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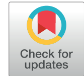
Research paper

Design and synthesis of new potent *N*,*N*-bis(arylalkyl)piperazine derivatives as multidrug resistance (MDR) reversing agents



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A B S T R A C T

A series of 1,4-substituted arylalkyl piperazine derivatives were synthesized and studied with the aim to obtain potent P-gp-dependent multidrug-resistant (MDR) reversers. The new compounds were designed on the basis of the structures of our previous arylamine ester derivatives endowed with high P-gp-dependent multidrug resistance reversing activity. All new compounds were active in the pirarubicin uptake assay on the doxorubicin–resistant erythroleukemia K562 cells (K562/DOX). In particular, compounds bearing methoxy aromatic moieties showed fairly high reversal activities. The most potent compounds, **8**, **9**, **10** and **13**, were further studied by evaluating their doxorubicin cytotoxicity enhancement (reversal fold, RF) and the inhibition of P-gp-mediated rhodamine-123 (Rhd 123) efflux on the K562/DOX cell line. The results of all pharmacological assays indicated that the combination of a basic piperazine scaffold with arylalkyl residues allowed us to obtain very interesting P-gp modulating compounds. Two long-lasting P-gp pump modulators (**9** and **10**) were identified; they were able to inhibit remarkably the P-gp substrate rhodamine-123 efflux on the resistant K562/DOX cell line after 60 min. Overall compound **9** appeared the most promising compound being a potent and long-lasting P-gp–dependent MDR modulator.

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1. Introduction

The success of the anticancer chemotherapy is frequently impaired by drug resistance that is the main defense mechanism that the tumor cells develop against chemotherapeutic drugs. Multidrug resistance (MDR) is a type of acquired drug resistance to multiple classes of structurally and mechanistically unrelated anticancer drugs [1]. The cells exhibiting MDR accumulate a lower intracellular concentration of drug as a result of an accelerated efflux of the antitumor agents mediated by an ATP-dependent process. The main mechanism responsible of this transport is the overexpression of ABC (ATP Binding Cassette) transmembrane proteins such as P-glycoprotein (P-gp, ABCB1), the multidrug-resistance-associated protein-1 (MRP1, ABCC1) and the breast cancer resistance protein (BCRP, ABCG2).

P-glycoprotein is the most studied of the ABC transporters. It is a membrane glycoprotein expressed in several important tissues and blood-tissue barriers where it plays important physiological roles as the regulation of the secretion of lipophilic molecules and the extrusion of exogenous toxic agents that enter the organism [2,3]. Nevertheless, P-gp is overexpressed in cancer cells as a result of an upregulation of the human gene expression MDR1 that causes an accelerated efflux of the chemotherapeutic drugs inducing the classical multidrug resistance (MDR) [4–6].

Circumvention of multidrug resistance through the inhibition of

Abbreviations: P-gp, P-glycoprotein; MRP1, Multidrug Resistance associated Protein 1; BCRP, Breast Cancer Resistance Protein; DOX, Doxorubicin; EDCl, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimmide hydrochloride; DMAP, 4-Dimethylaminopyridine; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; Rhd 123, rhodamine-123.

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the functions of P-gp is considered an important field of investigation. MDR reversers (chemosensitizers) are P-gp modulators that administered in combination with cytotoxic agents, which are substrates of the efflux pump, could restore their efficacy in resistant cancer cells [7,8].

Drug possessing inhibitory properties have been and are actively being sought and many P-gp modulators, belonging to three generations of compounds have been identified [9–12]. A few of them have reached clinical trials [13,14], nevertheless, no drug has been approved for therapy. In fact, the development of safe MDR reversers is complicated by the P-gp physiological roles and the concurrent inhibitory action of these compounds on isoforms of cytochrome, like CyP3A4. Therefore, they could interfere with the pharmacokinetics of other substances, including the co-administered chemotherapeutic agents [15]. Thus, an ideal MDR reverser should be a potent and selective P-gp modulator without affecting the cytochrome activity.

The latest MDR reversing compounds, in addition to high potency and low toxicity, show a good specificity for P-gp displaying no pharmacokinetic interaction with chemotherapeutic drugs [16]. In particular, the third generation P-gp modulator Zosuquidar is a potent and selective P-gp inhibitor with almost no interaction with the multidrug-resistance-associated protein-1 (MRP1) or the breast cancer resistance protein (BCRP) and little effect on CyP3A4 [13].

Zosuquidar structure is characterized by the presence of a piperazine scaffold that is present also in other important MDR reversers as Dofequidar [17] (Chart 1). These compounds contain many of the features considered important for P-gp interaction, such as high lipophilicity, the presence of hydrogen bond acceptor groups, aromatic moieties and one or more protonable nitrogen atoms [18].

For many years our research group has been involved in the design and synthesis of several P-glycoprotein (P-gp) ligands with the aim to discover potent MDR modulators. Their structures are related to those of the tropane alkaloid pervilleine A, endowed with good MDR modulating activity and the first generation modulator verapamil [19] (Chart 2). Therefore these molecules are characterized by the presence of a basic nitrogen flanked at suitable distance by aromatic moieties The majority of these molecules are arylamine ester derivatives; likewise pervilleine A, they contain aromatic ester residues connected to a basic scaffold by linkers with different flexibility such as polymethylenic chains or cyclohexane rings (Chart 2, structure I). Most of these compounds have proven to be potent and efficacious P-gp-dependent multidrug resistance reversers [19–23]. The ester functions present in these molecules may be susceptible to enzymatic hydrolysis; therefore, to avoid any possible problem associated with the metabolic lability of this group, in the present study we reported the synthesis and preliminary pharmacological properties of a new series of derivatives carrying a piperazine nucleus as basic scaffold, and different arylalkyl groups on the two nitrogen atoms, avoiding the presence of ester functions in analogy to the structure of verapamil (compounds **1**–**13**, Chart 2).

These compounds were designed on the basis of the above mentioned structural requirements that are considered important for P-gp interaction (Chart 3). Therefore, aromatic substituents were chosen such as the cinnamyl (**a**), the 2-phenoxyethyl (**b**), the 2-isopropyl-2-phenylpentanenitrile (**c**) and the 4,4-diphenylbutyl (**d**) groups, that contain unsubstituted phenyl rings. The corresponding methoxylated moieties **g**, **h**, **i**, **l**, respectively, were inserted to evaluate the role of the hydrogen bond acceptor methoxy group, that is considered important for the MDR-reversing activity and is present in many well-known P-gp modulating compounds [9]. For the same reason the 3,4,5-trimethoxybenzyl group (**m**) was also inserted. The nucleus **e** was chosen to investigate the importance of the presence of fluorine, and the 9-methylanthracene nucleus **f** because it was present in compounds previously studied that proved to be very potent multidrug resistance reversers [19–21].

The reversal activity of the new compounds was evaluated in a preliminary screening by the pirarubicin uptake assay on doxorubicin-resistant erythroleukemia K562 cells. The pharmacological profile of the most potent compounds, **8**, **9**, **10** and **13**, was further studied by evaluating their doxorubicin cytotoxicity enhancement (reversal fold, RF) and the inhibition of P-gp-mediated rhodamine-123 (Rhd 123) efflux on the K562/DOX cell line.

In addition, an investigation about the stability of derivatives **8**, **9**, **10** and **13** was performed by evaluating the concentration of their solutions in phosphate buffer solution (PBS) at different incubation times. Moreover, the behaviour of these compounds in the presence of rat and human plasma was also studied to establish the actual concentration in biological conditions. In fact, their available concentration can be affected by media solubility or high affinity plasma protein binding.

2. Chemistry

The reaction pathways used to synthesized piperazine derivatives **1**–**13**, are described in Scheme 1. Reaction of *N*-*t*-BOC-piperazine [24] with the suitable arylalkyl halide in CH_3CN under basic conditions (K_2CO_3) gave compounds **15**–**19**. The arylalkyl halides are commercially available ((*E*)-(3-bromoprop-1-enyl)benzene) or were synthesized according to the literature (5-bromo-2-isopropyl-2-phenyl-pentanenitrile [25], 2-bromoethoxy benzene [25] and 9-(chloromethyl)anthracene [26]). The arylalkyl halide 5-(2-bromoethoxy)-1,2,3-trimethoxybenzene (**14**), used to synthesize compound **19**, was obtained by reaction of 3,4,5-trimethoxyphenol with 1,2-dibromoethane in anhydrous DMF, in a different way with respect to Drain [27] (Scheme 2). Finally, compound **20** was obtained by reaction of *N*-*t*-BOC-piperazine [24] with (*E*)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-ol [28] in the presence of CH_3SO_2Cl and anhydrous Et_3N, in anhydrous CH_2Cl_2. Cleavage of the protective group on compounds **15**–**20** was performed with CF_3COOH to obtain compounds **21**–**26**. These intermediates, together with 1-(3,4,5-trimethoxybenzyl)piperazine **27**, already described [29], were *N*-alkylated with the suitable arylalkyl halide and K_2CO_3 in CH_3CN to yield final compounds **1**–**13**. The arylalkyl halides are mentioned above or synthesized according to the literature (1-bromo-4,4-diphenylbutane [30], 1-bromo-4,4-bis(4-fluorophenyl)butane [31], 5-bromo-2-(3,4-dimethoxyphenyl)-2-isopropylpentanenitrile [32], 1-bromo-4,4-bis(4-methoxyphenyl)butane [33] and 5-(chloromethyl)-1,2,3-trimethoxybenzene [34].

3. Results and discussion

3.1. Modulation of pirarubicin uptake

The P-gp modulating ability of compounds **1**–**13** was evaluated on K562/DOX doxorubicin resistant cells that overexpress only the membrane glycoprotein P-gp [35–38]. K562 is a human leukemia cell line established from a patient with chronic myelogenous leukemia in blast transformation [39]. The uptake of THP-adriamycin (pirarubicin) was measured by a continuous spectrofluorometric signal of anthracycline at 590 nm ($\lambda_{ex} = 480$ nm) after cell incubation, following the protocols reported in previous papers [32,40]. The P-gp modulating activity of the studied compounds on the pirarubicin uptake test is expressed by: *i*) $[I]_{0.5}$, which measures the potency of the modulator and represents the concentration that causes a half-maximal increase ($\alpha = 0.5$) in the nuclear

N	Ar
15	c
16	b
17	a
18	f
19	h
20	g

N	Ar
21	c
22	b
23	a
24	f
25	h
26	g
27[a]	m

N	Ar	Ar_1
1	c	a
2	c	b
3	b	d
4	a	d
5	a	e
6	g	i

N	Ar	Ar_1
7	h	i
8	h	l
9	g	l
10	m	l
11	f	l
12	g	m
13	g	f

Ar, Ar_1 =

Scheme 1. Reagents and conditions: (i) ArBr or ArCl, K_2CO_3, CH_3CN, or (E)-3-(3,4,5-trimethoxyphenyl)-prop-2-en-1-ol [28], CH_3SO_2Cl, anhydrous Et_3N, anhydrous CH_2Cl_2; (ii) CF_3COOH, CH_2Cl_2; (iii) Ar_1Br or Ar_1Cl, K_2CO_3, CH_3CN. [a] See Ref. [29].

Scheme 2. Reagents and conditions: (i) $BrCH_2CH_2Br$, K_2CO_3, anhydrous DMF.

concentration of pirarubicin, and *ii*) α_{max}, which represents the efficacy of the modulator and is the maximum increase in the nuclear concentration of pirarubicin in resistant cells that can be obtained with a given compound. The value of α varies between 0 (in the absence of the modulator) and 1 (when the amount of pirarubicin in resistant cells is the same as in sensitive cells).

The results obtained are reported in Table 1 together with those of verapamil, the gold standard of P-gp activity inhibition, used as reference compound. All the newly synthesized compounds were able to inhibit the activity of P-gp and their potencies and efficacies were higher than those of verapamil, with the exception of compound **5** which was as potent as verapamil. All molecules showed potency values ($[I]_{0.5}$) in the submicromolar range and were able to completely reverse P-gp-dependent pirarubicin extrusion (α_{max} close to 1), except compound **5**.

These results fulfill our expectations on the positive effect of the presence of the piperazine scaffold on the P-gp modulating activity. In general, compounds bearing methoxy aromatic moieties (**6**–**13**) showed higher potencies than those lacking the methoxy groups (**1**–**5**). In particular, compounds **8**, **9**, **10** and **13** displayed an outstanding activity in the nanomolar range ($[I]_{0.5} = 0.01$, 0.09, 0.03 and 0.06 μM, respectively). The most active compounds have the 4,4-bis(4-methoxyphenyl)butyl moiety (**l**) combined with the residues **h**, **g** and **m** (compounds **8**, **9** and **10**, respectively) or the 9-(methyl)anthracene moiety (**f**) combined with the (*E*)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-yl group (**g**) (compound **13**). The presence of fluorine as substituent on the aromatic rings (see moiety **e**), appears to be not positive for the activity, in fact compound **5** was nearly 6 times less potent than the non-substituted compound **4** ($[I]_{0.5} = 1.71$ and 0.28 μM, respectively).

These results are not unexpected since methoxy substituted aromatic residues were present in the most active compounds of the previously synthesized series [19–23], as well as in pervilleine A and verapamil. In fact, the activities of compounds **8**, **9**, **10** and **13** ($[I]_{0.5} = 0.01$–0.09 μM and $\alpha_{max} = 0.90$–0.99), that contain a combination of the residues **f**, **g**, **h**, **l** and **m**, are within the same values range of the most potent diester compounds of the polymethylenic or cyclohexylic series characterized by the presence of these aromatic residues [20–23]. These data proved that the removal of the ester function, which is present in our previous derivatives, did not compromise the good P-gp inhibitory activity of this new series of compounds.

3.2. Effects on doxorubicin cytotoxicity

The most interesting compounds in the pirarubicin uptake test, **8**, **9**, **10** and **13**, were further studied to assess their ability in the enhancement of doxorubicin cytotoxicity on the resistant K562/DOX cell line. First of all the compounds were studied to evaluate their intrinsic cytotoxicity in the absence of doxorubicin on both K562 and K562/DOX cell lines, at 0.1, 1.0 and 3.0 μM concentrations, using the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay [41]. The compounds had no intrinsic toxicity on the parental and resistant cell lines, at the three concentrations tested, except compound **10** which showed an intrinsic toxicity, not exceeding 30%, at 3.0 μM concentration on the resistant cell line (data not shown).

The MDR-reversal activity of the four compounds was assessed evaluating their ability to decrease the doxorubicin IC_{50} value on both cell lines, at the same three concentrations. Fig. 1 shows the cytotoxicity curves related to the effects of doxorubicin alone and in combination with compounds **8**, **9**, **10** and **13**, at 1.0 μM concentration, on K562-parental cells (A) and the K562/DOX-resistant subline (B).

As shown in Fig. 1A, the compounds did not change the IC_{50}

Table 1
MDR-reversing activity of compounds **1**–**13**.

N	Ar	Ar_1	$[I]_{0.5}$ μM[a]	α_{max}[b]
1	c	a	0.31 ± 0.08	0.98 ± 0.02
2	c	b	0.25 ± 0.03	0.97 ± 0.03
3	b	d	0.23 ± 0.05	0.99 ± 0.01
4	a	d	0.28 ± 0.03	0.98 ± 0.02
5	a	e	1.71 ± 0.40	0.84 ± 0.06
6	g	i	0.27 ± 0.03	0.91 ± 0.03
7	h	i	0.49 ± 0.10	0.99 ± 0.01
8	h	l	0.01 ± 0.02	0.95 ± 0.06
9	g	l	0.09 ± 0.03	0.90 ± 0.04
10	m	l	0.03 ± 0.01	0.99 ± 0.01
11	f	l	0.12 ± 0.04	0.98 ± 0.02
12	g	m	0.50 ± 0.15	0.99 ± 0.01

Table 1 (*continued*)

N	Ar	Ar_1	$[I]_{0.5}$ μM[a]	α_{max}[b]
13	**g**	**f**	0.06 ± 0.01	0.99 ± 0.01
verapamil			1.60 ± 0.30	0.70 ± 0.07

[a] Concentration of the modulator 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. Results are expressed as the mean ± SE of three independent experiments done at least three times.

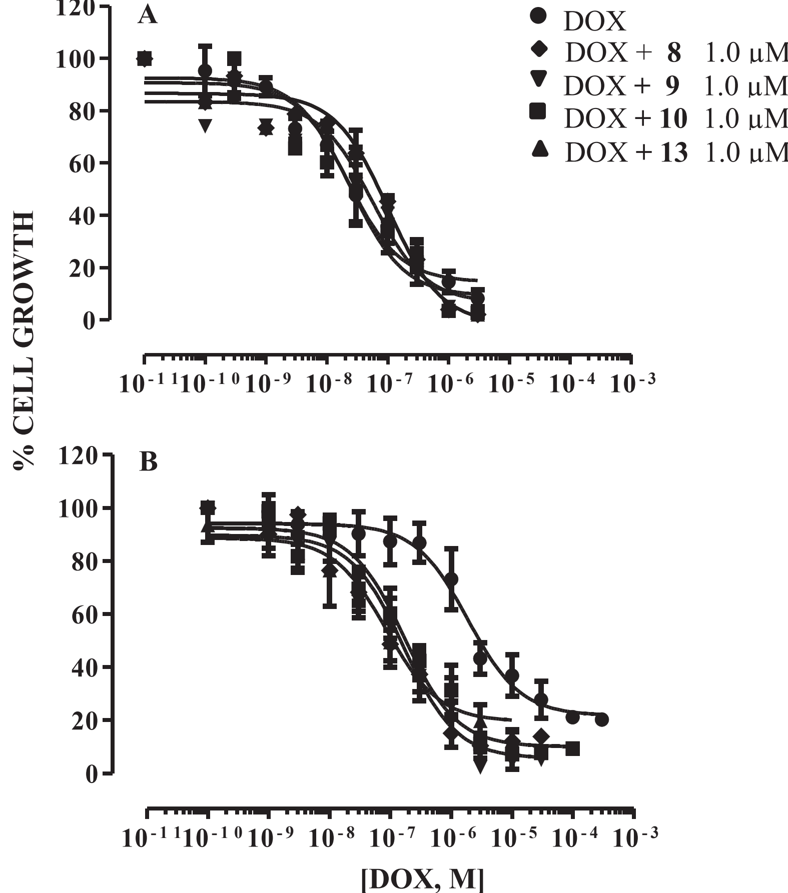


Fig. 1. Cytotoxicity curves related to the effects of doxorubicin alone and in combination with compounds **8**, **9**, **10** and **13**, at 1.0 μM concentration, on K562-parental cells (A) and K562/DOX-resistant subline (B). Each point of the curves is the mean ± SE of individual points determined in at least three experiments performed in quadruplicate.

value of doxorubicin on the parental cell line. Instead, in the resistant cell line they were able to decrease the IC_{50} value of doxorubicin restoring its cytotoxicity (Fig. 1B). These results indicate that these compounds have a good *in vitro* selectivity for the resistant cell line.

The IC_{50} values of doxorubicin tested alone and in combination with compounds **8**, **9**, **10** and **13**, on the K562/DOX cell line were shown in Table 2. The reversal-fold (RF) values, which indicate the MDR reverser potency of the tested compounds, are also reported. The RF values were obtained as the ratio between the doxorubicin IC_{50} values on K562/DOX cells in the absence and in presence of modulators. At the three concentrations tested (0.1, 1.0 and 3.0 μM), the four compounds caused no significant modification of the doxorubicin IC_{50} value (0.022 ± 0.004 μM) in the parental doxorubicin sensitive K562 line (data not shown).

Verapamil and the third generation chemosensitizer tariquidar, tested at 3.0 and 1.0 μM concentrations, respectively, were used as reference compounds.

As reported in Table 2, these compounds were able to restore a high cytotoxic effect of doxorubicin in the resistant cell line, showing concentration-dependent activities. All compounds had scarce activity at 0.1 μM concentration showing RF values ranging between 1.2 and 2.4, however at 1.0 μM and 3.0 μM concentrations they showed remarkable RF values.

Actually, the concentration-dependent activity of compound **8** was not very pronounced since its RF values at 0.1, 1.0 and 3.0 μM concentrations were 2.4, 4.5 and 8.8, respectively. Compounds **9**, **10** and **13** showed similar RF values at 1.0 μM concentration (12.9, 10.5

Table 2
Effects of compounds **8**, **9**, **10** and **13** on doxorubicin cytotoxicity on K562/DOX cells, at 0.1, 1.0 and 3.0 μM concentrations.

Compounds	K562/DOX cells	
	IC_{50} μM[a]	RF[b]
DOX	2.20 ± 0.060	
DOX + **8** (0.1 μM)	0.91 ± 0.005[c]	2.4
DOX + **8** (1.0 μM)	0.49 ± 0.020[c]	4.5
DOX + **8** (3.0 μM)	0.25 ± 0.050[c]	8.8
DOX + **9** (0.1 μM)	1.14 ± 0.570[c]	1.9
DOX + **9** (1.0 μM)	0.17 ± 0.050[c]	12.9
DOX + **9** (3.0 μM)	0.05 ± 0.008[c]	44.0
DOX + **10** (0.1 μM)	1.87 ± 0.130	1.2
DOX + **10** (1.0 μM)	0.21 ± 0.060[c]	10.5
DOX + **10** (3.0 μM)	0.03 ± 0.008[c]	73.3
DOX +**13** (0.1 μM)	1.28 ± 0.069	1.7
DOX + **13** (1.0 μM)	0.25 ± 0.056[c]	8.8
DOX + **13** (3.0 μM)	0.10 ± 0.019[c]	22.0
DOX + verapamil (3.0 μM)	0.74 ± 0.020[c]	3.0
DOX + tariquidar (1.0 μM)	0.07 ± 0.005[c]	31.4

[c] $p < 0.05$ vs control doxorubicin-treated.
[a] Mean ± SE of at least three determinations performed with quadruplicate cultures at each drug concentration tested and measured as described in Experimental section.
[b] Values of reversal fold (RF) of MDR obtained as the ratio between the doxorubicin IC_{50} value on K562/DOX cells in the absence and in presence of modulators.

and 8.8, respectively) whereas, at 3.0 μM concentration, the values were quite different (44.0, 73.3, 22.0, respectively) and the most potent compound, at this concentration, appears to be derivative **10** with a RF value of 73.3. In any case it should be considered that this compound showed, at the same concentration, some intrinsic cytotoxicity toward the resistant cell line. All compounds were more potent than verapamil, tested at 3.0 μM concentration, but less potent than tariquidar at 1.0 μM concentration; in fact, its RF value is higher than those of the four compounds at the same concentration.

The two most potent compounds **9** and **10** were also studied to assess their EC_{50} values on doxorubicin cytotoxicity enhancement, by evaluating the effect of increasing doses of modulators on a fixed doxorubicin dose. Verapamil was used as reference compound. For this experiment, we chose the 0.3 μM concentration of doxorubicin, which was non-toxic in K562/DOX cells if used in absence of modulators. The obtained results confirmed the good activity of compounds **9** and **10** that were able to cause the 100% death of K562/DOX cells when administered with the non-toxic 0.3 μM doxorubicin concentration, showing EC_{50} values of 2.7 and 1.9 μM, respectively. Differently, verapamil was unable to cause the death of 50% of K562/DOX cells, thus it was impossible to evaluate its EC_{50} value at the tested concentrations (from 0.003 μM to 30 μM) (experimental details are reported in the Supplementary material).

3.3. *Inhibition of P-gp-mediated rhodamine-123 (Rhd 123) efflux*

The ability of compounds **8**, **9**, **10** and **13** to inhibit the transport activity of the pump were measured using the P-gp specific substrate rhodamine-123 on the resistant K562/DOX cell line, following a previously described method with minor modifications [23]. The efflux of the fluorochrome was evaluated at two different times, 30 and 60 min, both in the presence and in absence of modulators at 1.0 μM concentration. On the parental cell line (K562), the studied compounds did not affect the fluorescence intensity, both in the accumulation and in the efflux phase, that was equal to that of the untreated control (data not shown).

The inhibition of P-gp-mediated Rhd 123 efflux of compounds **8**, **9**, **10** and **13** on K562/DOX cells is reported in Fig. 2. Verapamil, tested at 3.0 μM concentration, was used as reference compound.

Fig. 2A shows the mean fluorescence intensity (MFI) ratio values of compounds **8**, **9**, **10** and **13**, at 30 and 60 min of efflux (table and histograms), obtained as the ratio between the median fluorescence intensity value of samples treated with modulators, and the untreated control sample, as reported in the Experimental Section. MFI ratio values near the unit were indicative of absence of modulation, values greater than 1 represented inhibition of pump activity due to the presence of the compounds.

The MFI ratio values showed that all the tested compounds were able to inhibit the efflux of Rhd 123 at both efflux times, to different extent. The most potent compounds were **9** and **10** that were able to increase the Rhd 123 intracellular fluorescence at 30 min, by nearly 100 and 30 times, respectively. Remarkable MFI ratio values were showed also at 60 min, since these two compounds were able to maintain a Rhd 123 intracellular fluorescence by 50 and 10 times, respectively, higher than the untreated control.

All compounds tested at 1.0 μM concentration, were more potent than verapamil, tested at the concentration of 3.0 μM, at both efflux times. In fact, the increase of the Rhd 123 intracellular fluorescence, at 30 min, of compounds **8** and **13** was two or three times higher than verapamil (MFI ratio = 5.5, 7.5 and 2.3, respectively); after 60 min the Rhd 123 intracellular fluorescence in the cells treated with verapamil is the same as the control (MFI ratio = 1.0), while compounds **8** and **13** were still able to inhibit the efflux since their MFI ratio values were 1.4 and 2.4, respectively.

Fig. 2B shows the fluorescence curves corresponding to the Rhd 123 uptake after 30 min of incubation, and to the Rhd 123 efflux after 60 min in Rhd 123-free medium, in the absence (panel a) and in presence of verapamil or the studied compounds (panels b–f).

The treatment with verapamil and compounds **8**, **9**, **10** and **13**, caused a Rhd 123 intracellular retention higher than the untreated control, since the corresponding curves (red) were shifted to the right respect to the curve of the control (green). Compounds **9** and **10** showed a higher shift of the curves than compounds **8** and **13**, and all four compounds were more potent than verapamil in the accumulation phase.

The fluorescence curves corresponding to the Rhd 123 efflux showed that compounds **8**, **9**, **10** and **13** were able to inhibit the pump even after 60 min in Rhd 123-free medium, whereas verapamil caused an intracellular amount of Rhd 123 similar to the control (as also shown in Fig. 2A). In the case of compounds **9** and **10**, the curves were less shifted to the left, indicating a higher retention of the intracellular fluorescence.

The results obtained suggested that the four studied compounds were able to modulate the transport activity of the P-gp through a long duration pump inhibition; in particular, compounds **9** and **10** appear to be effective long-lasting P-gp pump modulators.

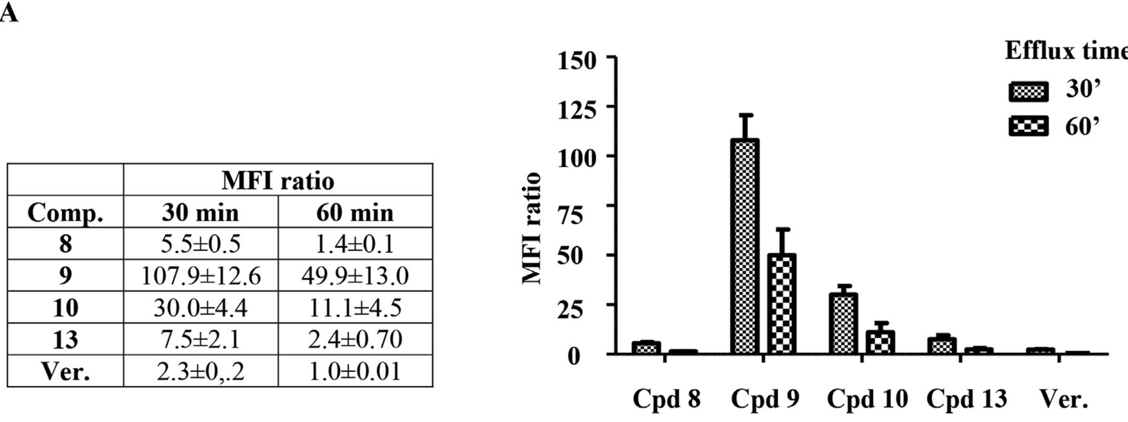
3.4. *Chemical stability test*

A stability study was performed to evaluate the actual concentration of compounds **8**, **9**, **10** and **13** in phosphate buffer solution (PBS), human and rat plasma samples.

The samples were analyzed by LC-MS/MS method operating in Multiple Reaction Monitoring (MRM) mode [42]. The solution stability of each studied compound was verified by monitoring the variation of analyte concentration at different incubation times in phosphate buffer solution (PBS), human or rat plasma samples. Each set of samples was incubated at 37 °C in triplicate at four different times, 0, 30, 60 and 120 min. The human plasma batch was collected from a pool of healthy volunteers and the rat plasma batch was collected from a pool of Sprague Dawley male rats (see Experimental Section). The raw data processing and evaluation of calibration results are reported in Supplementary material.

The obtained solution stability profiles showed that the

A



	MFI ratio	
Comp.	30 min	60 min
8	5.5±0.5	1.4±0.1
9	107.9±12.6	49.9±13.0
10	30.0±4.4	11.1±4.5
13	7.5±2.1	2.4±0.70
Ver.	2.3±0,.2	1.0±0.01

B

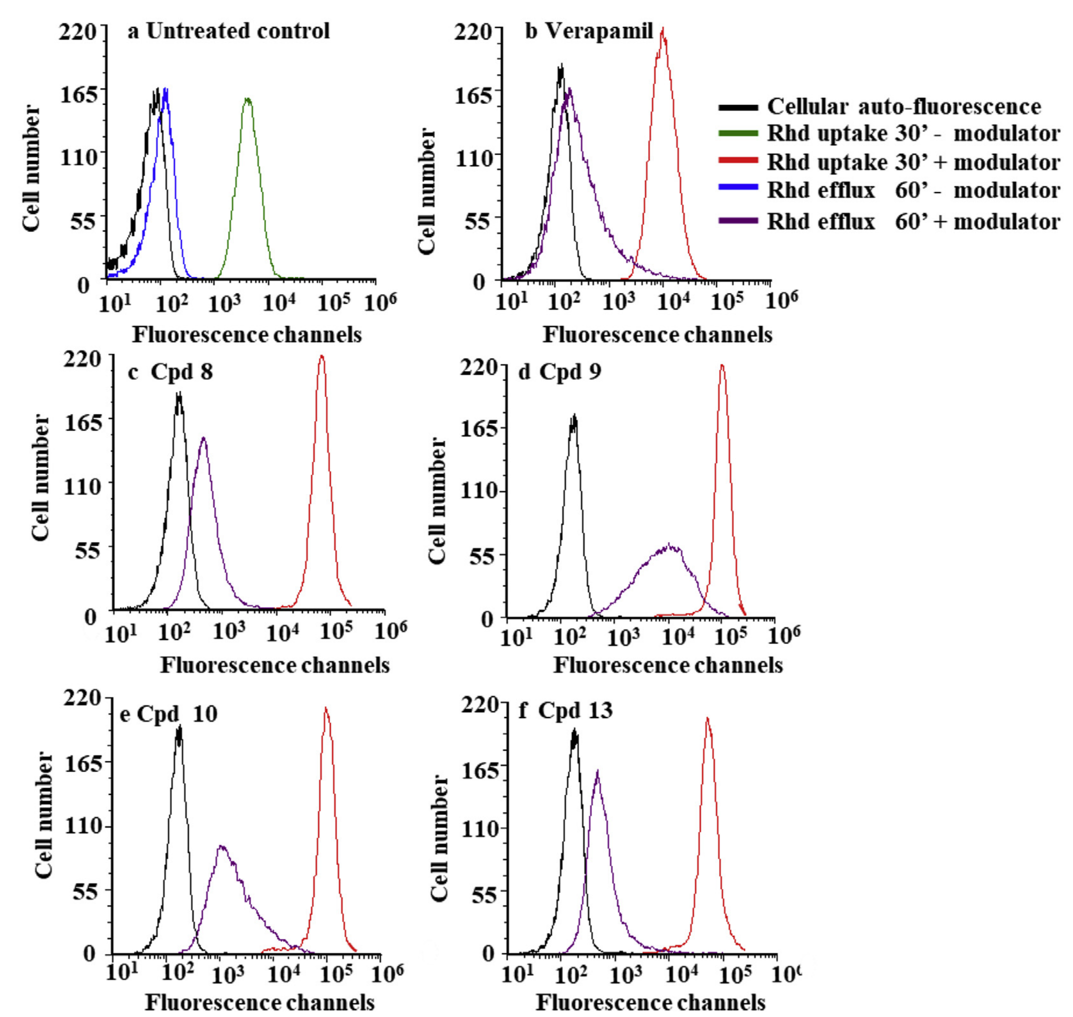


Fig. 2. Inhibition of P-gp-mediated Rhd 123 efflux of compounds **8**, **9**, **10** and **13** on K562/DOX cells. (A) Mean fluorescence intensity (MFI) ratio values in K562/DOX cells incubated with Rhd 123 in the presence of compounds **8**, **9**, **10** and **13**, tested at 1.0 μM concentration, and verapamil tested at 3.0 μM concentration. The data were calculated at two different efflux times, 30 and 60 min, as reported in the Experimental Section, and expressed as the mean ± SE of three independent experiments done at least three times. (B) Effects of compounds **8**, **9**, **10** and **13,** tested at 1.0 μM concentration, on Rhd 123 uptake and efflux in K562/DOX cells. The fluorescence curves corresponding to the Rhd 123 efflux were calculated after 60 min of incubation in Rhd 123-free medium. Each peak is identified by a fluorescence channel number and designates the amount of intracellular Rhd 123 in each sample in the absence and in presence of the tested compounds.

Chart 1. Third generation P-gp modulators.

Chart 2. Structures of lead and designed compounds.

Chart 3. General structure of the newly synthesized piperazine derivatives.

concentrations of compounds **8**, **9**, **10** and **13** did not varied until 120 min in the studied media (see Supplementary material). These data indicate that the solutions of compounds **8**, **9**, **10** and **13** were not susceptible to degradation in phosphate buffer solution (PBS), human or rat plasma samples. Moreover, the steady concentration of these compounds in human and rat plasma revealed that their available concentrations were not affected by high affinity plasma protein binding; this protein interaction could cause a loss of a fraction of analyte in the pellet precipitation during plasma proteins denaturation [43].

4. Conclusions

A series of derivatives containing a piperazine scaffold 1,4-substituted with different arylalkyl groups on the two nitrogen atoms, were synthesized and evaluated for P-gp inhibitory activity on doxorubicin-resistant erythroleukemia K562 cell line (K562/DOX). These new compounds showed good P-gp modulating ability in the pirarubicin assay with potency values that are, in general, higher than that of the reference compound verapamil. The efficacy values (α_{max}) are in most cases close to 1, meaning that they can nearly completely reverse P-gp-dependent pirarubicin extrusion.

The potency was positively influenced by the presence of methoxy groups in the arylalkyl residues, in fact, compounds **6**–**13**, bearing methoxy aromatic moieties, were more potent than those with methoxy unsubstituted aromatic moieties (**1**–**5**). In particular, compounds **8**, **9**, **10** and **13** displayed an outstanding activity with $[I]_{0.5}$ values in the nanomolar range. These compounds are characterized by the presence of the trimethoxy substituted arylalkyl groups **g**, **h** or **m** combined with a polyaryl group as **l** or **f**, on the two piperazine nitrogen atoms. The pharmacological profile of compounds **8**, **9**, **10** and **13** was further evaluated. Thus, compounds

8, **9**, **10** and **13** were tested in the doxorubicin cytotoxicity enhancement test on K562/DOX cells, that revealed that they were able to restore the cytotoxicity of doxorubicin in a concentration-dependent manner. In particular, compounds **9** and **10** showed good reversal activities at 1.0 and 3.0 μM concentrations; in fact they were able to reduce the doxorubicin IC_{50} values by at least 10 times at 1.0 μM concentrations and by 44 times and 73 times-, respectively, at 3.0 μM concentrations. Also in the rhodamine-123 efflux test the most interesting compounds were **9** and **10** that were able to reduce the efflux of Rhd 123 due to the P-gp transport activity, maintaining a high inhibition of the pump at least up to 60 min in Rhd 123-free medium.

The solution stability profiles performed showed that these compounds were stable in phosphate buffer solution (PBS), human and rat plasma and that their available concentrations were not affected by high affinity plasma protein binding. Therefore, under the experimental conditions employed, their half-life values were estimated over 4 h.

In summary, these studies suggest that the combination of a basic piperazine scaffold with aromatic residues, that characterized our previous polymethylenic or cyclohexylic series, appears to be a fruitful approach. The presence of an alkylamine function in place of a potentially metabolic unstable ester function did not compromise the P-gp inhibitory activity of this series of compounds that were able to inhibit the P-gp pump with good potencies and efficacies; in fact, their activity values were within the same values of the most potent diester compounds of our previous series. The remarkable activity of derivatives with methoxy substituted arylalkyl moieties on the 1,4-piperazine nitrogen atoms confirmed the positive influence of this hydrogen bond acceptor group on the P-gp modulation as it had been shown by the lead compounds and many well-known potent P-gp modulating compounds. Two long-lasting P-gp pump modulators were identified since compounds **9** and **10** were able to inhibit remarkably the P-gp substrate rhodamine-123 efflux on the resistant K562/DOX cell line after 60 min. Among the piperazine derivatives evaluated in this study, compound **9** presented the best pharmacological profile since it had no intrinsic toxicity to the parental and resistant cell line, and showed a outstanding activity in all pharmacological assays. Moreover it showed a peculiar binding kinetics that allowed it to inhibit the P-gp extrusion activity in an noteworthy way even after 60 min. Further development of compound **9** could be promising to the identification of potent and long-lasting efficacious P-gp–dependent MDR modulators.

5. Experimental section

5.1. Chemistry

All melting points were taken on a Büchi apparatus and are uncorrected. Infrared spectra were recorded with a Perkin-Elmer Spectrum RX I FT-IR spectrophotometer in Nujol mull for solids and neat for liquids. NMR spectra were recorded on a Bruker Avance 400 spectrometer (400 MHz for ^{1}H NMR, 100 MHz for ^{13}C NMR) using residual solvent such as chloroform (δ = 7.26) as internal standard. Chromatographic separations were performed on a silica gel column by gravity chromatography (Kieselgel 40, 0.063–0.200 mm; Merck) or flash chromatography (Kieselgel 40, 0.040–0.063 mm; Merck). Yields are given after purification, unless otherwise stated. When reactions were performed in anhydrous conditions, the mixtures were maintained under nitrogen. The combustion analyses of compounds **1**–**13** are indicated by symbols, and the analytical results are within ±0.4% of the theoretical values. ESI-MS spectra were obtained using a Varian 1200L triple quadrupole system (Palo Alto, CA, USA) equipped by Elettrospray Source (ESI) operating in both positive and negative ions. Except for compounds **3** and **4**, the free bases form of the final compounds were transformed into the dihydrochloride by treatment with 2.2 eq of acetyl chloride in anhydrous CH_3OH. The salts were crystallized from abs. ethanol/petroleum ether. Compounds were named following IUPAC rules as applied by ChemBioDraw Ultra 14.0 software.

*5.1.1. 5-(2-Bromoethoxy)-1,2,3-trimethoxybenzene **14***

To a solution of 3,4,5-trimethoxyphenol (1.0 g, 5.43 mmol) in anhydrous DMF, K_2CO_3 (6.76 g, 48.90 mmol) and 1,2-dibromoethane (1.22 g, 6.38 mmol) were added. The mixture was heated to 60 °C for 24 h and then treated with a saturated solution of NaCl and extracted three times with ethyl acetate. After drying with Na_2SO_4, the solvent was removed under reduced pressure. The crude product was purified by flash chromatography using cyclohexane/ethyl acetate 7:3 as eluting system. The title compound (1.15 g, 75% yield) was obtained as a colorless oil.

^{1}H NMR ($CDCl_3$): δ 6.16 (s, 2H, arom.); 4.26 (t, J = 6.0 Hz, 2H, OCH_2); 3.84 (s, 6H, OCH_3); 3.78 (s, 3H, OCH_3); 3.62 (t, J = 6.0 Hz, 2H, CH_2Br) ppm.

*5.1.2. General procedure for the synthesis of N-arylalkylpiperazine-N-carboxylic acid tert-butyl esters **15**–**19***

To a 1 mmol portion of the appropriate arylalkyl halide (5-bromo-2-isopropyl-2-phenylpentanenitrile [25], 2-bromoethoxy benzene [25], (E)-(3-bromoprop-1-enyl)benzene, 9-(chloromethyl)anthracene [26] and 5-(2-bromoethoxy)-1,2,3-trimethoxybenzene (**14**). and anhydrous K_2CO_3 (1.2 mmol) in 5 mL of anhydrous CH_3CN a solution of *N-t*-BOC-piperazine [24] (185 mg, 1 mmol) was added. The reaction mixture was heated at 60 °C for 5–6 h and stirred overnight at room temperature. Then the organic layer was washed three times with a saturated solution of $NaHCO_3$. After drying with Na_2SO_4, the solvent was removed under reduced pressure. The substances obtained were purified by flash chromatography or used as such for the next reaction, in the case of **19**.

*5.1.2.1. 4-(4-Cyano-5-methyl-4-phenylhexyl)piperazine-1-carboxylic acid tert-butyl ester **15***. Oil. Chromatographic eluent: abs EtOH/NH_4OH/CH_2Cl_2/ethyl ether/petroleum ether 42:2.5:180:180:560. Yield: 72.6%. IR (neat): ν 2234 (CN), 1696 (CO) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 7.35 (d, J = 3.5 Hz, 4H, arom.); 7.28 (d, J = 3.0 Hz, 1H, arom.); 3.35 (s, 4H, CH_2); 2.26–2.21 (m, 6H, CH_2); 2.16–2.08 (m, 3H, *CH*$_2$-*CH*H); 1.93–1.87 (td, J = 8.0 Hz, J = 4.3 Hz, 1H, CH*H*); 1.54–1.52 (m, 1H, CH); 1.42 (s, 9H, CH_3); 1.18 (d, J = 6.7 Hz, 3H, CH_3); 0.75 (d, J = 6.7 Hz, 3H, CH_3) ppm.

*5.1.2.2. 4-(2-Phenoxyethyl)piperazine-1-carboxylic acid tert-butyl ester **16***. Oil. Chromatographic eluent: abs EtOH/NH_4OH/CH_2Cl_2/ethyl ether/petroleum ether 42:2.5:180:180:560. Yield: 72.6%. IR (neat): ν 2234 (CN), 1696 (CO) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 7.35 (d, J = 3.5 Hz, 4H, arom.); 7.28 (d, J = 3.0 Hz, 1H, arom.); 3.35 (s, 4H, CH_2); 2.26–2.21 (m, 6H, CH_2); 2.16–2.08 (m, 3H, *CH*$_2$-*CH*H); 1.93–1.87 (td, J = 8.0 Hz, J = 4.3 Hz, 1H, CH*H*); 1.54–1.52 (m, 1H, CH); 1.42 (s, 9H, CH_3); 1.18 (d, J = 6.7 Hz, 3H, CH_3); 0.75 (d, J = 6.7 Hz, 3H, CH_3) ppm.

*5.1.2.3. 4-Cinnamylpiperazine-1-carboxylic acid tert-butyl ester **17***. Oil. Chromatographic eluent: abs EtOH/NH_4OH/CH_2Cl_2/ethyl ether/petroleum ether 180:9.9:360:360:900. Yield: 63%. IR (neat): ν 1692 (CO) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 7.39–7.33 (m, 2H arom.); 7.32–7.26 (m, 2H arom.); 7.23–7.19 (m, 1H arom.); 6.52 (d, J = 15.8 Hz, 1H, *CH*=CH); 6.30–6.21 (m, 1H, CH=*CH*); 3.46 (s, 4H, CH_2); 3.15 (d, 2H, CH_2N, J = 6.8 Hz); 2.44 (s, 4H, CH_2); 1.46 (s, 9H, CH_3) ppm.

*5.1.2.4. Anthracen-9-ylmethylpiperazine-1-carboxylic acid tert-butyl ester **18**.* Orange solid, mp: 148–151 °C. Yield: 89%. IR (neat): ν 1692 (CO) cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.48 (s, 1H, arom.); 8.44 (d, *J* = 12.4 Hz, 2H, arom.); 8.01 (d, *J* = 8.0 Hz, 2H, arom.); 7.58–7.42 (m, 4H, arom.); 4.44 (s, 2H, CH_2); 3.38 (bs, 4H, CH_2); 2.57 (bs, 4H, CH_2); 1.58–1.37 (m, 9H, CH_3) ppm.

5.1.2.5. 4-[2-(3,4,5-Trimethoxyphenoxy)ethyl]piperazine-1-carboxylic acid tert-butyl ester 19. Oil. Chromatographic eluent: CH2Cl2/MeOH/NH4OH 95:5:0.5. Yield: 85%. IR (neat): ν 1692 (CO) cm−1. 1H NMR (CDCl3) δ: 6.14 (s, 2H, arom.); 4.05 (t, J = 5.6 Hz, 2H, OCH2); 3.82 (s, 6H, OCH3); 3.77 (s, 3H, OCH3); 3.45 (t, J = 5.6 Hz, 4H, CH2); 2.78 (t, J = 6.0 Hz, 2H, CH2); 2.50 (t, J = 6.0 Hz, 4H, CH2); 1.44 (s, 9H, CH3) ppm.

*5.1.2.6. (E)-4-[3-(3,4,5-Trimethoxyphenyl)allyl]piperazine-1-carboxylic acid tert-butyl ester **20**.* A solution of 501.0 mg of (E)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-ol [28] (2.24 mmol) and anhydrous Et_3N (0.37 mL, 2.68 mmol) in 6 mL of anhydrous CH_2Cl_2 was cooled to 0 °C and CH_3SO_2Cl (0.21 mL, 2.24 mmol) was added. The reaction mixture was stirred at 0 °C for 45 min and then at room temperature for 3 h. Then solid *N-t*-BOC-piperazine [24] (417.0 mg, 2.24 mmol) was added and the reaction mixture was stirred for 18 h at room temperature and heated to 60 °C for 6 h. Then the organic layer was washed three times with a saturated solution of $NaHCO_3$. After drying with Na_2SO_4, the solvent was removed under reduced pressure. The crude product was purified by chromatography using CH_2Cl_2/MeOH 98:2 as eluting system. The title compound (251.0 mg, 29% yield) was obtained as a yellow oil. IR (neat): ν 1692 (CO) cm^{-1}. ^1H NMR ($CDCl_3$): δ 6.57 (s, 2H, arom.); 6.41 (d, *J* = 16.0 Hz, 1H, *CH*=CH); 6.13 (dt, *J* = 16.0 Hz, 6.8 Hz, 1H, CH=*CH*); 3.82 (s, 6H, OCH_3); 3.80 (s, 3H, OCH_3); 3.52–3.38 (m, 4H, CH_2); 3.11 (d, *J* = 6.8 Hz, 2H, CH_2); 2.53–2.36 (m, 4H, CH_2); 1.42 (s, 9H, CH_3) ppm.

5.1.3. General procedure for the synthesis of N-arylalkylpiperazines **21–26**

A 1 mmol portion of the suitable *N*-arylalkyl-piperazine-*N*-carboxylic acid *tert*-butyl esters (**15–20**) was dissolved in 10 mL of anhydrous CH_2Cl_2 and 2 mL of CF_3COOH were added. The reaction mixture was stirred for 2 h at room temperature. The solvent was removed under reduced pressure and the residue was washed three times with a saturated solution of $NaHCO_3$. The organic layer was dried with Na_2SO_4, and the solvent was removed under reduced pressure yielding a substance which was used as such for the next reaction.

*5.1.3.1. 4-(4-Cyano-5-methyl-4-phenylhexyl)piperazine **21**.* Oil. Yield: 100%. IR (neat): ν 2233 (CN), 3272 (NH) cm-1. 1H NMR (CDCl3): δ 7.26 (t, *J* = 6.4 Hz, 4H, arom.); 7.20–7.16 (m, 1H, arom.); 2.73 (t, *J* = 4.7 Hz, 4H, CH2); 2.15 (t, *J* = 7.2 Hz, 6H, CH2); 2.09–2.01 (m, 2H, CH2); 1.96 (s, 1H, NH); 1.80 (td, *J* = 8.0 Hz, *J* = 4.4 Hz, 1H, CHH); 1.50–1.42 (m, 1H, CHH); 1.10 (d, *J* = 6.8 Hz, 3H, CH3); 1.06–0.99 (m, 1H, CH); 0.67 (d, *J* = 6.8 Hz, 3H, CH3) ppm.

*5.1.3.2. 1-(2-Phenoxyethyl)piperazine **22**.* Oil. Yield: 93%. IR (neat): ν 3300 (NH) cm-1. 1H NMR (CDCl3): δ 7.31–7.24 (m, 2H, arom.); 6.96–6.90 (m, 3H, arom.); 4.11 (t, *J* = 5.9 Hz, 2H, CH2O); 2.97–2.86 (m, 4H, CH2); 2.80 (t, *J* = 5.9 Hz, 2H, CH2N); 2.55 (s, 4H, CH2); 1.83 (bs, 1H, NH) ppm.

*5.1.3.3. 1-Cinnamylpiperazine **23**.* Oil. Yield: 99%. IR (neat): ν 3300 (NH) cm-1. 1H NMR (CDCl3): δ 7.40–7.30 (m, 1H arom.); 7.31–7.27 (m, 3H arom.); 7.24–7.19 (m, 1H arom.); 6.51 (d, *J* = 15.8 Hz, 1H, CH=CH); 6.31–6.22 (m, 1H, CH=CH); 3.14 (d, *J* = 5.64 Hz, 2H, CH2); 2.95–2.83 (m, 4H, CH2); 2.47 (s, 4H, CH2); 2.29 (bs, 1H, NH) ppm.

*5.1.3.4. 1-Anthracen-9-ylmethylpiperazine **24**.* Brown oil. Yield: 93%. IR (neat): ν 3300 (NH) cm-1. 1H NMR (CDCl3): δ 8.49 (d, *J* = 8.8 Hz, 2H, arom.); 8.42 (s, 1H, arom.); 8.00 (d, *J* = 8.0 Hz, 2H, arom.); 7.58–7.42 (m, 4H, arom.); 4.42 (s, 2H, CH2); 2.82 (t, *J* = 4.8 Hz, 4H, CH2); 2.72–2.52 (m, 4H, CH2); 2.14 (bs, 1H, NH) ppm.

*5.1.3.5. 1-[2-(3,4,5-Trimethoxyphenoxy)ethyl]piperazine **25**.* Brown solid, mp: 76–80 °C. Yield: 94%. IR (neat): ν 3300 (NH) cm-1. 1H NMR (CDCl3): δ 6.12 (s, 2H, arom.); 4.04 (t, *J* = 5.6 Hz, 2H, OCH2); 3.80 (s, 6H, OCH3); 3.74 (s, 3H, OCH3); 2.89 (t, *J* = 5.6 Hz, 4H, CH2); 2.79 (t, *J* = 5.6 Hz, 2H, NCH2); 2.52 (bs, 4H, CH2); 2.02 (bs, 1H, NH) ppm.

*5.1.3.6. (E)-1-[3-(3,4,5-Trimethoxyphenyl)allyl]piperazine **26**.* Yellow-orange oil. Yield: 67%. IR (neat): ν 3300 (NH) cm^{-1}. ^1H NMR ($CDCl_3$): δ 6.59 (s, 2H, arom.); 6.43 (d, *J* = 16.0 Hz, 1H, *CH*=CH); 6.17 (dt, *J* = 16.0 Hz, *J* = 6.8 Hz, 1H, CH=*CH*); 3.85 (s, 6H, OCH_3); 3.82 (s, 3H, OCH_3); 3.12 (d, *J* = 6.8 Hz, 2H, CH_2); 3.00–2.79 (m, 4H, CH_2); 2.48 (bs, 4H, CH_2); 2.18 (bs, 1H, NH) ppm.

5.1.4. General procedure for the synthesis of N,N-bis(arylalkyl) piperazines **1–13**

To a mixture of a 1 mmol portion of the suitable *N*-arylalkylpiperazine (**21–27**) and anhydrous K_2CO_3 (1.2 mmol), the appropriate arylalkyl halide (1 mmol) ((E)-(3-bromoprop-1-enyl)benzene, 2-bromo-ethoxy benzene [25], 1-bromo-4,4-diphenylbutane [30], 1-bromo-4,4-bis(4-fluorophenyl)butane [31], 5-bromo-2-(3,4-dimethoxyphenyl)-2-isopropylpentanenitrile [32], 1-bromo-4,4-bis(4-methoxyphenyl)butane [33], 5-(chloromethyl)-1,2,3-trimethoxybenzene [34], 9-(chloromethyl)anthracene [26]) dissolved in 5 mL of anhydrous CH_3CN, was added. The reaction mixture was heated at 60 °C for 5–6 h and stirred overnight at room temperature. Then CH_2Cl_2 was added and the organic layer was washed three times with a saturated solution of $NaHCO_3$. After drying with Na_2SO_4, the solvent was removed under reduced pressure. The crude product was then purified by flash chromatography using the appropriate eluting system, yielding the desired compound as an oil or solid. The compounds were transformed into the corresponding dihydrochloride as white solid or used as free base (**3** and **4**). The salts were crystallized from abs. ethanol/petroleum ether.

*5.1.4.1. 5-(4-Cinnamylpiperazin-1-yl)-2-isopropyl-2-phenylpentanenitrile **1**.* Free base: chromatographic eluent: CH2Cl2/MeOH 95:5. Yield: 95%. IR (neat): ν 2234 (CN) cm-1. 1H NMR (CDCl3): δ 7.38 (t, *J* = 3.5 Hz, 5H, arom.); 7.34–7.22 (m, 5H, arom.); 6.53 (d, *J* = 15.8 Hz, 1H, CH=CH); 6.31–6.24 (m, 1H, CH=CH); 3.16 (d, *J* = 6.7 Hz, 2H, CH2); 2.50 (bs, 4H, CH2); 2.40 (bs, 4H, CH2); 2.30 (t, *J* = 7.0 Hz, 2H, CH2); 2.20–2.10 (m, 2H, CH2); 1.91 (td, *J* = 8.0 Hz, *J* = 4.5 Hz, 1H, CHH); 1.60–1.54 (m, 1H, CHH); 1.22 (d, *J* = 6.7 Hz, 3H, CH3); 1.18–1.13 (m, 1H, CH); 0.79 (d, *J* = 6.7 Hz, 3H, CH3) ppm. 13C NMR (CDCl3): δ 138.14 (CH2); 136.85 (C); 133.31 (CH); 128.74 (CH arom.); 128.65 (CH arom.); 128.57 (CH arom.); 126.35 (CH arom.); 126.17 (CH); 121.21 (C); 60.92 (C); 57.79 (C); 53.72 (CH2); 52.91 (CH2); 37.73 (CH); 35.47 (CH2); 22.18 (CH2); 18.95 (CH3); 18.61 (CH3). Dihydrochloride. Mp: 234–235 °C. Anal: C27H37Cl2N3 (C, H, N).

5.1.4.2. 2-Isopropyl-5-(4-(2-phenoxyethyl)piperazin-1-yl)-2-phenylpentanenitrile 2. Free base: chromatographic eluent: CH2Cl2/MeOH 95:5. Yield: 70%. IR (neat): ν 2233 (CN), cm-1. 1H NMR (CDCl3): δ 7.38–7.26 (m, 7H, arom.); 6.97–6.90 (m, 3H, arom.); 4.10 (t, *J* = 5.9 Hz, 2H, CH2); 2.80 (t, *J* = 5.9 Hz, 2H, CH2);

2.60 (bs, 4H, CH2); 2.40 (bs, 4H, CH2); 2.31–2.20 (m, 2H, CH2); 2.19–2.10 (m, 2H, CH2); 1.91 (td, *J* = 8.0 Hz, *J* = 4.4 Hz, 1H, CHH); 1.60–1.53 (m, 1H, CHH); 1.22 (d, *J* = 6.7 Hz, 3H, CH3); 1.18–1.12 (m, 1H, CH); 0.79 (d, *J* = 6.7 Hz, 3H, CH3) ppm. 13C NMR (CDCl3): δ 138.00 (C); 129.45 (CH arom.); 128.79 (CH arom.); 127.66 (CH arom.); 126.36 (CH arom.); 121.17 (CN); 120.90 (CH arom.); 114.58 (CH arom.); 65.53 (CH2); 57.54 (C); 56.97 (C); 53.68 (CH2); 52.97 (CH2N); 52.95 (CH2); 37.74 (CH); 35.34 (CH2); 22.52 (CH2); 18.95 (CH3); 18.61 (CH3) ppm. Dihydrochloride. Mp: 208–212 °C. Anal: C26H37Cl2N3O (C, H, N).

5.1.4.3. 1-(4,4-Diphenylbutyl)-4-(2-phenoxyethyl)piperazine ***3***. Free base: chromatographic eluent: abs. EtOH./NH_4OH/CH_2Cl_2/ethyl ether/petroleum ether 180:9.9:360:360: 900. Mp: 75–77 °C. Yield: 50%. ^{1}H NMR ($CDCl_3$): δ 7.32–7.26 (m, 10H, arom.); 7.21–7.17 (m, 2H arom.); 6.99–6.92 (m, 3H arom.); 4.13 (t, *J* = 5.9 Hz, 2H, CH_2O); 3.93 (t, *J* = 7.8 Hz, 1H, CH); 2.84 (t, *J* = 5.9 Hz, 2H, CH_2); 2.63 (bs, 4H, CH_2); 2.48 (bs, 4H, CH_2); 2.40 (t, *J* = 7.5 Hz, 2H, CH_2); 2.10 (q, *J* = 7.8 Hz, 2H, CH_2); 1.52–1.47 (m, 2H, CH_2) ppm. ^{13}C NMR ($CDCl_3$): δ 158.77 (C); 145.04 (C); 129.45 (CH arom.); 128.44 (CH arom.); 127.86 (CH arom.); 126.12 (CH arom.); 120.80 (CH arom.); 114.62 (CH arom.); 65.79 (CH_2); 58.55 (CH_2); 57.25 (CH_2); 53.62 (CH_2N); 53.14 (CH_2N); 51.36 (CH); 33.61 (CH_2); 25.38 (CH_2) ppm. Anal: $C_{28}H_{34}N_2O$ (C, H, N).

5.1.4.4. 1-Cinnamyl-4-(4,4-diphenylbutyl)piperazine ***4***. Free base: chromatographic eluent: $CHCl_3$/MeOH 95:5. Mp: 105–107 °C. Yield: 56%. ^{1}H NMR ($CDCl_3$): δ 7.42–7.38 (m, 2H arom.); 7.37–7.26 (m, 11H arom.); 7.22–7.18 (m, 2H arom.); 6.58–6.56 (d, *J* = 15.9 Hz, 1H, *CH*=CH); 6.35–6.28 (m, 1H, CH=*CH*); 3.94 (t, *J* = 7.8 Hz, 1H, CH); 3.19 (d, *J* = 6.8 Hz, 2H, CH_2); 2.50 (bs, 6H, CH_2); 2.41 (t, *J* = 7.5 Hz, 4H, CH_2); 2.10 (q, *J* = 7.8 Hz, 2H, CH_2); 1.55–1.47 (m, 2H, CH_2) ppm. ^{13}C NMR ($CDCl_3$): δ 145.04 (C); 136.96 (C); 133.08 (CH); 128.57 (CH arom.); 128.43 (CH arom.); 127.86 (CH arom.); 127.49 (CH); 126.59 (CH arom.); 126.33 (CH arom.); 126.11 (CH arom.); 61.08 (CH_2); 58.56 (CH_2); 53.19 (CH_2N); 51.36 (CH); 33.63 (CH_2); 25.40 (CH_2) ppm. Anal: $C_{29}H_{34}N_2$ (C, H, N).

5.1.4.5. 1-(4,4-Bis(4-fluorophenyl)butyl)-4-cinnamylpiperazine ***5***. Free base: chromatographic eluent: $CHCl_3$/MeOH 95:5.Yield: 56%. ^{1}H NMR ($CDCl_3$): δ 7.41–7.37 (m, 2H arom.); 7.35–7.28 (m, 2H arom.); 7.26–7.23 (m, 1H arom.); 7.20–7.16 (m, 4H arom.); 7.01–6.96 (m, 4H arom.); 6.54 (d, *J* = 15.8 Hz, 1H, *CH*=CH); 6.33–6.26 (m, 1H, CH=*CH*); 3.89 (t, *J* = 7.7 Hz, 1H, CH); 3.18 (d, *J* = 6.8 Hz, 2H, CH_2); 2.55–2.48 (bs, 6H, CH_2); 2.39 (t, *J* = 7.4 Hz, 4H, $2CH_2$); 2.02 (q, *J* = 7.8 Hz, 2H, CH_2); 1.47 (q, *J* = 8.0 Hz, 2H, CH_2) ppm. ^{13}C NMR ($CDCl_3$): δ 161.35 (d, *J* = 243 Hz, CF); 140.53 (C); 136.94 (C); 133.08 (CH); 129.10 (d, *J* = 7 Hz, CHF); 128.57 (CH arom.); 127.50 (CH); 126.57 (CH arom.); 126.32 (CH arom.); 115.26 (d, CHF, *J* = 21 Hz); 61.06 (CH_2); 58.45 (CH_2); 53.20 (CH_2-N); 49.76 (CH); 33.84 (CH_2); 25.31 (CH_2) ppm. Dihydrochloride. Mp: 244–248 °C. Anal: $C_{29}H_{34}Cl_2F_2N_2$ (C, H, N).

5.1.4.6. (E)-2-(3,4-Dimethoxyphenyl)-2-isopropyl-5-{4-[3-(3,4,5-trimethoxyphenyl)allyl]piperazin-1-yl}pentanenitrile ***6***. Free base: chromatographic eluent: CH_2Cl_2/MeOH/NH_4OH 95:5:0.5. Yield: 62%. IR (neat): ν 2233 (CN), cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 6.92–6.90 (m, 1H, arom.); 6.89 (s, 2H, arom.); 6.59 (s, 2H, arom.); 6.42 (d, *J* = 16.0 Hz, 1H, *CH*=CH); 6.17 (dt, *J* = 16.0 Hz, *J* = 6.8 Hz, 1H, CH=*CH*); 3.91–3.82 (m, 15H, OCH_3); 3.12 (d, *J* = 6.8 Hz, 2H, CH_2); 2.65–2.32 (m, 8H, CH_2); 2.29 (t, *J* = 7.2 Hz, 2H, CH_2); 2.18–2.02 (m, 2H, CH_2); 1.81–1.70 (m, 2H, CH_2); 1.49–1.62 (m, 1H, CH); 1.18 (d, *J* = 6.4 Hz, 3H, CH_3); 0.78 (d, *J* = 6.8 Hz, 3H, CH_3) ppm. ^{13}C NMR ($CDCl_3$): δ 153.29 (C); 149.03 (C); 148.30 (C); 132.61 (CH); 132.59 (C); 130.65 (C); 126.07 (C=C); 121.35 (C); 118.69 (CH arom.); 111.14 (CH arom.); 109.58 (CH arom.); 103.35 (CH arom.); 60.88 (OCH_3); 60.88 (CH_2); 57.92 (CH_2); 56.03 (OCH_3); 56.01 (OCH_3); 55.99 (OCH_3); 55.90 (OCH_3); 53.37 (C); 53.13 (CH_2); 53.02 (CH_2); 37.84 (CH); 35.68 (CH_2); 22.93 (CH_2); 18.96 (CH_3); 18.59 (CH_3) ppm. Dihydrochloride. Low melting solid. Anal: $C_{32}H_{47}Cl_2N_3O_5$ (C, H, N).

5.1.4.7. 2-(3,4-Dimethoxyphenyl)-2-isopropyl-5-{4-[2-(3,4,5-trimethoxyphenoxy)ethyl]piperazin-1-yl}-pentanenitrile ***7***. Free base: chromatographic eluent: CH_2Cl_2/MeOH/NH_4OH 95:5:0.5. Yield: 80%. IR (neat): ν 2233 (CN), cm^{-1}.^{1}H NMR ($CDCl_3$): δ 6.94–6.87 (m, 1H, arom.); 6.86–6.78 (m, 2H, arom.); 6.14 (s, 2H, arom.); 4.02 (t, *J* = 6.0 Hz, 2H, OCH_2); 3.87 (s, 3H, OCH_3); 3.86 (s, 3H, OCH_3); 3.81 (s, 6H, OCH_3); 3.76 (s, 3H, OCH_3); 2.75 (t, *J* = 6.0 Hz, 2H, CH_2); 2.55 (bs, 4H, CH_2); 2.37 (bs, 4H, CH_2); 2.27 (t, *J* = 7.2 Hz, 2H, CH_2); 2.14–1.77 (m, 4H, CH_2); 1.54–1.23 (m, 1H, CH); 1.15 (d, *J* = 3.4 Hz, 3H, CH_3); 0.77 (d, *J* = 3.4 Hz, 3H, CH_3) ppm. ^{13}C NMR ($CDCl_3$): δ 155.38 (C); 153.67 (C); 149.02 (C); 148.30 (C); 130.65 (C); 121.37 (CN); 118.71 (CH arom.); 111.12 (CH arom.); 109.53 (CH arom.); 92.36 (CH arom.); 66.15 (OCH_2); 61.01 (OCH_3); 57.93 (CH_2N); 57.21 (NCH_2); 56.08 (OCH_3); 55.99 (OCH_3); 53.59 (CH_2); 53.38 (CCN); 52.99 (CH_2); 37.86 (CH); 35.76 (CH_2); 22.95 (CH_2); 18.97 (CH_3); 18.61 (CH_3) ppm. Dihydrochloride. Mp: 90–92 °C. Anal: $C_{31}H_{47}Cl_2N_3O_6$ (C, H, N).

5.1.4.8. 1-[4,4-Bis-(4-methoxyphenyl)-butyl]-4-[2-(3,4,5-trimethoxyphenoxy)ethyl]piperazine ***8***. Free base: chromatographic eluent: CH2Cl2/MeOH/NH4OH 95:5:0.5. Yield: 73%. 1H NMR (CDCl3): δ 7.14 (d, *J* = 4.4 Hz, 4H, arom.); 6.80 (d, *J* = 4.4 Hz, 4H, arom.); 6.15 (s, 2H, arom.); 4.05 (t, *J* = 7.6 Hz, 2H, OCH2); 3.82 (s, 7H, CH and OCH3); 3.78 (s, 3H, OCH3); 3.75 (s, 6H, OCH3); 2.78 (t, *J* = 6.8 Hz, 2H, CH2); 2.58 (bs, 4H, CH2); 2.44 (bs, 4H, CH2); 2.35 (t, *J* = 6.8 Hz, 2H, CH2); 2.04–1.92 (m, 2H, CH2); 1.52–1.48 (m, 2H, CH2) ppm. 13C NMR (CDCl3): δ 157.81 (C); 155.42 (C); 153.68 (C); 137.60 (C); 128.62 (CH arom.); 113.79 (CH arom.); 92.39 (CH arom.); 66.15 (OCH2); 61.02 (OCH3); 58.58 (CH2N); 57.25 (CH2N); 56.09 (OCH3); 55.22 (OCH3); 53.66 (CH2); 53.14 (CH2); 49.58 (CH); 33.95 (CH2); 25.42 (CH2) ppm. Dihydrochloride. Mp: 161–163 °C. Anal: C33H46Cl2N2O6 (C, H, N).

5.1.4.9. (E)-1-[4,4-Bis-(4-methoxyphenyl)butyl]-4-[3-(3,4,5-trimethoxyphenyl)allyl]piperazine ***9***. Free base: chromatographic eluent: CH2Cl2/MeOH/NH4OH 95:5:0.5. Yield: 60%. 1H NMR (CDCl3): δ 7.12 (d, *J* = 8.8 Hz, 4H, arom.); 6.84 (d, *J* = 8.8 Hz, 4H, arom.); 6.60 (s, 2H, arom.); 6.43 (d, *J* = 15.6 Hz, 1H, CH=CH); 6.19 (dt, *J* = 15.6 Hz, 6.8 Hz, 1H, CH=CH); 3.85 (s, 6H, OCH3); 3.83 (s, 3H, OCH3); 3.78–3.82 (m, 1H, CH); 3.75 (s, 6H, OCH3); 3.13 (d, *J* = 6.8 Hz, 2H, CH2); 2.47 (bs, 8H, CH2); 2.36 (t, *J* = 7.6 Hz, 2H, CH2); 2.05–1.93 (m, 2H, CH2); 1.58–1.48 (m, 2H, CH2) ppm. 13C NMR (CDCl3): δ 157.81 (C); 133.31 (C); 137.60 (C); 132.88 (C=C); 132.66 (C); 128.61 (CH arom.); 126.22 (C=C); 113.78 (CH arom.); 103.34 (CH arom.); 60.96 (OCH3); 60.92 (CH2); 58.59 (CH2); 55.21 (OCH3); 53.24 (OCH3); 53.24 (CH2); 53.19 (CH2); 49.58 (CH); 33.97 (CH2); 25.42 (CH2) ppm. Dihydrochloride. Mp: 101–103 °C. Anal: C34H46Cl2N2O5 (C, H, N).

5.1.4.10. 1-[4,4-Bis(4-methoxyphenyl)butyl]-4-(3,4,5-trimethoxybenzyl)piperazine ***10***. Free base: chromatographic eluent: CH2Cl2/MeOH/NH4OH 97:3:0.3. Yield: 52%. 1H NMR (CDCl3): δ 7.12 (d, *J* = 8.4 Hz, 4H, arom.); 6.80 (d, *J* = 8.4 Hz, 4H, arom.); 6.55 (s, 2H, arom.); 3.85 (s, 6H, OCH3); 3.83 (s, 3H, OCH3); 3.80 (t, *J* = 8.0 Hz, 1H, CH); 3.75 (s, 6H, OCH3); 3.42 (s, 2H, CH2); 2.44 (bs, 6H, CH2); 2.38–2.29 (m, 4H, CH2); 2.03–1.94 (m, 2H, CH2); 1.49–1.38 (m, 2H, CH2) ppm. 13C NMR (CDCl3): δ 157.80 (C); 153.06 (C); 137.51 (C); 136.87 (C); 134.05 (C); 128.61 (CH arom.); 113.78 (CH arom.); 105.83 (CH arom.); 63.28 (CH2); 60.85 (OCH3);

58.64 (CH2); 56.13 (OCH3); 55.22 (OCH3); 53.26 (CH2); 53.11 (CH2); 49.57 (CH); 34.00 (CH2); 25.45 (CH2) ppm. ESI-MS: 535.4 [M+H]+. Dihydrochloride. Mp: 249–251 (dec) °C. Anal: C32H44Cl2N2O5 (C, H, N).

5.1.4.11. 1-Anthracen-9-ylmethyl-4-[4,4-bis(4-methoxyphenyl)butyl] piperazine ***11***. Free base: chromatographic eluent: CH2Cl2/MeOH/NH4OH 99:1:0.1. Yield: 20%. 1H NMR (CDCl3): δ 8.48 (d, $J = 8.8$ Hz, 2H, arom.); 8.41 (s, 1H, arom.); 8.00 (d, $J = 8.0$ Hz, 2H, arom.); 7.61–7.40 (m, 4H, arom.); 7.12 (d, $J = 6.8$ Hz, 4H, arom.); 6.80 (d, $J = 6.8$ Hz, 4H, arom.); 4.43 (s, 2H, CH2); 3.88–3.66 (m, 7H, CH and OCH3); 2.65 (bs, 4H, CH2); 2.50–2.22 (m, 6H, CH2); 2.08–1.90 (m, 2H, CH2); 1.52–1.39 (m, 2H, CH2) ppm. 13C NMR (CDCl3): δ 157.87 (C); 137.67 (C); 131.46 (C); 129.86 (C); 128.99 (CH arom.); 128.67 (CH arom.); 127.44 (CH arom.); 125.62 (CH arom.); 125.22 (CH arom.); 124.91 (CH arom.); 113.84 (CH arom.); 58.54 (CH2); 55.25 (OCH3); 54.32 (CH2); 53.37 (CH2); 53.21 (CH2); 49.59 (CH); 33.98 (CH2); 25.39 (CH2) ppm. Dihydrochloride. Mp: 120–122 °C. Anal: C37H42Cl2N2O2 (C, H, N).

5.1.4.12. (E)-1-(3,4,5-Trimethoxybenzyl)-4-[3-(3,4,5-trimethoxyphenyl)allyl]piperazine ***12***. Free base: chromatographic eluent: CH_2Cl_2/MeOH/NH_4OH 98:2:0.2. Yield: 61%. ^{1}H NMR ($CDCl_3$): δ 6.58 (s, 2H, arom.); 6.54 (s, 2H, arom.); 6.42 (d, $J = 15.6$ Hz, 1H, *CH*=CH); 6.18 (dt, $J = 15.6$ Hz, $J = 6.8$ Hz, 1H, CH=*CH*); 3.90–3.75 (m, 18H, OCH_3); 3.44 (s, 2H, CH_2); 3.15 (d, $J = 6.4$ Hz, 2H, CH_2); 2.52 (bs, 8H, CH_2) ppm. ^{13}C NMR ($CDCl_3$): δ 153.31 (C); 153.09 (C); 137.82 (C); 137.00 (C); 133.78 (C); 133.02 (CH); 132.59 (C); 126.00 (CH); 105.91 (CH arom.); 103.40 (CH arom.); 63.16 (CH_2); 60.90 (CH_2); 60.81 (OCH_3); 56.13 (OCH_3); 55.04 (OCH_3); 53.20 (CH_2); 52.96 (CH_2) ppm. Dihydrochloride. Mp: 87 (dec) °C. Anal: $C_{26}H_{38}Cl_2N_2O_6$ (C, H, N).

5.1.4.13. (E)-1-Anthracen-9-ylmethyl-4-[3-(3,4,5-trimethoxyphenyl) allyl]piperazine ***13***. Free base: chromatographic eluent: CH_2Cl_2/MeOH/NH_4OH 98:2:0.2. Yield: 35%. ^{1}H NMR ($CDCl_3$): δ 8.48 (d, $J = 8.8$ Hz, 2H, arom.); 8.41 (s, 1H, arom.); 8.00 (d, $J = 8.4$ Hz, 2H, arom.); 7.68–7.40 (m, 4H, arom.); 6.59 (s, 2H, arom.); 6.41 (d, $J = 16.0$ Hz, 1H, *CH*=CH); 6.21 (dt, $J = 16.0$ Hz, $J = 6.4$ Hz, 1H, CH=*CH*); 4.47 (s, 2H, CH_2); 3.85 (s, 6H, OCH_3); 3.84 (s, 3H, OCH_3); 3.15 (d, $J = 6.8$ Hz, 2H, CH_2); 2.72 (bs, 4H, CH_2); 2.52 (bs, 4H, CH_2) ppm. ^{13}C NMR ($CDCl_3$): δ 153.32 (C); 133.25 (CH); 132.53 (C); 131.40 (C); 129.55 (C); 128.96 (CH arom.); 127.47 (CH arom.); 125.62 (CH); 125.07 (CH arom.); 124.86 (CH arom.); 103.43 (CH arom.); 60.92 (OCH_3); 60.75 (CH_2); 56.07 (OCH_3); 54.18 (CH_2); 53.25 (CH_2); 52.97 (CH_2) ppm. Dihydrochloride. Mp: 90 (dec) °C. Anal: $C_{31}H_{36}Cl_2N_2O_3$ (C, H, N).

5.2. Biology

5.2.1. Cell lines and cultures

K562 is an undifferentiated erythroleukemia cell line originally derived from a patient with chronic myelogenous leukemia [39]. The K562 leukemia cells and the P-gp over-expressing K562/DOX cells were obtained from Prof. J.P. Marie (Hopital Hotel-Dieu, Paris, France). These cells were cultured following the previously reported protocol [19].

5.2.2. Modulation of pirarubicin uptake

Purified pirarubicin and verapamil were provided by Sigma-Aldrich (Milan-Italy). Concentrations were determined by diluting stock solutions to approximately 10^{-5} M and using $\varepsilon_{480} = 11500\ M^{-1}\ cm^{-1}$. 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.

The uptake of pirarubicin in cells was followed by monitoring the decrease in the fluorescence signal at 590 nm ($\lambda_{ex} = 480$ nm) according to the previously described method [44,45].

5.2.3. Effects on doxorubicin cytotoxicity

Doxorubicin hydrochloride (DOX), verapamil, dimethylsulphoxide (DMSO) and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Milan-Italy). Tariquidar was purchased from Medchemexpress Europe (Stockholm SWEDEN). MTT stock solution was prepared following the previously described method [19]. Tested compounds and tariquidar solutions were prepared in DMSO at 10^{-2} M, DOX stock, verapamil solutions were prepared in water at 10^{-2} M. Drugs and studied compounds were then diluted with complete medium to obtain the 10x desired final maximum test concentrations. Verapamil and tariquidar were used as standard P-gp modulators and were evaluated at 3 and 1 μM concentrations, respectively.

To evaluate the reversing activity of the tested compounds, the cells, in exponential growth phase (3×10^5 cells/mL), were seeded at 10^4 cells/well and solutions of either tested compounds or doxorubicin, or a solution of doxorubicin in combination with the tested compounds, were added to the wells. Then the plates were incubated at 37 °C for 72 h in 5% CO_2 incubator. The compounds were evaluated for MDR reversal activity at 0.1, 1.0 and 3.0 μM and the corresponding doxorubicin concentrations tested were between 0.0001 and 100 μM. The MTT working solution 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 acidified isopropanol solution. The absorbance of the solution was then read on an automated plate reader at a wavelength of 570 nm.

The potency of the MDR reversers was expressed by the reversal fold (RF) values obtained as the ratio between the doxorubicin IC_{50} values on K562/DOX cells in the absence and in the presence of modulators.

5.2.4. Inhibition of P-gp-mediated rhodamine-123 (Rhd123) efflux

P-gp activity was evaluated by measuring the efflux of the P-gp substrate rhodamine 123 (Rhd 123), in K562/DOX cells, in the absence or in the presence of compounds **8**, **9**, **10** and **13**, following a previously described method with minor modification [23]. Briefly, cells were loaded with 5.0 μM rhodamine 123 for 30 min at 37 °C in a humidified atmosphere of 5% CO_2, with or without (control) tested compounds at 1.0 μM. All compounds were added 15 min before rhodamine-123. After the accumulation period of 30 min, the cells were washed and suspended in Rhd 123-free medium with or without compounds **8**, **9**, **10** and **13** at 1.0 μM concentration and reference compound verapamil at 3.0 μM concentration, for the efflux measures.

The efflux was carried out for different times, 30 and 60 min, at 37 °C in 5% CO_2. At the end of each efflux period, cells were sedimented, washed twice with ice-cold phosphate buffer saline (PBS) and placed in PBS on ice, and kept in the dark until flow cytometric analysis. Samples were analyzed on a FACSCanto flow cytometer (Becton Dickinson, san Jose CA,USA) equipped with 488 and 633 nm lasers and FACSDIVA software. Samples were gated on forward scatter versus side scatter to exclude cell debris and at least 20,000 events were acquired; the green fluorescence of Rhd 123 was collected after a 530 nm band pass filter. Data were analyzed by FCS Express (De Novo Software). For each sample, the intracellular amount of Rhd 123 after the efflux times was expressed by the mean fluorescence intensity (MFI) ratio obtained as the ratio between the median fluorescence intensity value of samples treated

with Rhd 123 and modulators, and the control sample. The histograms were generated by program GraphPad Prism 5 (GraphPad Prism software, Inc. CA).

5.3. Statistical analysis

All experiments were carried out in triplicate or quadruplicate and were independently done at least three times. 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).

5.4. Chemical stability tests

5.4.1. Materials and methods

5.4.1.1. Chemicals. Acetonitrile (Chromasolv), formic acid and ammonium formate (MS grade), NaCl, KCl, Na_2HPO_4 $2H_2O$, KH_2PO_4 (Reagent grade) and verapamil hydrochloride (analytical standard, used as internal standard), ketoprofen and enalapril (analytical standard) were purchased by Sigma-Aldrich (Milan, Italy). Ketoprofen Ethyl Ester (KEE) were obtained by Fisher's reaction from ketoprofen and ethanol.

MilliQ water 18 MΩ was obtained from Millipore's Simplicity system (Milan - Italy).

Phosphate buffer solution (PBS) was prepared by adding 8.01 $g\,L^{-1}$ of NaCl, 0.2 $g\,L^{-1}$ of KCl, 1.78 $g\,L^{-1}$ of Na_2HPO_4 $2H_2O$ and 0.27 $g\,L^{-1}$ of KH_2PO_4. Human plasma was collected from healthy male volunteer and the rat plasma batch was collected from Sprague Dawley male rats and kept at −80 °C until use.

5.4.1.2. Instrumental. The LC-MS/MS analysis was carried out using a Varian 1200L triple quadrupole system (Palo Alto, CA, USA) equipped by two Prostar 210 pumps, a Prostar 410 autosampler and an Elettrospray Source (ESI) operating in positive ions. Raw-data were collected an processed by Varian Workstation Vers. 6.8 software.

G-Therm 015 thermostatic oven was used to maintained the samples at 37 °C during the test of degradation. ALC micro centrifugette 4214 was employed to centrifuge plasma samples.

5.4.2. Preparation of samples

Each sample was prepared adding 10 μL of working solution 1–100 μL of tested matrix (PBS or rat plasma or human plasma) in microcentrifuge tubes. The obtained solutions correspond to 1 μM of analyte.

Each set of samples was incubated in triplicate at four different times, 0, 30, 60 and 120 min at 37 °C. Therefore the degradation profile of each analyte was represented by a batch of 12 samples (4 incubation times x 3 replicates). After the incubation, the samples were added with 300 μL of ISTD solution and centrifuged (room temperature for 5 min at 10000 rpm). The supernatants were transferred in autosampler vials and dried under a gentle stream of nitrogen.

The dried samples were dissolved in 1.0 mL of 10 mM of formic acid in mQ water:acetonitrile 80:20 solution. The obtained sample solutions were analyzed by LC-MS/MS method described in Supplementary material.

The hydrolytic activity of human and rat plasma batches were checked using ketoprofene ethylester (KEE, half-life < 2 h) or enalapril (half-life < 0.5 h) as reference compounds, respectively.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejmech.2018.01.092.

Evaluation of EC_{50} values of compounds **9** and **10** on K562/DOX cells. ^{1}H NMR and ^{13}C-APT-NMR spectra of compounds **9** and **10**. Chemical stability data of compounds **8**, **9**, **10** and **13**.

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