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Diphenylcyclohexylamine derivatives as new potent multidrug resistance (MDR) modulators

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Abstract—A series of compounds with a diphenylmethyl cyclohexyl skeleton, loosely related to verapamil, has been synthesized and tested as MDR modulators on anthracycline-resistant erythroleukemia K 562 cells. Their residual cardiovascular action (negative inotropic and chronotropic activity as well as vasorelaxant activity) was evaluated on guinea-pig isolated atria preparations and on guinea-pig aortic strip preparations. Most compounds of the series possess a good MDR-reverting activity together with a low cardiovascular action. Among them, compounds **3a$_1$**, **7a**, and **8a** are more potent than verapamil as MDR reverters and lack any cardiovascular action; they can represent useful leads for the development of new safe MDR reversing drugs.

1. Introduction

Drug resistance is a phenomenon that frequently impairs proper treatment of infections and cancer with chemotherapeutics. Multidrug resistance (MDR) is a kind of acquired drug resistance of cancer cells to multiple classes of chemotherapic drugs that can be structurally and mechanistically unrelated.[1,2] MDR can be the result of a variety of mechanisms that are not fully understood, but the most widely implicated mechanism is concerned with altered membrane transport in tumour cells.[3] This mechanism is often referred to as classical MDR[4] and is related to a lower cell concentration of cytotoxic drugs associated with accelerated efflux of antitumour agents, due to the over expression of a variety of proteins that act as ATP-dependent extrusion pumps. At the moment, the best-known extrusion proteins are P-glycoprotein (P-gp) and MRP1;[5] both belong to the ABC superfamily of transporters. It is important to notice that, in addition to their role in cancer cell resistance, these proteins seem to have multiple physiological functions as well[6,7] since they are expressed also in many important non-tumoural tissues, such as the blood–brain barrier (BBB), intestinal epithelium and hepatic cells, and similar transporting proteins of the ABC superfamily are largely present in procariotic organisms.[8]

Due to the unprecedented variety of substrates extruded, the mechanism of action of these transporters is still controversial. As a matter of fact, a number of models have been proposed to explain the involvement of these extrusion pumps in MDR:[9] the dominant 'drug pump' model has been questioned but, at present, it seems that P-gp and related proteins are indeed directly involved in the extrusion of multiple drugs.[10–12]

Whatever the mechanism of action of P-gp, inhibition of its functions has been rapidly recognized as a possible

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approach to circumvent MDR and drugs possessing inhibitory properties have been and are actively being sought.[13,14] Although verapamil, the first drug able to reverse MDR, was recognized more than 20 years ago as a chemosensitizer, a potent, specific and safe drug able to reverse multidrug resistance and to assist in the chemotherapy of cancer is still lacking. In fact, most of the molecules initially found active (the so called 'first generation' chemosensitizers) were known drugs with a definite pharmacological action that induced unwanted side effects. Therefore, the problem of the specific design of chemosensitizers was approached, and a second generation of drugs able to revert MDR appeared on the stage, but the fairly heterogeneous chemical structure of the compounds found active in reverting MDR has made difficult to establish structure–activity relationships, and made the design of new candidates difficult.[13] Indeed, the broad substrate selectivity of P-gp suggests that there might be more than a single binding mode within a large binding site[15–17] and it is not surprising that the efforts to establish SAR has led only to qualitative, generic indications.[18–20] Also the role of lipophilicity, a characteristic that is generally considered important for MDR-reverting properties, is quite elusive, since till now no significant quantitative relationships have been established between partition coefficients and activity,[21–23] and successful correlations were found only for highly homogeneous sets of molecules.[24,25]

As pointed out above, verapamil, a calcium channel antagonist (Chart 1), was one of the most studied first generation chemosensitizers, both in vitro and in vivo, but a limiting factor of its therapeutic use are the pronounced cardiovascular effects, which occur at the plasma concentrations required to efficiently block P-gp transport. From a clinical point of view, it is therefore important to find analogues with low calcium channel blocking activity and high MDR-reverting action. Indeed, verapamil has been used as the lead molecule in several attempts to identify more potent and selective drugs. In a previous study,[21] we identified some rigid verapamil analogues that were, as chemosensitizers, slightly more potent than the lead and, at the same time, showed lower activity on the cardiovascular system in some cases. On the basis of these results, for the last few years we have been synthesizing different sets of compounds, in which the backbone of our derivatives has been substituted with several kinds of large and lipophilic amines.[26–28] Our research has led to the discovery

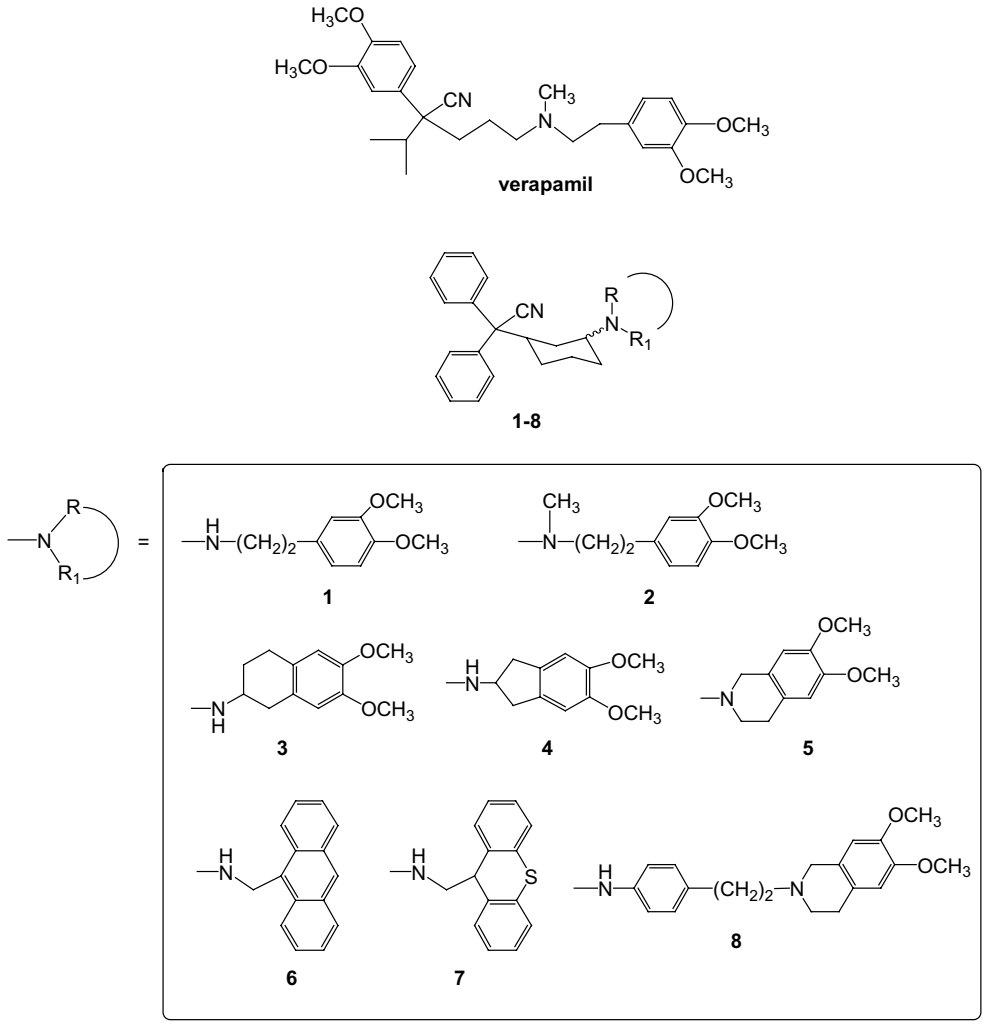


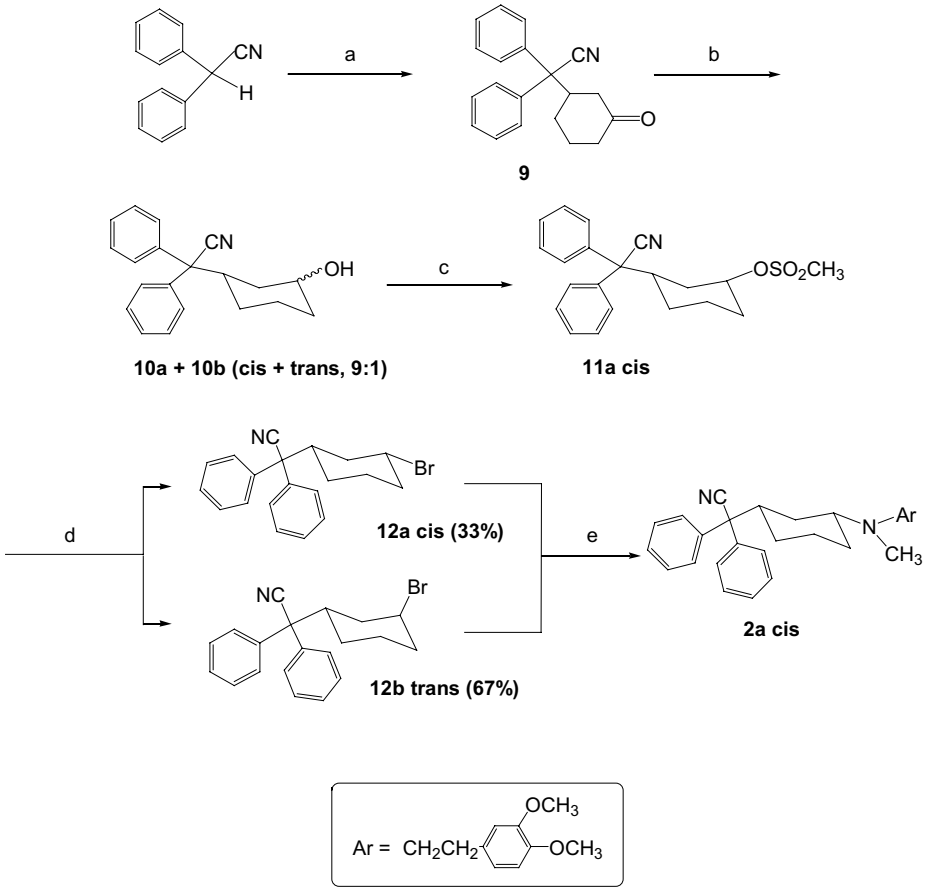
Chart 1.

of some promising derivatives that are more potent as MDR reverters, with respect to the lead verapamil, and are endowed with reduced cardiovascular activity. Among them compound MM36 is active at a nanomolar concentration on anthracycline-resistant erythroleukemia K 562 cell lines.[26]

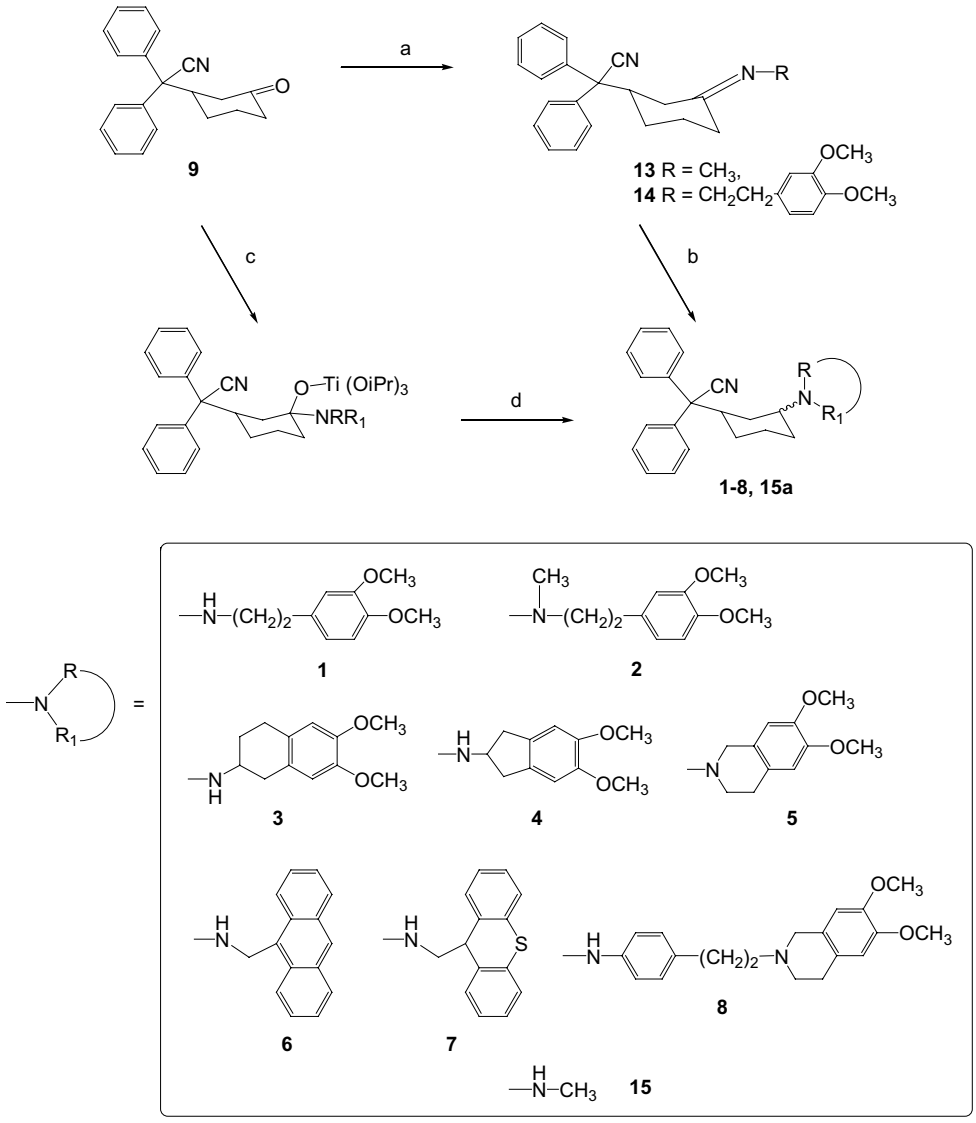
Since the cyclohexyl moiety present in some of our rigid derivatives seemed to reduce the residual cardiovascular action of verapamil-derived compounds, we have decided to synthetize and study the series of compounds shown in Chart 1. There are indications that weak polar interactions such as those produced by the overlapping of π orbitals of aromatic rings can play an important role in stabilizing the binding of MDR-reverting agents to P-gp protein:[29] on this basis, all the newly designed derivatives bear a diphenyl group on the carbon in position 1 of the cyclohexane, instead of the dimethoxyphenylisopropyl moiety characteristic of verapamil and present in the original derivatives. This general structure was modulated by inserting different lipophilic amines in position 3 (Chart 1). *N*-Methylhomoveratrylamine was chosen because it is present in the lead compound verapamil; also the corresponding desmethyl amine was utilized in order to evaluate the activity of secondary amine derivatives that often result more effective[21] in comparison with the corresponding N-methylated ones. 5,6-Dimethoxy-2-aminoindane, 6,7-dimethoxy-2-aminotetraline and 6,7-dimethoxy-1,2,3,4-tetrahydro-2-isoquinoline can be considered restricted flexibility analogues of the previous amines, and have already been successfully inserted in another series of MDR modulators.[21] The 9-aminomethylanthracene and 9-aminomethylthioxantene moieties are present in the previously mentioned MM36[30] and in its thioxantene analogue,[28] which is almost completely devoid of cardiovascular activity. Finally, compound **8** was synthesized to insert in this series some of the features of XR 9576,[31] a potent modulator of MDR.

2. Chemistry

The synthetic pathways used to obtain the desired compounds are shown in Schemes 1 and 2. The first step of the procedure is the Michael addition of diphenylacetonitrile to 2-cyclohexen-1-one, leading to compound **9** (Scheme 1). This compound is among the many claimed in a Japanese patent;[32] no details on the synthesis have been, however, reported. We performed this reaction both using butyllithium and LDA (lithium diisopropylamide) at a low temperature. The best yield (47%) was obtained with butyllithium at −78 °C, but even in this case side reactions could not be eliminated, and the reaction of butyllithium with the CN group gave rise to the by-product 1,1-diphenyl-2-hexanone.



Scheme 1. Reagents and conditions: (a) BuLi, 2-cyclohexen-1-one, −78 °C; (b) $NaBH_4$; (c) CH_3SO_2Cl; (d) LiBr, chromatographic separation; (e) *N*-methylhomoveratrylamine.



Scheme 2. Reagents and conditions: (a) **13**: methylamine, toluene, *p*-toluenesulfonic acid, **14**: homoveratrylamine, benzene, *p*-toluenesulfonic acid; (b) **15a**: $NaBH_4$ in MeOH, **1a**: H_2, Pd/C in EtOH or $NaBH_4$ in MeOH; (c) titanium(IV) isopropoxide, the suitable amine; (d) $NaBH_3CN$.

In a first attempt, looking for a general procedure that could be applied to all our derivatives, cyclohexanone **9**, was reduced with $NaBH_4$ yielding two isomeric alcohols (**10a** *cis* and **10b** *trans*, Scheme 1) in a 9:1 ratio. The alcohol mixture was transformed into the corresponding mesyl derivatives **11** in almost the same isomeric ratio; crystallization of this mixture yielded the *cis* isomer **11a**, which was reacted with LiBr. Bromination occurred with partial inversion, giving the two isomeric bromides **12b** (*trans*) and **12a** (*cis*) in a 2:1 ratio. This mixture was separated by chromatography. The stereochemistry of these compounds was attributed on the basis of the NMR characteristics of the C3 proton (see next section). However, both isomers **12a** and **12b**, when reacted with *N*-methylhomoveratrylamine, gave only the most stable isomer **2a** (*cis*).

Therefore, in order to obtain both isomers, we decided to explore the reduction of the Schiff bases according to Scheme 2. Accordingly, compound **9** was reacted with methylamine and homoveratrylamine and the Schiff bases obtained reduced with $NaBH_4$ or H_2/Pd/C to obtain compounds **15** and **1**. From both **1** and **15** it was possible, using the proper reagents (methylamine and 2-(3,4-dimethoxyphenyl)ethyl bromide, respectively) to obtain compound **2**. However, using this procedure yields were very poor and only the *cis* isomers **1a** and **15a** were obtained.

The desired amines **1**–**8** were eventually obtained in a good yield, according to the Mattson procedure[33] by reductive alkylation of **9** with the suitable amine, using titanium(IV) isopropoxide as Lewis acid catalyst and $NaBH_3CN$ as reducing agent (Scheme 2). The amines used are commercially available (6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline.HCl, 2-(3,4-dimethoxyphenyl)ethylamine or homoveratrylamine, 2-[(3,4-dimethoxyphenyl)ethyl]methylamine) or *N*-methylhomo-veratrylamine or synthesized according to the literature ((9H-thioxanthen-9-yl)methylamine),[28] 4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolyl)-ethyl]benzenamine,[34] anthracen-9-ylmethylamine,[26] 5,6-

dimethoxyindan-2-ylamine,[35] 6,7-dimethoxy-1,2,3,4-tetrahydronaphthalen-2-ylamine, procedure slightly modified with the addition of molecular sieves.[36] This procedure made it possible to obtain each diastereoisomer in a sufficient quantity to perform the pharmacological and biochemical tests. In fact, the compounds of the series possess two stereogenic centres (three in the case of **3**) and, therefore, four optical isomers are possible (eight for **3**) as two diastereoisomeric racemic mixtures. At this stage of the research, also considering the modest impact of stereochemistry in the MDR activity of verapamil-like compounds,[37] we decided to postpone this problem until complete evaluation of their pharmacological profiles.

In the case of **3** (the only compound that presents three stereocentres), two out of the four possible racemates were isolated in good yield, the others being present only in traces. NMR spectroscopy indicated that the two diastereoisomers isolated possess a *cis* geometry with respect to the cyclohexane ring.

3. Stereochemistry

The relative stereochemistry of the substituents in position 1,3 on the cyclohexane ring of the synthesized compounds was attributed on the basis of the ^{1}H NMR characteristics of the C1 and C3 protons. In fact, in most cases, it was possible to extract the J_{aa} (and sometimes the J_{ae} and J_{ee}) constants; when the signal does not allow extraction of the coupling constants, the chemical shift, as well as the half-height amplitude of the signal ($w/2$),[27,38] allow confident attribution of their equatorial or axial nature. Moreover, the chemical shift of the signals is diagnostic, because axial protons resonate at higher fields, while the corresponding equatorial ones are less shielded.

In the case of the two isomeric alcohols (**10a** *cis* and **10b** *trans*), the proton in position 1 in both compounds shows axial characteristics (δ = 2.55 ppm, J_{aa} = 11.4 Hz, J_{ae} not detectable), suggesting that, as expected, the bulky substituent prefers an equatorial position. On the other hand, the proton in position 3 shows prevalent axial characteristics (δ = 3.60 ppm, $w/2$ = 26.5 Hz) in the most abundant isomer (**10a**), as compared with that of the minor isomer (**10b**) which shows equatorial characteristics (δ = 4.20 ppm, $w/2$ = 8.2 Hz). This indicates that **10a** has the hydroxy group in an equatorial position, implying that the bulky diphenylacetonitrile residue and the hydroxy group are *cis* to each other. Accordingly, **10b** will have the bulky residue and the hydroxy group *trans* to each other. The same applies to the bromo derivatives **12b** (*trans*) and **12a** (*cis*). Also in this case, in both compounds, the C1 protons show axial characteristics (δ = 3.20 ppm, J_{aa} = 11.6 Hz, J_{ae} = 3.2 Hz for **12b** and δ = 2.58 ppm, J_{aa} = 12.0 Hz, J_{ae} = 4.0 Hz for **12a**), whereas the C3 protons show equatorial characteristics in the case of the *trans* isomer (δ = 4.77 ppm, J_{ae} = 2.8 Hz) and axial characteristics for the *cis* one (δ = 4.00 ppm, J_{aa} = 11.8 Hz, J_{ae} = 4.2 Hz).

As regards the final compounds **1**–**8**, structure attribution was less simple, because in some cases the diagnostic signals are partially obscured (Table 1). Also here, the proton in position 1 always shows axial characteristics, suggesting that the bulky substituent prefers an equatorial position. As regards the protons in 3 however, while for the couples of compounds **1**, **5**, **6**, **7** and **8** attribution is quite straightforward, in the case of **2a** and **2b** the coupling constant of the less abundant isomer (**2b**) is too high to safely attribute a *trans* stereochemistry. To solve this problem, we performed X-ray crystallography (see Fig. 1), which confirmed that the most abundant compound, **2a**, has a *cis* geometry, leaving to compound **2b** a *trans* stereochemistry. In the case of couple **4**, where the diagnostic signals are obscured, the attribution was based mainly on the fact that in this series of compounds, the *cis* isomer is consistently found to be the most abundant one in the mixture. This attribution is also supported by the δ values of the C3 protons. In fact, their signals are at higher field in compound **4a**, with respect to the corresponding **b** isomers, suggesting a *cis* configuration for the former, and a *trans* geometry for the latter. As regards compound **3**, which presents three stereocentres, NMR spectroscopy allowed us to establish that both the diastereoisomeric mixtures isolated (**3a$_1$** and **3a$_2$**) possess a *cis* geometry with respect to the cyclohexane ring (H3: **3a$_1$** δ = 3.16 ppm, J_{aa} = 11.4 Hz; **3a$_2$** δ = 3.05 ppm, $w/2$ = 25.8 Hz).

4. Pharmacology

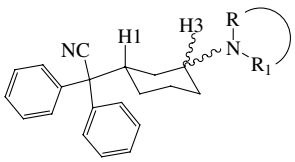
4.1. MDR-reverting activity

The ability of the compounds to revert MDR was evaluated on anthracycline-resistant erythroleukemia K 562 cells, measuring the uptake of THP-adriamycin (pirarubicin) by continuous spectrofluorometric signal of the anthracycline at 590 nm (λ_{ex} = 480 nm) after incubation of the cells, following the protocols reported in previous papers.[21,26,27] Greater detail is provided in the experimental part of the present paper. MDR-reverting activity is described by (i) α, which represents the fold increase in the nuclear concentration of pirarubicin in the presence of the MDR-reverting agent and varies between 0 (in the absence of the inhibitor) and 1 (when the amount of pirarubicin in resistant cells is the same as in sensitive cells); (ii) α_{max}, which expresses the efficacy of MDR-modulator and is the maximum increase that can be obtained in the nuclear concentration of pirarubicin in resistant cells with a given inhibitor; (iii) $[i]_{0.5}$, which measures the potency of MDR-reverting agent and represents the concentration of the inhibitor that causes a half-maximal increase in nuclear concentration of pirarubicin at α = 0.5 (see Table 2).

4.2. Cardiovascular activity

Inotropic and chronotropic activities were tested on guinea-pig isolated atria preparations, and vasodilator activity was tested on guinea-pig aortic strip preparations following standard procedures,[39] details of which

Table 1. [1]H NMR signals due to C1 and C3 protons at 200 MHz



Compound	H1	Axial H3 (*cis*)	Equatorial H3 (*trans*)
1a	δ = 2.58 ppm[a,b] J_{aa} = 11.0 Hz J_{ae} = 4.0 Hz	δ = 2.60 ppm[a,b] J_{aa} = 12.0 Hz J_{ae} = 4.0 Hz	
1b	δ = 2.82–2.70 ppm[a,c]		δ = 3.08 ppm[a] $w/2$ = 18.0 Hz[d]
2a	δ = 2.76–2.53 ppm[c]	δ = 2.76–2.53 ppm[c]	
2b	δ = 2.76–2.54 ppm[c]		δ = 3.00 ppm J = 10.4 Hz
3a$_1$	δ = 2.96–2.47 ppm[c]	δ = 3.16 ppm J_{aa} = 11.4 Hz	
3a$_2$	δ = 2.95–2.38 ppm[c]	δ = 3.05 ppm $w/2$ = 25.8 Hz[d]	
4a	δ = 2.68–2.56 ppm[c]	δ = 2.68–2.56 ppm[c]	
4b	δ = 3.16–3.02 ppm[c]		δ = 3.16–3.02 ppm[c]
5a	δ = 2.70–2.45 ppm[c]	δ = 3.20 ppm J_{aa} = 11.5 Hz	
5b	δ = 2.83–2.58 ppm[c]		δ = 3.75 ppm $w/2$ = 12.0 Hz[d]
6a	δ = 2.71–2.49 ppm[c]	δ = 2.87 ppm J_{aa} = 15.6 Hz	
6b	δ = 2.60–2.21 ppm[c]		δ = 3.10 ppm[c] $w/2$ = 11.0 Hz[d]
7a	δ = 2.43 ppm[b] J_{aa} = 11.0 Hz	δ = 2.49 ppm[b] J_{aa} = 10.8 Hz	
7b	δ = 2.95–2.60 ppm[c]		δ = 2.99 ppm $w/2$ = 10.0 Hz[d]
8a	δ = 2.43 ppm J_{aa} = 13.5 Hz J_{ae} = 2.2 Hz	δ = 3.00–2.60 ppm[c]	
8b	δ = 2.43 ppm J_{aa} = 11.6 Hz		δ = 3.14 ppm J_{ae} = 4.2 Hz

[a] Evaluated on the 400 MHz [1]H NMR spectra.
[b] The attribution of H1 and H3 signal can be interchanged.
[c] Signal obscured.
[d] The use of $w/2$ (half-height width) to estimate the equatorial or axial properties of cyclohexane protons is a simple way of evaluating the size of coupling constants when the exact value cannot be obtained (see text).

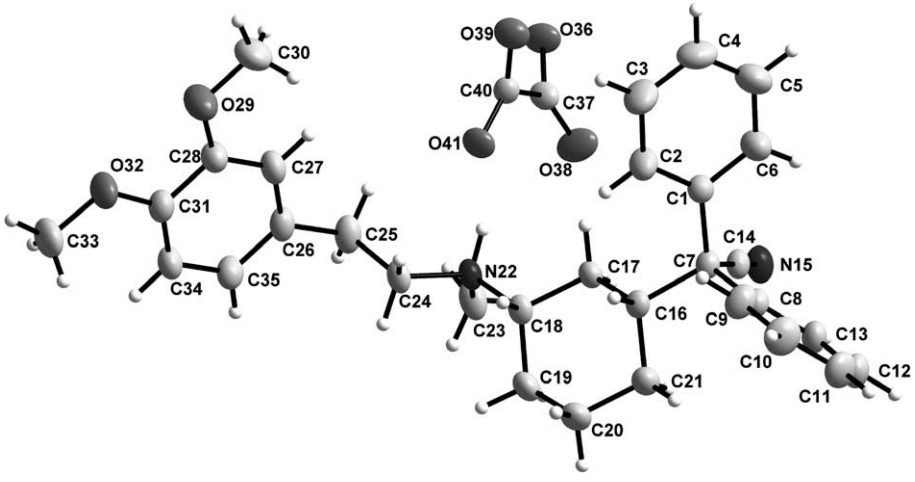


Figure 1. Thermal ellipsoid plot (30% ellipsoids) of compound **2a oxalate**.

Table 2. Cardiovascular and chemosensitizer activity of compounds **1–8**

	Cardiovascular activity									MDR activity	
	Negative inotropy			Negative chronotropy			Vasorelaxant activity				
Comp	Ia %[a] (±SEM)	EC_{50} (μM)[b]	(95% cl)	Ia %[c] (±SEM)	EC_{30} (μM)[b]	(95% cl)	Ia %[d] (±SEM)	IC_{50} (μM)[b]	(95% cl)	$[i]_{0.5}$ μM	α max
1a	50 ± 3.6			68 ± 3.4	2.07	(1.82–2.15)	40 ± 2.5			0.44 ± 0.09	0.86
1b	46 ± 2.4			67 ± 2.5	1.42	(1.31–1.62)	15 ± 0.7			0.34 ± 0.07	0.88
2a	63 ± 3.5	1.49	(1.06–2.11)	90 ± 1.4	3.75	(3.39–4.41)	41 ± 2.3			0.10 ± 0.02	0.87
2b	33 ± 1.7			73 ± 2.5[e]	1.67	(1.31–1.92)	23 ± 1.6			0.68 ± 0.14	0.85
$3a_1$	46 ± 1.4			49 ± 3.8			20 ± 1.3			0.36 ± 0.07	0.90
$3a_2$	40 ± 0.8[f]			68 ± 3.4	1.57	(1.25–1.90)	26 ± 1.5			6.2 ± 1.2	0.80
4a	81 ± 3.6	1.84	(1.36–2.91)	75 ± 4.5[f]	1.68	(1.45–2.02)	31 ± 2.1			0.14 ± 0.03	0.90
4b	45 ± 1.4			73 ± 3.2	6.09	(5.81–6.38)	25 ± 1.5			0.29 ± 0.06	0.90
5a	75 ± 2.6[f]	0.41	(0.27–0.60)	21 ± 1.9			15 ± 1.1			1.30 ± 0.26	0.83
5b	61 ± 1.5	1.58	(1.12–2.23)	65 ± 2.7	4.97	(4.66–4.72)	22 ± 0.2			0.50 ± 0.1	0.86
6a	56 ± 1.2	1.02	(0.79–1.21)	18 ± 0.8			6 ± 0.3			1.5 ± 0.3	0.85
6b	54 ± 3.4	0.83	(0.62–0.98)	22 ± 1.8			5 ± 0.4			50 ± 10	~1
7a	38 ± 1.5			24 ± 1.7			2 ± 0.2			0.90 ± 0.18	0.83
7b	32 ± 2.3[f]			14 ± 1.1[f]			2 ± 0.1			1.70 ± 0.2	0.77
8a	41 ± 1.4[e]			41 ± 2.6			37 ± 1.3			0.54 ± 0.11	0.88
8b	16 ± 0.4[g]			31 ± 2.4			19 ± 0.3			1.40 ± 0.30	~1
VRP	84 ± 2.1[f]	0.61	(0.40–0.80)	94 ± 3.4[g]	0.07	(0.05–0.10)	95 ± 1.7[g]	0.38	(0.20–0.70)	1.6 ± 0.3	0.70
MM36	55 ± 0.2	1.11	(0.85–1.45)	65 ± 4.3	0.86	(0.76–1.00)	29 ± 2.3			0.05 ± 0.01	0.70

[a] Intrinsic activity: decrease in the developed tension in isolated guinea-pig left atrium at 5×10^{-5} M, expressed as percent changes from the control ($n = 4–6$). The left atria were driven at 1 Hz. The 5×10^{-5} M concentration gave the maximum effect for most compounds.

[b] Calculated from log concentration–response curves (Probit analysis by Litchfield and Wilcoxon with $n = 5–6$). When the maximum effect was <50%, the EC_{50} ino., EC_{30} chrono., IC_{50} values were not calculated.

[c] Intrinsic activity: decrease in the atrial rate on guinea-pig spontaneously beating isolated right atrium at 5×10^{-5} M, expressed as percent changes from the control ($n = 6–7$). The pretreatment heart rate ranged from 165 to 190 beats/min. 5×10^{-5} M gave the maximum effect for most compounds.

[d] Intrinsic activity: percent inhibition of calcium-induced contraction on K^+-depolarized guinea-pig aortic strip at 5×10^{-5} M ($n = 5–6$). The 5×10^{-5} M concentration gave the maximum effect for most compounds.

[e] At the 5×10^{-6} mol/L concn.

[f] At the 10^{-5} mol/L concn.

[g] At the 10^{-6} mol/L concn.

are reported in the experimental section. Potency of the drug is defined as EC_{50} (negative inotropic activity), IC_{50} (vasodilator activity), and EC_{30} (negative chronotropic activity). Activity is defined as the percent decrease in developed tension on isolated left atrium (negative inotropic activity), percent decrease in atrial rate of spontaneously beating isolated right atrium (negative chronotropic activity), and percent inhibition of calcium-induced contraction on K^+-depolarized aortic strips (vasodilator activity) at the concentrations indicated in the footnotes of Table 2.

5. Results and discussion

The results of MDR-reverting activity and cardiovascular effect of our compounds are reported in Table 2 where the corresponding values for verapamil and MM36 are reported as reference. In general, all compounds show good MDR-reverting activity with a potency that is comparable to that of verapamil and, in several cases, higher. Only **3a$_2$** and **6b** show a decrease in the chemosensitizing effect with respect to the reference compound. On the contrary, cardiovascular activity is, as expected, always lower. Actually, some compounds show very low intrinsic activity and may be considered inactive, both on myocardial and vascular preparations. As a consequence, when the maximum effect, that is intrinsic activity (evaluated up to the 5×10^{-5} M concentration), was lower than 50%, the corresponding EC_{50} inotropic, EC_{30} chronotropic, and IC_{50} values were not calculated.

Considering this new series of derivatives, compounds **2a** ($[i]_{0.5} = 0.10$ μM) and **4a** ($[i]_{0.5} = 0.14$ μM) show the highest chemosensitizing effect, with a $[i]_{0.5}$ that is one order of magnitude lower with respect to the reference compound verapamil. They lack vasorelaxant activity, but maintain a residual cardiac effect, both on inotropy and chronotropy. Compounds **1a** ($[i]_{0.5} = 0.44$ μM), **1b** ($[i]_{0.5} = 0.34$ μM) and **2b** ($[i]_{0.5} = 0.68$ μM) also possess good anti-MDR activity showing only a modest chronotropic effect. The best results were obtained with compound **4b** ($[i]_{0.5} = 0.29$ μM) showing only a residual negative chronotropic effect (100 times lower than that of verapamil) and with compounds **7a** ($[i]_{0.5} = 0.90$ μM), **8a** ($[i]_{0.5} = 0.54$ μM) and **3a$_1$** ($[i]_{0.5} = 0.36$ μM), that lack any cardiovascular effect. Therefore, the first result to mention is that, as hypothesized, the diphenylmethyl cyclohexane moiety decreases cardiovascular activity without affecting MDR-reverting properties which, on the contrary, are in most cases exalted in the new derivatives.

Analysis of MDR structure–activity relationships does not give univocal answers. A first aspect to evaluate is the influence of the amine moieties on the pharmacological profile of our derivatives. Comparing the activity of compounds **1**–**5**, there is no apparent trend relating MDR-reverting activity and selectivity with the number of alkyl substituents on the nitrogen, contrary to the suggestions of previous works which indicate a higher MDR activity and a lower cardiovascular activity for secondary amines.[21] For example, compounds **1** and **2** show more or less the same profile, with $[i]_{0.5}$ ranging from 0.10 (**2a**) to 0.68 (**2b**) μM, and with a similar cardiovascular activity. Restricting the conformational freedom of the amine moiety does not ameliorate the pharmacological profile of the compounds and does not seem to induce major changes, as shown by the similar profiles of **3**, **4**, **5** for both MDR and cardiovascular activity, with the notable exception of **3a$_1$**, that completely loose any cardiovascular action. At the contrary, the introduction of flat and bulky groups on the nitrogen atom, as happens in compounds **6, 7**, and **8** produces interesting effects: compound **6** shows only a residual negative inotropic activity and, as previously observed in other series,[28] introduction of the thioxantene moiety (**7**) does abolish cardiovascular activity. The same happens for compound **8**. These results confirm the importance of lipophilic moiety to separate cardiovascular and anti-MDR activity.

In accordance with previously reported results, stereochemistry does not seem to play a major role.[21] In fact, *cis* isomers seem to be more effective as MDR antagonists with respect to the *trans* ones, but the differences are not so impressive, the largest difference being found between compounds **6a** and **6b**. The tetrahydronaphthalene derivatives are an exception that will require further studies: **3a$_1$** is 20 times more potent than **3a$_2$** as chemosensitizer and completely lacks cardiovascular activity, while its less MDR-potent isomer maintains a residual negative chronotropic action.

In conclusion, we have synthesized a new series of potent MDR reverters that are only loosely related to verapamil. Three compounds of the series (**3a$_1$**, **7a**, **8a**), which are more potent than verapamil and lack any cardiovascular action, deserve further studies and can be useful leads for the development of new MDR reversing drugs.

6. Experimental

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 in Nujol mull for solids and neat for liquids. Mass spectra were acquired by a triple quadrupole mass spectrometer API 365 equipped with a TurboIon Spray interface from Applied Biosystems. Spectra were recorded in positive ion mode in the mass range from 120 to 620 Th, at unit mass resolution. GC–MS spectra were acquired by a Perkin–Elmer 8420 capillary gas chromatograph, connected to a Perkin–Elmer Ion Trap detector. Unless otherwise stated, NMR spectra were recorded on a Gemini 200 spectrometer (200 MHz for ^{1}H NMR, 50.3 MHz for ^{13}C), and 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). Although the IR

spectra data are not always included, they were obtained for all compounds reported and are consistent with the assigned structures. Yields are given after purification, unless otherwise stated. Where analyses are indicated by symbols, the analytical results are within ±0.4% of the theoretical values. Compounds were named following IUPAC rules as applied by Beilstein-Institut AutoNom (version 2.1), a software for systematic names in organic chemistry. When reactions were performed in anhydrous conditions, the mixtures were maintained under nitrogen.

6.1.1. (3-Oxocyclohexyl)diphenylacetonitrile (9). A solution of butyllithium (12 mL of a 1.6 M solution in hexane, 19.2 mmol) was added to a solution of diphenylacetonitrile (3 g, 15.52 mmol) in anhydrous THF at −78 °C. The mixture turned yellow and was kept at −78 °C for 1 h. Then 1.8 mL (1.79 g, 18.59 mmol) of 2-cyclohexen-1-one were added, and the reaction was left to reach room temperature overnight. To the crude reaction mixture, kept at 0 °C, a saturated aqueous NH_4Cl solution was added, the layers were separated, the aqueous solution was re-extracted with diethyl ether, and the two organic extracts were combined, washed with water and dried over Na_2SO_4. The solvent was removed in vacuo, and the crude product was purified by flash chromatography on silica gel, using cyclohexane/ethyl acetate (70:30) as the eluent. Yield 2.11 g (7.30 mmol, 47%).

^{1}H NMR ($CDCl_3$) δ 7.52–7.25 (m, 10H, aromatics), 3.04–2.86 (m, 1H, CH–C–CN), 2.50–2.20 (m, 3H, cyclohexane protons), 2.18–2.04 (m, 1H, cyclohexane proton), 2.00–1.60 (m, 4H, cyclohexane protons); MS: 289 ($M^{+\cdot}$), 271 ($M^{+\cdot}-16$), 193 ($M^{+\cdot}-96$, 100%). Anal. ($C_{20}H_{19}NO$) C, H, N.

6.1.2. (3-Hydroxycyclohexyl)diphenylacetonitrile (10). A portion of **9** (1.11 g, 3.84 mmol) in anhydrous THF was added dropwise to a suspension of 436 mg (3 equiv, 11.52 mmol) of $NaBH_4$ in anhydrous THF at 0 °C. The mixture was kept 2 h at 0 °C, then was allowed to warm to room temperature overnight. The mixture was then cooled at 0 °C and 5 mL of a 30% solution of NH_4OH added. The organic layer was separated, dried and evaporated to give a white solid (mp 48–50 °C). TLC and GC–MS showed the presence of two isomers. The mixture was then column chromatographed, using ethyl acetate/cyclohexane (30:70) as the eluent. The chromatography yielded 810 mg (2.78 mmol, 73% yield) of a mixture of the two isomers, which could not be separated.

^{1}H NMR δ 7.50–7.20 (m, 10H, aromatics), 4.20 (br s, $w/2$ = 8.2 Hz, 0.1H), 3.60 (br s, $w/2$ = 26.5 Hz, 0.9H) (CH–O), 2.55 (t, J = 11.4 Hz, 1H, CH–C–CN), 2.10–1.77 (m, 3H, OH and cyclohexane protons), 1.70–1.47 (m, 2H, cyclohexane protons), 1.43–1.15 (m, 4H, cyclohexane protons); GC–MS first eluted compound **10b** (*trans*): 291 ($M^{+\cdot}$), 274 ($M^{+\cdot}-17$), 247 ($M^{+\cdot}-44$), 193 ($M^{+\cdot}-98$, 100%), second eluted compound **10a** (*cis*): 291 ($M^{+\cdot}$), 274 ($M^{+\cdot}-17$), 247 ($M^{+\cdot}-44$), 193 ($M^{+\cdot}-98$, 100%). Anal. ($C_{20}H_{21}NO$) C, H, N.

6.1.3. *cis* Methanesulfonic acid 3-(cianodiphenylmethyl)-cyclohexyl ester (11a). A solution of **10** (as the mixture of the *cis* and the *trans* isomer) (890 mg, 3.06 mmol) and triethylamine (0.6 mL, 440 mg, 4.32 mmol) in anhydrous CH_2Cl_2 was treated with 0.36 mL (500 mg, 4.32 mmol) of methanesulfonyl chloride dissolved in CH_2Cl_2 at −20 °C and kept at this temperature for 2 h. The mixture was then allowed to warm to room temperature overnight. The solution was washed with water, dried, and evaporated under vacuum to give a solid that was purified by crystallization from ethanol (mp 90–92 °C). Yield: 1.06 g (2.66 mmol, 87%). TLC and GC–MS indicated a small amount of the *trans* isomer that remained in the mater liquor.

^{1}H NMR δ 7.52–7.23 (m, 10H, aromatics), 4.69 (tt, J = 4.6 Hz, J = 11.0 Hz, 1H, $CHOSO_2$) 2.94 (s, 3H, CH_3SO_2) 2.62 (tt, J = 3.2 Hz, J = 12.0 Hz, 1H, CH–C–CN), 2.30–1.85 (m, 3H, cyclohexane protons), 1.74–1.22 (m, 5H, cyclohexane protons); GC–MS of the mixture: first eluted compound **11a** (*cis*): 399 ($M^{+\cdot}$), 274 ($M^{+\cdot}-125$), 247 ($M^{+\cdot}-152$), 193 ($M^{+\cdot}-206$, 100%), second eluted compound **11b** (*trans*): 399 ($M^{+\cdot}$), 274 ($M^{+\cdot}-125$), 247 ($M^{+\cdot}-152$), 193 ($M^{+\cdot}-206$, 100%). Anal. ($C_{21}H_{23}NO_3S$) C, H, N.

6.1.4. *cis* (3-Bromocyclohexyl)diphenylacetonitrile (12a) and *trans* (3-bromocyclohexyl)diphenylacetonitrile (12b). Compound **11a** (650 mg, 1.63 mmol) was dissolved in dry acetone, and a solution of LiBr (984 mg, 7 equiv, 11.41 mmol) in dry acetone was added. The mixture was heated to reflux for 38 h, then the solvent was removed and the residue dissolved in CH_2Cl_2, washed with water and dried. Evaporation of the solvent gave a thick oil which was a mixture of the two isomers (TLC) that were separated by column chromatography using ethyl acetate/cyclohexane (10:90) as eluting system.

The *trans* isomer **12b** was eluted first (240 mg, 42% yield). ^{1}H NMR δ 7.57–7.21 (m, 10H, aromatics), 4.77 (t, J = 2.8 Hz, 1H, CHBr) 3.20 (tt, J = 3.2 Hz, J = 11.6 Hz, 1 H, CH–C–CN), 2.40–1.20 (m, 8H, cyclohexane protons); MS: 355/353 (1:1, $M^{+\cdot}$), 329–327 (1:1, $M^{+\cdot}-26$), 273 ($M^{+\cdot}-82/80$), 193 ($M^{+\cdot}-162/160$, 100%). Anal. ($C_{20}H_{20}BrN$): C, H, N.

The second fraction was the *cis* isomer **12a** (130 mg, 23% yield). ^{1}H NMR δ 7.56–7.21 (m, 10H, aromatics), 4.00 (tt, J = 4.2 Hz, J = 11.8 Hz, 1H, CHBr) 2.58 (tt, J = 4.0 Hz, J = 12.0 Hz, 1H, CH–C–CN), 2.36–1.32 (m, 8H, cyclohexane protons); MS: 355/353 (1:1, $M^{+\cdot}$), 329/327 (1:1, $M^{+\cdot}-26$), 273 ($M^{+\cdot}-82/80$), 193 ($M^{+\cdot}-162/160$, 100%). Anal. ($C_{20}H_{20}BrN$) C, H, N.

6.1.5. *cis* (3-Methylaminocyclohexyl)diphenylacetonitrile (15a). A 20% toluene solution of methylamine (2 mL), **9** (0.5 g, 1.73 mmol) and *p*-toluenesulfonic acid monohydrate (150 mg) was kept 24 h at 110 °C in a small steel bomb. Evaporation of the solvent gave an oil that was dissolved in CH_2Cl_2, washed with water and dried. Removal of the solvent gave 420 mg of the Schiff base (3-methyliminocyclohexyl)diphenylacetonitrile (**13**) as an

oil that was used as such in the following reaction. IR (neat) ν 2250 (CN), 1670 (C=N) cm^{-1}. Then $NaBH_4$ (60 mg) was cautiously added to a solution of the crude Schiff base **13** in hot methanol (10 mL) over a period of 0.5 h. The mixture was then heated to reflux for 5 h. After cooling, the mixture was treated with a few drops of water and the excess solvent removed. The residue was dissolved in CH_2Cl_2, washed with a saturated solution of $NaHCO_3$ and dried. Evaporation of the solvent gave an oil that was separated by column chromatography with chloroform/methanol (97:03) as the eluent, yielding 40 mg of the title compound.

^1H NMR δ 7.60–7.30 (m, 10H, aromatics), 2.52 (s, 3H, NCH_3), 2.46 (br s, $w/2 = 22$ Hz, 1H, CHN), 2.20–1.60 (m, 10H, cyclohexane protons and NH) ppm. Anal. ($C_{21}H_{24}N_2$) C, H, N.

6.1.6. [3-[2-(3,4-Dimethoxyphenyl)ethylamino]cyclohexyl]diphenylacetonitrile (1). A solution of **9** (530 mg, 1.83 mmol), homoveratrylamine (0.34 mL, 360 mg, 1.99 mmol) and *p*-toluenesulfonic acid monohydrate (150 mg) in anhydrous benzene was heated to reflux for 24 h and water was removed from the reaction with the aid of a Dean–Stark trap. The solvent was distilled off and the residue dissolved in CH_2Cl_2, washed with a saturated solution of $NaHCO_3$ and dried. Evaporation of the solvent gave 800 mg of Schiff base [3-[2-(3,4-dimethoxyphenyl)ethylimino]cyclohexyl]diphenylacetonitrile (**14**) that is quite unstable and was used as such in the next reaction. IR (neat): ν 2250 (CN), 1670 (C=N) cm^{-1}. The Schiff base was then reduced by two different procedures.

6.1.6.1. Procedure A. To a solution of crude **14** (400 mg) in hot methanol (10 mL), $NaBH_4$ (68 mg) was cautiously added over a period of 0.5 h. The mixture was then heated to reflux for 5 h. After cooling, the mixture was treated with a few drops of water and the excess solvent removed. The residue was dissolved in CH_2Cl_2, washed with a saturated solution of $NaHCO_3$ and dried. Evaporation of the solvent gave 380 mg of an oil that was converted into the oxalate to eliminate non-basic side products. The mixture of amines obtained from the oxalate (aqueous NaOH and CH_2Cl_2 extraction) (300 mg) was separated by column chromatography with chloroform/methanol (90:10). The third fraction constituted about 7% of the chromatographed mixture and was the *cis* isomer **1a**. Its ^1H NMR spectrum is consistent with the proposed structure and is reported in the supplementary material.

6.1.6.2. Procedure B. To a solution of crude **14** (400 mg) in abs ethanol (10 mL), Pd/C (120 mg) was added and the mixture was maintained for 4 days at 82 psi at room temperature in a Parr apparatus. The mixture was then filtered. Evaporation of the solvent gave 350 mg of an oil that was separated by column chromatography with chloroform/methanol (90:10). The second fraction (70 mg) was constituted by a mixture of the two isomers **1a** *cis* and **1b** *trans* (TLC), which was not purified further.

6.1.7. *cis*[3-[2-[2-(3,4-Dimethoxyphenyl)ethyl]methylamino]cyclohexyl]diphenylacetonitrile (2a). Compound *trans* **12b** (220 mg, 0.62 mmol) were added to 0.11 mL (120 mg, 0.62 mmol) of *N*-methylhomoveratrylamine in triethylamine. The mixture was refluxed for 20 h. CH_2Cl_2 was then added, the organic layers were washed with water and a saturated solution of $NaHCO_3$, and dried. Evaporation of the solvent gave the crude product that was purified by column chromatography using chloroform/methanol (97:03) as the eluent. Yield: 50 mg of isomer **2a** (0.11 mmol, 17%).

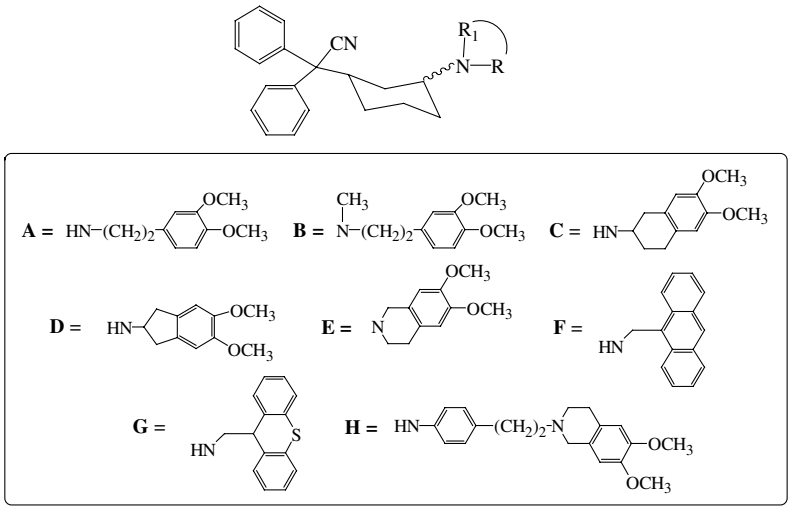
With the same procedure described above, and starting from the *cis* isomer **12a**, the same compound **2a** was obtained in 11% yield.

6.1.8. General procedure for the synthesis of diphenylcyclohexane derivatives (1–8). A mixture of **9** (300 mg, 1.09 mmol), an equimolar amount of the suitable amine (1.09 mmol) and an excess of titanium(IV) isopropoxide (0.45 mL, 1.36 mmol) was stirred with a drying tube at room temperature for 2–10 h and heating at 60 °C if necessary, depending on the starting compound. When necessary, a few drops of benzene were added in order to make the mixture homogeneous. After a suitable time, the IR spectrum of the mixture showed no ketone band, and the viscous solution was diluted with abs ethanol (2 mL). Sodium cyanoborohydride (60 mg, 0.74 mmol) was added, and the solution was stirred for 20 h. Water (0.5 mL) was then added, and the mixture was concentrated in vacuo. The crude product was dissolved in CH_2Cl_2, filtered to remove the solids, washed with a solution of $NaHCO_3$ and water, dried over Na_2SO_4 and concentrated in vacuo. The crude substance was then purified by column chromatography. Total yield ranged from 40% to 85%. The eluents used and the diastereoisomers yield ratios are reported in Table 3. The obtained diastereoisomers were transformed into the corresponding oxalates by treatment of the resulting compounds with an equimolar amount of oxalic acid in ethyl acetate.

The chemical and physical characteristics of the compounds **1–8** are reported in Table 3. Their IR spectra are consistent with the proposed structures; their ^1H NMR spectra are reported in the supplementary material. The ^1H NMR spectra of the two isomers of *cis* {3-[2-(3,4-dimethoxyphenyl)ethylamino]cyclohexyl}diphenylacetonitrile (**1a**) and *trans* {3-[2-(3,4-dimethoxyphenyl)ethylamino]cyclohexyl}diphenylacetonitrile (**1b**) were performed also at 400 MHz, and these spectra are reported as an example.

1a: ^1H NMR, 400 MHz ($CDCl_3$) δ: 7.51–7.45 (m, 4H, aromatics); 7.37–7.31 (m, 4H, aromatics); 7.29–7.21 (m, 2H, aromatics); 6.85–6.68 (m, 3H, aromatics); 3.85 (s, 3H, OCH_3); 3.84 (s, 3H, OCH_3); 2.95–2.69 (m, 4H, CH_2–CH_2); 2.60 (tt, $J = 12.0$ Hz, $J = 4.0$ Hz, 1H, CH–N); 2.58 (tt, $J = 11.0$ Hz, $J = 4.0$ Hz, 1H, CH–C–CN); 2.20 (br s, 1H, NH); 2.02 (d, $J = 12.0$ Hz, 1H); 1.95 (d, $J = 16.0$ Hz, 1H); 1.88–1.80 (m, 1H); 1.66–1.60 (m, 1H); 1.38–1.18 (m, 3H); 1.12–1.04 (m, 1H) (cyclohexane protons).

Table 3. Chemical and physical characteristics of derivatives **1**–**8**



Compd	–NRR$_1$	Mass (M+H)$^{+a}$	Eluent	Isomers order of elution and yield ratio	Mp (°C)[b]	Analysis[c]
1b	A	455	$CHCl_3$/MeOH	*trans*, *cis*	235–237	$C_{32}H_{36}N_2O_6$
1a	A	455	(95:05)	4:6	200–201	$C_{32}H_{36}N_2O_6$
2b	B	469	$CHCl_3$	*trans*, *cis*	103–105	$C_{33}H_{38}N_2O_6$
2a	B	469	(100)	4:6	179–181	$C_{33}H_{38}N_2O_6$
3a1	C	481	$CHCl_3$	*cis*, *cis*	257–260	$C_{34}H_{38}N_2O_6$
3a2	C	481	(100)	4:4[d]	200–204	$C_{34}H_{38}N_2O_6$
4b	D	467	CH_2Cl_2	*trans*, *cis*	233–235	$C_{33}H_{36}N_2O_6$
4a	D	467	(100)	3:7	239–241	$C_{33}H_{36}N_2O_6$
5a	E	467	$CHCl_3$/MeOH	*cis*, *trans*	135–136	$C_{33}H_{36}N_2O_6$
5b	E	467	(99:01)	7:3	155–157	$C_{33}H_{36}N_2O_6$
6b	F	481	$CHCl_3$/petr. ether	*trans*, *cis*	95–97	$C_{37}H_{34}N_2O_4$
6a	F	481	(90:10)	4:6	155–156	$C_{37}H_{34}N_2O_4$
7b	G	501	$CHCl_3$	*trans*, *cis*	218–220	$C_{36}H_{34}N_2O_4S$
7a	G	501	(100)	3:7	170–171	$C_{36}H_{34}N_2O_4S$
8a	H	586	$CHCl_3$/MeOH	*cis*, *trans*	180–181	$C_{41}H_{45}N_3O_6$
8b	H	586	(98:02)	9:1	163–164	$C_{41}H_{45}N_3O_6$

[a] As free base. Spectra were recorded in positive ion mode at unit mass resolution (see experimental part for details).
[b] As oxalate. Crystallization solvent:absolute ethanol.
[c] As oxalate. All compounds have been analyzed for C, H, N after vacuum drying at a temperature below the melting point; the results obtained range within ±0.4% of the theoretical values.
[d] The two *trans* isomers represent 20% of the mixture and were not characterized.

1b: ^{1}H NMR, 400 MHz ($CDCl_3$) δ: 7.53–7.43 (m, 4H, aromatics); 7.38–7.21 (m, 6H, aromatics); 6.85–6.73 (m, 3H, aromatics); 3.89 (s, 6H, OCH_3); 3.08 (br s, $w/2$ = 18 Hz, 1H, CH–N); 2.90 (br s, 1H, NH); 2.82–2.70 (m, 5H, CH–C–CN and CH_2–CH_2); 1.82–1.61 (m, 4H, cyclohexane protons); 1.61–1.20 (m, 4H, cyclohexane protons).

6.1.9. Crystal structure determination and refinement of 2a oxalate. Diffraction data were collected at 293 K on CuKα radiation, using a rotating anode source and a 1K CCD detector from Bruker equipped with Göbel mirrors. Data were processed using the program SAINT from Bruker and intensities were corrected for absorption (SADABS).[40] The crystal data and details of the data collection and structure refinement are summarized in Table 4. The crystal structure was solved by direct methods using the SIR97 program[41] which gave the position of most of the non-hydrogen atoms. The remaining atoms were identified by successive Fourier difference syntheses. One independent compound molecule and one oxalate molecule were found in the asymmetric unit. Refinement was carried out on F^2 by full-matrix least square techniques, using the SHELX97 program package.[42] Hydrogen atoms, except for the non-salified hydrogen of oxalic acid, were added in the model constrained to idealized positions and refined using a riding model with riding isotropic displacement parameters. All the non-hydrogen atoms were refined anisotropically.

In Figure 1 a thermal ellipsoid representation of **2a oxalate** is shown. Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre with number CCDC 228890.

6.2. Pharmacology

6.2.1. MDR-reverting activity. 6.2.1.1. Drugs and chemicals. Purified pirarubicin was provided by Laboratoire

Table 4. Crystal data and structure refinement for **2a oxalate**

Formula[a]	$C_{33}H_{37}N_2O_6$
Formula weight[a]	557.65
Space group	*P*-1
Unit cell dimensions	
a, Å	9.1353(10)
b, Å	11.2413(12)
c, Å	15.0484(17)
α, deg	83.957(5)
β, deg	87.607(6)
γ, deg	78.541(6)
Volume, Å^3	1505.80
Z	2
Density (calcd), g/cm^3	1.230
Abs coeff, mm^{-1}	0.69
Radiation	CuKα
Reflns collected	4971
No. of unique reflns	3193
Completeness %	81.6
Value of R_{int}	0.0284
Final *R* indices [$I > 2\text{sigma}(I)$]	$R1 = 0.0508$
R indices (all data)	$R1 = 0.0647$, $wR2 = 0.1306$
Extinction coeff	0.001573
Largest diff. peak and hole, e Å^{-3}	0.32, −0.12

[a] The non-salified hydrogen of oxalic acid was not introduced in the refinement (see the experimental part).

Roger Bellon (France). Concentrations were determined by diluting stock solutions to approximately 10^{-5} M and using $\varepsilon_{480} = 11{,}500\ M^{-1}\ cm^{-1}$. 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 7.25.

6.2.1.2. Cell lines and cultures. K 562 is a human leukemia cell line.[43] Cells resistant to doxorubicin were obtained by continuous exposure to increasing doxorubicin concentrations and were maintained in medium containing doxorubicin (400 nM) until 1–4 weeks before experiments. This subline expresses a unique membrane glycoprotein with a molecular weight of 180,000 Da.[44] Doxorubicin-sensitive and -resistant erythroleukemia K 562 cells were grown in suspension in RPMI 1640 (Sigma) medium supplemented with L-glutamine and 10% FCS at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cultures, initiated at a density of 10^5 cells/mL grew exponentially to $8–10 \times 10^5$ cells/mL in three days. For the spectrofluorometric assays, in order to have cells in the exponential growth phase, culture was initiated at 5×10^5 cells/mL and cells were used 24 h later, when the culture had grown to about $8–10 \times 10^5$ cells/mL. Cell viability was assessed by trypan blue exclusion. The cell number was determined by Coulter counter analysis.

6.2.1.3. Cellular drug accumulation. The uptake of pirarubicin cells was followed by monitoring the decrease in the fluorescence signal at 590 nm ($\lambda_{ex} = 480$ nm) following the method previously described.[45] Using this method it is possible to accurately quantify the kinetics of the drug uptake by the cells and the amount of anthracycline intercalated between the base pairs in the nucleus at the steady state, as incubation of the cells with the drug proceeds without compromising cell viability. All experiments were conducted in 1 cm quartz cuvettes containing 2 mL of buffer at 37 °C using a circulating thermostated water bath. Cells, 2×10^6, were suspended in 2 mL of glucose containing HEPES buffer at pH 7.3, under vigorous stirring; 20 μL of the stock anthracycline solution was quickly added to this suspension yielding an anthracycline concentration C_T equal to 1 μM. The decrease of the fluorescence intensity F at 590 nm was followed as a function of time. After about 20 min, the curve $F = f(t)$ reached a plateau and the fluorescence intensity was equal to F_n. The drug-cells system was thus in a steady state and the overall concentration C_n of drug intercalated between the base pairs in the nucleus was $C_n = C_T \cdot (F_0 - F_n)/F_0$. Once the steady state was reached, the inhibitor at concentration [i] ([i] was varied from 0.05 to 10 μM) was added and a new steady state was reached, the fluorescence intensity being F_n^i The overall concentration C_n^i of drug intercalated between the base pairs in the nucleus was then $C_n^i = C_T \cdot (F_0 - F_n^i)/F_0$. An aliquot of the solution was then taken away and cell viability was assessed by trypan blue exclusion. Cell membranes were then permeabilized by the addition of 0.05% Triton X-100 yielding the equilibrium state which was characterized by a new value F_N of the fluorescence intensity. The overall concentration C_N of drug intercalated between the base pairs in the nucleus was then $C_N = C_T \cdot (F_0 - F_N)/F_0$. We checked that tested compounds did not affect the fluorescence of THP-adriamycin. α was measured with the following expression: $\alpha = (C_n^i - C_n)/(C_N - C_n)$.

6.2.2. Cardiovascular activity. The pharmacological profile of compounds was tested on guinea-pig isolated left and right atria to evaluate their inotropic and chronotropic effects, respectively, and on K^+-depolarized guinea-pig aortic strips to assess calcium antagonist activity. At first all compounds were checked at increasing doses to evaluate the percent decrease on developed tension on isolated left atrium driven at 1 Hz (negative inotropic activity), the percent decrease in atrial rate on spontaneously beating right atrium (negative chronotropic activity) and the percent inhibition of calcium-induced contraction on K^+-depolarized aortic strips (vasorelaxant activity). Data were analyzed by Student's *t*-test. The potency of drugs defined as EC_{50}, EC_{30} and IC_{50} was evaluated from log concentration–response curves (Probit analysis by Litchfield and Wilcoxon, $n = 6–8$) in the appropriate pharmacological preparations. All data are presented as mean ± SEM.[46]

6.2.2.1. Guinea-pig atrial preparations. Guinea pigs (300–400 g female) were sacrificed by cervical dislocation. After thoracotomy the heart was immediately removed and washed by perfusion through the aorta with oxygenated Tyrode solution of the following composition (mM): 136.9 NaCl, 5.4 KCl, 2.5 $CaCl_2$, 1.0 $MgCl_2$, 0.4 $NaH_2PO_4 \times H_2O$, 11.9 $NaHCO_3$ and 5.5 glucose. The physiological salt solution (PSS) was buffered at pH 7.4 by saturation with 95% O_2–5% CO_2 gas, and the temperature was maintained at 35 °C. Isolated guinea-pig heart preparations were used, spontaneously beating right atria and left atria driven at 1 Hz. For each

preparation, the entire left and right atria were dissected from the ventricles, cleaned of excess tissue, and hung vertically in a 15-mL organ bath containing the PSS continuously bubbled with 95% O_2–5% CO_2 gas at 35 °C, pH 7.4. The contractile activity was recorded isometrically by means of a force transducer (FT 0.3, Grass Instruments, Quincy, MA) using Power Lab software (Basile, Italy). The left atria were stimulated by rectangular pulses of 0.6–0.8 ms duration and about 50% threshold voltage through two platinum contact electrodes in the lower holding clamp (Grass S88 stimulator). The right atrium was in spontaneous activity. After the tissue was beating for several minutes, a length–tension curve was determined, and the muscle length was maintained at which elicited 90% of maximum contractile force observed at the optimal length. A stabilization period of 45–60 min was allowed before the atria were used to test compounds. During the equilibration period, the bathing solution was changed every 15 min and the threshold voltage was ascertained for the left atria. Atrial muscle preparations were used to examine the inotropic and chronotropic activity of the compounds (0.1, 0.5, 1, 5, 10, 50 and 100 μM), first dissolved in DMF and then diluted with PSS. According to this procedure, the concentration of DMF in the bath solution never exceeded 0.3%, a concentration that did not produce appreciable inotropic and chronotropic effects. During the construction of cumulative dose–response curves, the next higher concentration of the compounds was added only after the preparation reached a steady state.

6.2.2.2. Guinea-pig aortic strips. The thoracic aorta was removed and placed in a Tyrode solution of the following composition (mM): 118 NaCl, 4.75 KCl, 2.54 $CaCl_2$, 1.20 $MgSO_4$, 1.19 KH_2PO_4, 25 $NaHCO_3$ and 11 glucose equilibrated with 95% O_2–5% CO_2 gas at pH 7.4. The vessel was cleaned of extraneous connective tissue. Two helicoidal strips (10 mm × 1 mm) were cut from each aorta beginning from the end most proximal to the heart. Vascular strips were then tied with surgical thread (6–0) and suspended in a jacketed tissue bath (15 mL) containing aerated pharmacological salt solution (PSS) at 35 °C. Strips were secured at one end to a force displacement (FT 0.3, Grass) transducer for monitoring changes in isometric contraction. Aortic strips were subjected to a resting force of 1 g and washed every 20 min with fresh PSS for 1 h after the equilibration period; guinea-pig aortic strips were contracted by washing in PSS containing 80 mM KCl (equimolar substitution of K^+ for Na^+). After the contraction reached a plateau (about 45 min), the compounds (0.1, 0.5, 1, 5, 10, 50 and 100 μM) were added cumulatively to the bath allowing for any relaxation to obtain an equilibrated level of force. Addition of the drug vehicle had no appreciable effect on K^+-induced contraction (DMF for all compounds).

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