **6d**); in set **3** the two isomers **3a** and **3b**, that present *cis*/*trans* and *trans*/*trans* geometry respectively, have nearly identical potency. Isomer **7c** presenting *cis*/*cis* geometry is the most potent of set **7**. These results would suggest that, among the compounds of this class, *trans*/*cis* geometry[31] is the most suitable to interact with the P-gp recognition site. However, with the exception of set **1**, where the most active isomer **1d** is about 27 times more potent than **1b** ($[I]_{0.5}$ = 0.012 and 0.32 μM, respectively) and of set **7** where **7c** is approximately 10 times more potent than **7b** ($[I]_{0.5}$ = 0.05 and 0.50 μM, respectively), the differences in potency are generally small (3- to 5-fold) which makes it risky to generalize about the interaction geometry. As far as efficacy is concerned, the situation is nearly the same as only in sets **1**, **4**, **5**, **6** maximum efficacy (α_{max}) corresponds to the most potent isomer of each set.

(ii) A comparison of the different sets confirms that the *N,N*-bis(cyclohexanol)amine scaffold, when esterified with suitable aromatic acids, is definitely efficient in establishing productive interactions with the P-gp recognition site.[31,46] Activity was maintained with only modest variation in set **1** when the *trans*-2-(3,4,5-trimethoxyphenyl)vinyl moiety (A) was combined, to give sets **2** and **3**, with 4-cyano-4-(3,4-dimethoxyphenyl)-5-methylpentyl (C) and 9*H*-fluorene (D) moieties that characterized early MDR reversers active at nanomolar doses.[31,32] Results obtained with sets **4** and **5**, designed to evaluate the consequences of modulation of the geometry imposed by the double bond of *trans*-2-(3,4,5-trimethoxyphenyl)vinyl moiety present in set **1**, were particularly interesting. Clearly, the reduction of the double bond (set **4**) and the consequent increase of conformational freedom, and of the related entropy toll, are detrimental to both affinity and efficacy. On the contrary, further restriction of the conformational freedom of the

molecule, obtained through the introduction of the triple bond (set **5**) maintained the potency, smoothening the differences among the four isomers, and significantly increased efficacy. In both cases *trans*/*cis* isomers (**1d** and **5d**) are the most potent ones, suggesting that sets **1** and **5** bind in very similar binding sites.

Unfortunately, the set carrying the cis-2-(3,4,5-trimethoxyphenyl)vinyl moiety, where the double bond of set 1 would be in the cis geometry, could not be isolated for biological testing, since the final products isomerize to the more stable trans isomers (set 1), which precludes a more complete analysis of the geometric requests of the binding space.-Since 9H-fluorene-9-carboxylic acid can be considered a restricted conformational analogue of 2,2-bis(4-methoxyphenyl)acetic acid, sets 6 and 7 were synthesized following the good activity of the compounds of set 5, to collect information on the geometric requirements at both ends of the molecules. Further conformational restriction of set 6 to set 7 had a different effect on the activity of the isomers. The most potent isomer of set 6 is 6d ([I]0.5 = 0.02 μM trans/cis geometry) while the most potent isomer of set 7 is 7c ([I]0.5 = 0.05 μM cis/cis geometry). In this case reduction of conformational freedom slightly reduces the potency while maintaining good efficacy and seemingly forcing 7c to change binding mode.

(iii) Almost all compounds in Table 1 are very potent and efficacious reversers of P-gp-dependent-MDR. In terms of binding modes, the results presented above seem to indicate that the rather rigid *N,N*-bis(cyclohexanol)amine scaffold plays a major role, while the flexible arylester moieties produce quantitatively different effects on potency and efficacy. These results can be explained by proposing that the *N,N*-bis(cyclohexanol)amine scaffold provides much of the binding energy of the members of this class, while arylester moieties provide the fine tuning. As proposed at the very beginning of this research, it is likely that compounds of this series, that are quite different from most of known MDR modulators,[22,24] exploit at best the possibility, within the large and polymorphous recognition site, of adopting the most efficient binding modes within the recognition site in a mutual molding (induced fit) of their chemical and physical characteristics.[29,47]

The MDR reversal effects of selected compounds of the library (**2d**, **3a**, **3b**, **5a**–**d**) on the MDR phenotype were also investigated using the cytotoxicity test on doxorubicin-resistant erythroleukemia K562 cells (K562/DOX) overexpressing only Pgp and the results are reported in Table 2, together with those of previously reported compounds **1a**–**d**.[31]

As expected, all compounds, with the exception of **5b**, were fairly effective in restoring the sensitivity of K562/DOX cells to doxorubicin since, at both 0.1 and 1.0 μM concentrations they are more effective than verapamil at 2 and 20 μM. However, correlation with the results of the pirarubicin test is modest. Actually, the two most potent members of sets **1** and **5** (**1d** and **5d**) in the pirarubicin test are the most potent also in the doxorubicin test. However their RF values (20.3 and 8.5) do not seem closely related to potency and efficacy in the pirarubicin test ($[I]_{0.5}$ = 0.012, α_{max} = 0.98; $[I]_{0.5}$ = 0.02, α_{max} = 0.99). Compare also **2d** (RF = 10.7, $[I]_{0.5}$ = 0.08, α_{max} = 0.89), and **5c** (RF = 4.5, $[I]_{0.5}$ = 0.07, α_{max} = 0.99). It is difficult to indicate the reasons for these inconsistencies between the two tests. However, we must consider that two anthracyclines with different rates of cellular uptake, pirarubicin faster than doxorubicin, are used. Pirarubicin and doxorubicin intracellular concentrations depend on the kinetics of P-glycoprotein mediated efflux of the drugs but also on the kinetics of their uptake by the cells. These kinetics have a role not only in the cytotoxicity of the drug towards the resistant cells but also in the ability of inhibitors to restore intracellular incorporation to a level comparable with that observed in parent cells.[48] Moreover, metabolic processes on doxorubicin, during the long lasting cytotoxicity experiment (72 h) can alter the cytotoxic response of the cells and require higher concentrations of Pgp modulators. Studies are in progress to evaluate the metabolic profile of these compounds.

Neri et al.[49] have extended the study of P-gp modulation activity of sets **1** and **5** to the inhibition of rhodamine123 (R123) efflux in human MDR1-gene transfected mouse T-lymphoma L5178 cells. All the compounds were active at low concentration, **1c**, **1d**, **5c** and **5d** being active at nanomolar doses. These compounds were more potent than reference drugs GF120918[20] (Elacridar) and CSA[19] (Cyclosporin A), two of the most potent P-gp modulators studied. Their structure is reported in Figure 1.

The same two sets were also tested on the ATPase activity of human P-gp enriched intestinal *Spodoptera frugiperda* membranes (*Sf9*), both in the absence and presence of 0.1 μM epirubicin. Compounds **1c**, **1d**, **5c** and **5d** potently inhibited epirubicin-stimulated ATPase activity. Based on the results of their experiments, the authors suggest that this class of P-gp modulators might act as transport substrates.[49]

In conclusion, we have expanded SARs of a new class of potent *N,N*-bis(cyclohexanol)amine aryl esters which act as reversers of P-gp-mediated MDR by synthesizing several new compounds. Even if the potency of the parent compounds **1c** and **1d**,[31] remained unmatched, we have identified two other molecules (**5c** and **5d**) that, like **1c** and **1d**,[31] are potent and highly efficacious modulators of P-gp-dependent MDR and appear to be very promising leads for the development of MDR-reversal drugs.

6. Experimental section

6.1. Chemistry

All melting points were taken on a Büchi apparatus and are uncorrected. Infrared spectra were recorded with a Perkin–Elmer 681 or a Perkin–Elmer Spectrum RX I FT-IR spectrophotometer. ^{1}H NMR spectra were recorded on Bruker Avance 400 spectrometer. Chromatographic separations were performed on a silica gel column by flash chromatography (Kieselgel 40, 0.040–0.063 mm; Merck). Yields are given after purification, unless stated otherwise. Where analyses are indicated by symbols, the analytical results are within ±0.4% of the theoretical values. We have chosen to perform and report only the combustion analyses of final compounds. The identity and purity of the intermediates was ascertained through IR, ^{1}H NMR and TLC chromatography. When reactions were performed in anhydrous conditions, the mixtures were maintained under nitrogen.

Compounds were named following IUPAC rules as applied by Beilstein-Institut AutoNom 2000 4.01.305, a software for systematic names in organic chemistry.

6.2. 3-(3,4,5-Trimethoxyphenyl)propiolic acid 4-oxocyclohexyl ester (8)

To a solution of 4-hydroxycyclohexanone[35] (725 mg, 6.36 mmol) in dry CH_2Cl_2 cooled to 0 °C were added in this sequence: 3-(3,4,5-trimethoxyphenyl)propiolic acid[36] (2.25 g, 9.53 mmol), 4-dimethylaminopyridine (DMAP) (621 mg, 5.10 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCl) (2.19 g, 11.0 mmol). The reaction mixture was stirred at 0 °C for

1 h and then was kept at room temperature for 48 h. The mixture was treated with CH_2Cl_2 and the organic layer washed with water and then with a saturated solution of $NaHCO_3$. After drying with Na_2SO_4, the solvent was removed under reduced pressure and the residue purified by flash chromatography using cyclohexane/ethyl acetate (7:3) as eluting system. The title compound (658 mg, 34% yield) was obtained as a colourless oil. ^{1}H NMR ($CDCl_3$): δ 6.81 (s, 2H, aromatic); 5.29–5.27 (m, 1H, CHO); 3.84 (s, 3H, OCH_3); 3.82 (s, 6H, OCH_3); 2.67–2.59 (m, 2H, CH_2); 2.43–2.37 (m, 2H, CH_2); 2.24–2.11 (m, 4H, $2CH_2$) ppm.

6.3. *cis*/*trans*-3-(3,4,5-Trimethoxyphenyl)propiolic acid 4-(4-hydroxycyclohexylamino)cyclo-hexyl ester (9a) and *trans*/*trans*-3-(3,4,5-trimethoxyphenyl)propiolic acid 4-(4-hydroxy-cyclohexylamino)cyclohexyl ester (9b)

A mixture of compound **8** (658 mg, 2.14 mmol), *trans*-4-amino-cyclohexanol (292 mg, 2.54 mmol) and titanium (IV) isopropoxide (1.28 mL, 4.28 mmol) was stirred at room temperature. After 3 h, absolute ethanol (2.0 mL) and $NaBH_3CN$ (266 mg, 4.28 mmol) were added and the solution was stirred for 20 h. Water (10 mL) was added, the organic solvent was removed under reduced pressure then the mixture was treated with CH_2Cl_2 and the organic layer washed with a saturated solution of $NaHCO_3$ After drying with Na_2SO_4, the solvent was removed under reduced pressure and the residue purified by flash chromatography using CH_2Cl_2/MeOH/NH_4OH (95:5:0.5) as eluting system. Compound **9** (370 mg, 40% yield) as a approximately 50/50 mixture of *cis*/*trans* and *trans*/*trans* isomers, was obtained as a white solid. Mp: 67–69 °C. IR (neat): ν 3392 (OH and NH); 1705 (CO) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 6.82 (s, 2H, aromatic); 5.10 (br s, 0.5H, CHO); 4.83 (m, 0.5H, CHO); 3.88 (s, 3H, OCH_3); 3.85 (s, 6H, OCH_3); 3.60–3.52 (m, 1H, CHOH); 2.72–2.50 (m, 2H, 2NCH); 2.20–1.00 (m, 18H, $8CH_2$, OH and NH) ppm.

Flash chromatography of 370 mg of the mixture **9a**/**b**, using CH_2Cl_2/MeOH/NH_3 (97:3:0.3) as eluting system afforded the two isomers *cis*/*trans* and *trans*/*trans* (**9a** and **9b**).

6.3.1. *cis*/*trans* 9a

Hundred and thirty six milligram as a colourless oil; IR (neat): ν 3390 (OH and NH); 1706 (CO) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 6.82 (s, 2H, aromatic); 5.09 (br s, 1H, CHO); 3.86 (s, 3H, OCH_3); 3.85 (s, 6H, $2OCH_3$); 3.59 (m, 1H, CHOH); 2.71–2.51 (m, 2H, 2NCH); 2.08–1.10 (m, 18H, $8CH_2$, OH and NH) ppm.

6.3.2. *trans*/*trans* 9b

Hundred milligram as a colourless oil; IR (neat): ν 3390 (OH and NH); 1706 (CO) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 6.83 (s, 2H, aromatic); 4.85 (m, 1H, CHO); 3.88 (s, 3H, OCH_3); 3.85 (s, 6H, $2OCH_3$); 3.62 (m, 1H, CHOH); 2.70–2.52 (m, 2H, 2NCH); 2.12–1.10 (m, 18H, $8CH_2$, OH and NH) ppm.

6.4. *cis*/*cis*-3-(3,4,5-Trimethoxyphenyl)propiolic acid 4-(4-hydroxycyclohexylamino)cyclohexyl ester (9c) and *trans*/*cis*-3-(3,4,5-trimethoxyphenyl)propiolic acid 4-(4-hydroxycyclohexyl-amino)cyclohexyl ester (9d)

An approximately 50/50 mixture of *cis*/*cis* and *trans*/*cis* isomers **9c/d** was obtained in the same way as **9a**/**b** starting from **8** and using *cis*-4-aminocyclohexanol.[37] Its IR and ^{1}H NMR spectra are consistent with the proposed structure. The chromatographic separation of 0.25 g of the mixture afforded the two isomers **9c** and **9d.**

6.4.1. *cis*/*cis* 9c

Hundred and twenty milligram as a light yellow oil; IR (neat): ν 3390 (OH and NH); 1706 (CO) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 6.86 (s, 2H, aromatic); 5.12 (br s, 1H, CHO); 3.97 (br s, 1H, CHOH); 3.86 (s, 3H, OCH_3); 3.85 (s, 6H, $2OCH_3$); 2.98–2.72 (m, 2H, 2NCH); 2.07–2.01 (m, 2H, CH_2); 1.92–1.51 (m, 14H, $7CH_2$) ppm.

6.4.2. *trans*/*cis* 9d

Eighty milligram as a light yellow oil; IR (neat): ν 3390 (OH and NH); 1706 (CO) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 6.83 (s, 2H, aromatic); 4.88–4.83 (m, 1H, CHO); 3.93 (br s, 1H, CHOH); 3.87 (s, 3H, OCH_3); 3.84 (s, 6H, $2OCH_3$); 2.81–2.69 (m, 2H, 2NCH); 2.18–2.11 (m, 2H, CH_2); 2.08–2.01 (m, 2H, CH_2); 1.83–1.30 (m, 12H, $6CH_2$) ppm.

6.5. *cis*/*trans*-3-(3,4,5-Trimethoxyphenyl)propiolic acid 4-[(4-hydroxycyclohexyl)methylamino] cyclohexyl ester (11a)

To a solution of 80 mg (0.19 mmol) of **9a** in 1 mL of absolute ethanol, 0.12 mL of HCOOH and 0.03 mL of HCHO were added. The mixture was heated to 80 °C for 4 h and concentrated in vacuo. The residue was then dissolved in CH_2Cl_2 and the organic layer was washed with a saturated solution of $NaHCO_3$ and with water. After drying with Na_2SO_4, the solvent was removed under reduced pressure. Compound **11a** was obtained as an oil (76.4 mg, 94% yield). IR (neat): ν 3360 (OH); 1708 (CO) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 6.84 (s, 2H, aromatic); 5.11 (br s, 1H, CHO); 3.88 (s, 3H, OCH_3); 3.86 (s, 6H, $2OCH_3$); 3.61-3.51 (m, 1H, CHOH); 2.69-2.59 (m, 2H, 2NCH); 2.31 (s, 3H, NCH_3); 2.10-1.18 (m, 17H, $8CH_2$, and OH) ppm.

Compounds **11b**, **11c** and **11d** were obtained in the same way from **9b**, **9c** and **9d** respectively.

6.5.1. *trans*/*trans* 11b

IR (neat): ν 3360 (OH); 1708 (CO) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 6.81 (s, 2H, aromatic); 4.81–4.72 (m, 1H, CHO); 3.85 (s, 3H, OCH_3); 3.83 (s, 6H, $2OCH_3$); 3.60–3.50 (m, 1H, CHOH); 2.68–2.50 (m, 2H, 2NCH); 2.22 (s, 3H, NCH_3); 2.18–1.20 (m, 17H, $8CH_2$, and OH) ppm.

6.5.2. *cis*/*cis* 11c

IR (neat): ν 3360 (OH); 1708 (CO) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 6.85 (s, 2H, aromatic); 5.11 (br s, 1H, CHO); 4.05 (br s, 1H, CHOH); 3.92 (s, 3H, OCH_3); 3.90 (s, 6H, $2OCH_3$); 2.71–2.61 (m, 2H, 2NCH); 2.32 (s, 3H, NCH_3); 2.10–2.01 (m, 2H, CH_2); 1.83–1.50 (m, 15H, $7CH_2$, and OH) ppm.

6.5.3. *trans*/*cis* 11d

IR (neat): ν 3360 (OH); 1708 (CO) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 6.82 (s, 2H, aromatic); 4.83–4.74 (m, 1H, CHO); 3.97 (br s, 1H, CHOH); 3.86 (s, 3H, OCH_3); 3.84 (s, 6H, $2OCH_3$); 2.73–2.52 (m, 2H, 2NCH); 2.28 (s, 3H, NCH_3); 2.19–2.08 (m, 2H, CH_2); 1.92–1.42 (m, 15H, $7CH_2$, and OH) ppm.

6.6. *cis*/*trans*-9*H*-Fluorene-9-carboxylic acid 4-(methyl{4-[3-(3,4,5-trimethoxyphenyl)propyno-yl-oxy]cyclohexyl}amino)-cyclohexyl ester (7a)

Following the procedure described for compound **8**, to a solution of **11a** (76.4 mg, 0.17 mmol) in dry CH_2Cl_2 cooled at 0° C were added in this sequence: 9*H*-fluorene-9-carboxylic acid (54.1 mg, 0.26 mmol), 4-dimethylaminopyridine (DMAP) (16.7 mg, 0.14 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCl) (59.2 mg, 0.31 mmol). The reaction mixture was stirred at 0 °C for 1 h and then was kept at room temperature for 48 h. The mixture was treated with CH_2Cl_2 and the organic

layer washed with water and then with a satured solution of $NaHCO_3$. After drying with Na_2SO_4, the solvent was removed under reduced pressure and the residue purified by flash chromatography using CH_2Cl_2/MeOH/NH_4OH (97:3:0.3) as eluting system. The title compound (54 mg, 49% yield) was obtained as an oil. IR (neat): ν 1708 (CO) cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.76 (d, J = 7.6 Hz, 2H, aromatic); 7.64 (d, J = 7.6 Hz, 2H, aromatic); 7.41 (t, J = 7.6 Hz, 2H, aromatic); 7.32 (t, J = 7.6 Hz, 2H, aromatic); 6.85 (s, 2H, aromatic); 5.11 (br s, 1H, CHO); 4.82 (s, 1H, aliphatic); 4.75 (m, 1H, CHO); 3.88 (s, 3H, OCH_3); 3.86 (s, 6H, $2OCH_3$); 2.76–2.52 (m, 2H, 2NCH); 2.29 (s, 3H, NCH_3); 2.13–1.38 (m, 16H, $8CH_2$) ppm. ^{13}C NMR ($CDCl_3$): δ 170.27 (C); 153.67 (C); 153.16 (C); 141.43 (C); 140.74 (C); 128.08 (CH aromatic); 127.31 (CH aromatic); 125.54 (CH aromatic); 120.04 (CH aromatic); 114.32 (C); 110.38 (CH aromatic); 86.33 (C); 80.29 (C); 73.53 (CHO); 71.28 (CHO); 61.03 (OCH_3); 58.60 (NCH); 56.27 (OCH_3); 53.53 (CH aliphatic); 32.75 (NCH_3); 30.70 (CH_2); 29.01 (CH_2); 26.71 (CH_2) ppm. The oily product was transformed into the hydrochloride and recrystallized from absolute ethanol/anhydrous diethylether. Mp: 140–143 °C. Anal. ($C_{39}H_{44}ClNO_7$) C, H, N.

Compounds **7b**, **7c** and **7d** were obtained in the same way from **11b**, **11c** and **11d** respectively. Compounds **6a–d** were obtained in the same way from **11a–d** using 2,2-bis(4-methoxyphenyl)acetic acid[40] and compounds **2a–d** and **3a–d** were obtained in the same way from **10a–d**[31] using 4-cyano-4-(3,4-dimethoxyphenyl)-5-methylhexanoic acid[39] or 9*H*-fluorene-9-carboxylic acid, respectively. All compounds were transformed into the hydrochloride. Their chemical and physical characteristics are reported in Table S1; IR and ^1H and ^{13}C NMR spectra are reported in Tables S2 and S3 (Supplementary data).

6.7. Biology

6.7.1. Cell lines and cultures

The K562 cell line is a highly undifferentiated erythroleukemia originally derived from a patient with chronic myelogenous leukemia.[41] The K562 leukemia cells and the P-gp expressing K562/DOX cells were obtained from Professor J.P. Marie (Hopital Hotel-Dieu, Paris, France). These cells were cultured following the previously reported protocol.[31]

6.8. Drugs and chemicals

Purified pirarubicin was provided by Laboratoire Roger Bellon (France). Concentrations were determined by diluting stock solutions to approximately 10^{-5} M and using ε_{480} = 11,500 M^{-1} cm^{-1}. Stock solutions were prepared just before use. Buffer solutions were HEPES buffer containing 5 mM HEPES, 132 mM NaCl, 3.5 mM $CaCl_2$, 5 mM glucose, at pH 7.3.

Doxorubicin hydrochloride (DOX) and verapamil were obtained from Sigma; dimethylsulphoxide (DMSO) and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. MTT stock solution was prepared following the previously described method.[31]

Test compounds and DOX stock solutions were prepared in DMSO at 10^{-2} M. Verapamil was prepared in water at 10^{-2} M. Drugs and test compounds were then diluted with complete medium to obtain the 10× desired final maximum test concentrations. Test compounds were evaluated for MDR reversal activity at 0.1 and 1.0 μM and the corresponding doxorubicin concentrations tested were between 0.001 and 10 μM.

Verapamil was used as a standard chemomodulator and was evaluated at 2 and 20 μM. All experiments were carried out in quadruplicate.

6.9. Cellular drug accumulation and MTT assay.

The uptake of pirarubicin in cells was followed by monitoring the decrease in the fluorescence signal at 590 nm (λ_{ex} = 480 nm) according to the previously described method.[50]

To evaluate the reverting activity of test compounds, the cells, in exponential growth phase (3–5 × 10^5 cells/mL), were seeded at 3000 cells/well and either solutions of test compounds or solution of doxorubicin or combination of a solution of doxorubicin and test compounds were added to the wells and the plates were incubated at 37 °C for 72 h in 5% CO_2 incubator. The MTT working solution (50 μL) was added and plates were further incubated for 3 h. Following incubation cells and formazan crystals were inspected microscopically. The supernatant was then carefully removed by slow aspiration and the formazan crystals were dissolved in 150 μL of DMSO; the absorbance of the solution was then read on an automated plate reader at a wavelength of 540 nm.

All results are presented as means ± SE and statistical analysis was performed using the one-way Anova test and Bonferroni's multiple comparison test (GraphPad Prism software, Inc. CA, USA.

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Supplementary data

Supplementary data (chemical and physical characteristics, IR, ^1H NMR and ^{13}C NMR spectra, elemental analyses of compounds **2a–d**, **3a–d**, **6a–d** and **7a–d** and LC–ESI-MS) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.11.019.

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