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Inhibition of P-glycoprotein-mediated Multidrug Resistance (MDR) by *N,N*-bis(cyclohexanol)amine aryl esters: Further restriction of molecular flexibility maintains high potency and efficacy

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ABSTRACT

Conformational modulation of the aryl portion of a set of *N,N*-bis(cyclohexanol)amine aryl esters (**1a–d**) that are potent Pgp-dependent MDR inhibitors has been performed. Toward this end the *trans*-3-(3,4,5-trimethoxyphenyl)acrylic acid present in set **1** was substituted with 3-(3,4,5-trimethoxyphenyl)propanoic and 3-(3,4,5-trimethoxyphenyl)propiolic moieties to give sets **2** and **3**, respectively. While the introduction of 3-(3,4,5-trimethoxyphenyl)propanoic moiety resulted in a definite drop in potency and efficacy, esterification with 3-(3,4,5-trimethoxyphenyl)propiolic acid gave four isomers (**3a–d**) that maintain high potency and possess optimal efficacy. These results are discussed in terms of conformational flexibility of the different sets of compounds.

Drug transporter proteins are fundamental to the functions of living organisms, being involved in the absorption, translocation, secretion, and excretion of a variety of endogenous and xenobiotic molecules. Because of their physiological role, these proteins can modulate pharmacokinetic and toxic properties of nutrients and drugs.[1]

The ABC superfamily of multidrug transporter proteins is one of the largest in living organisms and its members are involved in several fundamental cellular processes.[2] ABCB1 (Pgp) is the most intensively studied member of the family since it has been found in several important tissues and blood–tissue barriers where, together with other family members, it seems to regulate the secretion of physiologically important lipophilic molecules[3] and the extrusion of xenobiotics that enter the organism.[4] Overexpression of Pgp and related proteins (mainly ABCC1 and ABCG2) that act as extrusion pumps, can induce classical multidrug resistance (MDR), a kind of acquired drug resistance of cancer cells and microorganisms to various chemotherapeutic drugs that usually are structurally and mechanistically unrelated.[5,6]

Even if there is no high-resolution crystal structure of human Pgp, homology models, build upon the resolved structure of other related proteins such as Sav 1866[7,8] and murine Pgp[9,10] integrated by the information obtained from other bacterial proteins such as QacR[11,12] suggest that Pgp recognition site is characterized by a polymorphous drug binding domain[13,14] where a variety of molecules can accommodate in a plurality of binding modes including π–π, ion–π, hydrogen bonds and hydrophobic interactions.

In principle, modulation of the functions of Pgp and like proteins would be one way of circumventing the appearance of MDR in cancer cells. This is the main reason prompting the design and synthesis of Pgp inhibitors,[15–19] although thus far, no drug of this class has been approved for cancer therapy.[20] However other potential uses of these agents are emerging, such as that of enhancing drug penetration through biologically protective barriers, such as the blood–brain and blood–cerebrospinal fluid.[21,22] Recent evidence indicates that Pgp plays a role in the inhibition of viral infectivity of the human immunodeficiency virus (HIV).[23] Recently, the important role of ABC transporter proteins like Pgp in stem cells has been evidenced.[24] Finally, the increasing interest in the functions and mechanism of action of Pgp and sister proteins requires

Abbreviations: EDCl, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimmide hydrochloride; DMAP, 4-dimethylaminopyridine; DOX, doxorubicin.

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the availability of new and potent molecules to be used as pharmacological tools.

In the search for efficient MDR reverters, we designed a new family of compounds[25] on the basis of the characteristics of the substrate recognition sites of Pgp, MRP1 and similar proteins, discussed above. Structurally, the compounds designed are *N,N*-bis(cyclohexanol)amine aryl esters formed by a scaffold where a basic linker tethers two aromatic moieties. Some of these novel derivatives showed low-nanomolar potency and high efficacy in inhibiting Pgp-dependent nuclear pirarubicin efflux in doxorubicin-resistant erythroleukemia K562 cells as well as rat intestinal mucosa ATPase activity mostly referable to MRP1 and in increasing the cytotoxicity of doxorubicin towards doxorubicin-resistant erythroleukemia K562 cells.[25]

The scaffold of *N,N*-bis(cyclohexanol)amine aryl esters is fairly rigid but the aryl substituents maintain some flexibility and we decided to evaluate the consequences of further conformational modulation of the *trans*-3-(3,4,5-trimethoxyphenyl)acrylic acid moiety in the most active members of the series, the **1a–d** set of isomers (Chart 1). Therefore we synthesized the **2a–d** and **3a–d** sets of isomers where *trans*-3-(3,4,5-trimethoxyphenyl)acrylic acid is substituted by 3-(3,4,5-trimethoxyphenyl)propanoic acid or 3-(3,4,5-trimethoxyphenyl)propiolic acid (Chart 1). Unfortunately, we were unable to obtain the set of isomers esterified with *cis*-3-(3,4,5-trimethoxyphenyl)acrylic acid since the compounds rapidly isomerize to the more stable *trans* isomers.

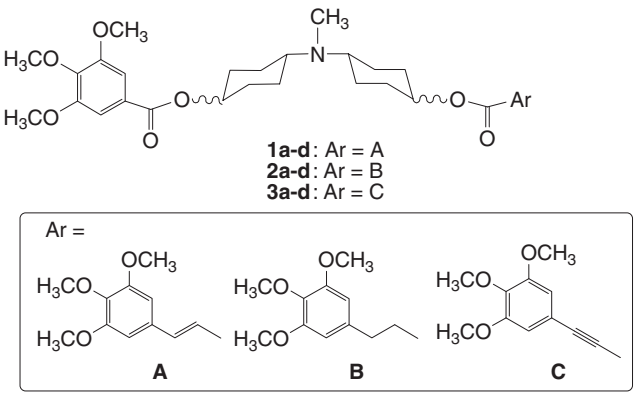


Chart 1. General structures of designed compounds.

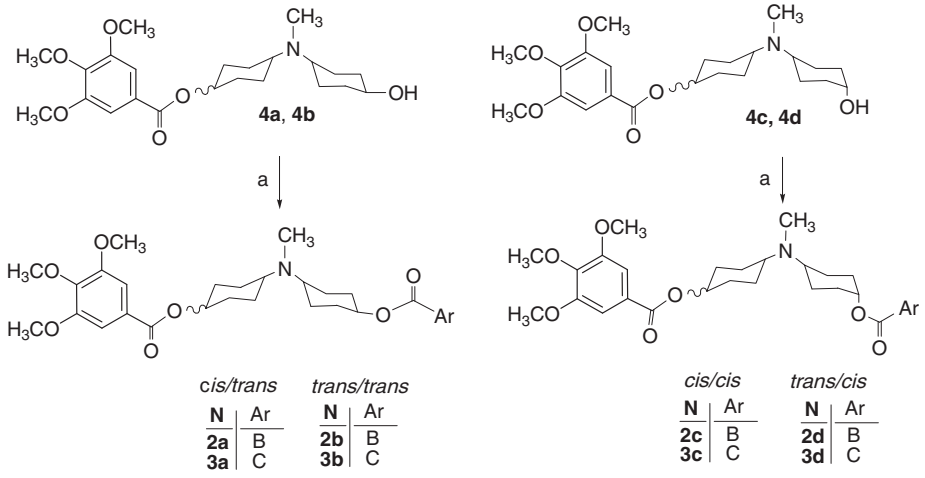
The synthesis of compounds **1a–d** has been previously reported.[25] Compounds **2a**–**d** and **3a**–**d** were obtained following the reaction pathways described in Scheme 1, starting from intermediates **4a–d** whose synthesis has been previously described as well as their configuration that was attributed on the basis of the ^{1}H NMR characteristics of the cyclohexane protons.[25] ^{1}H NMR spectra indicate that the two cyclohexane rings are in a *cis/trans* (series a), *trans/trans* (series b), *cis/cis* (series c) and *trans/cis* (series d) configuration.

Final compounds **2a–d** and **3a–d** were obtained by reaction of **4a–d** with 3-(3,4,5-trimethoxyphenyl)propanoyl chloride or with 3-(3,4,5-trimethoxyphenyl)propiolic acid[26] in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCl) and 4-dimethylaminopyridine (DMAP). Chemical and physical characteristics of the new compounds are reported in Supplementary data (Table 1).

The ^{1}H NMR data reported in Supplementary data (Table 2) show that the configurations of **4a–d** are maintained in the final products **2a–d** and **3a**–**d**, ruling out any isomerization in the subsequent reactions.

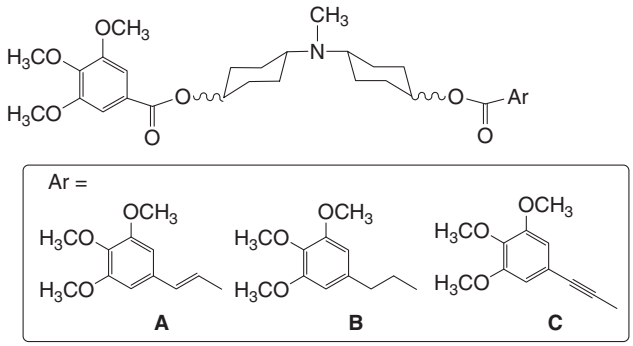
Modulation of pirarubicin uptake. The ability of isomers of sets **1–3** to modulate Pgp action was evaluated on doxorubicin-resistant erythroleukemia K562 cells (K562/DOX) that, as reported in the literature, overexpress only Pgp.[27,28] K562 is a human leukemia cell line established from a patient with chronic myelogeneous leukemia in blast transformation.[29] K562/DOX cells resistant to doxorubicin express a unique membrane glycoprotein with a molecular mass of 180,000 Da.[30] We measured 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 Letters.[31–34] Pgp-blocking activity is described by: (i) α, which represents the fold increase in the nuclear concentration of pirarubicin in the presence of the Pgp inhibitor 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 the Pgp inhibitor and is the maximum increase that can be obtained in the nuclear concentration of pirarubicin in resistant cells with a given compound; and (iii) $[I]_{0.5}$, which measures the potency of the inhibitor and represents the concentration that causes a half-maximal increase (α = 0.5) in the nuclear concentration of pirarubicin (see Table 1).

The results are reported in Table 1, together with those of compounds **1a–d**,[25] MM36, the most potent compound that we have



Scheme 1. Synthesis of compounds **2a–d** and **3a–d**. Reagents and conditions: (a) ArCOCl, $CHCl_3$ or ArCOOH, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCl), 4-dimethylaminopyridine (DMAP); for the meaning of Ar see Table 1. Compounds **4a–d** are described in Ref. 25.

Table 1
MDR-reverting activity of compounds **1a–d**, **2a–d**, and **3a–d**



Compound	Ar	$[I]_{0.5}$[a] (μM)	α_{max}[b]
1a (*cis*/*trans*)	A	0.092 ± 0.015[c]	0.85
1b (*trans*/*trans*)	A	0.32 ± 0.10[c]	0.81
1c (*cis*/*cis*)	A	0.03 ± 0.01[c]	0.80
1d (*trans*/*cis*)	A	0.012 ± 0.001[c]	0.98
2a (*cis*/*trans*)	B	0.63 ± 0.2	0.75
2b (*trans*/*trans*)	B	0.61 ± 0.18	0.76
2c (*cis*/*cis*)	B	1.10 ± 0.3	0.76
2d (*trans*/*cis*)	B	0.35 ± 0.1	0.77
3a (*cis*/*trans*)	C	0.08 ± 0.01	0.98
3b (*trans*/*trans*)	C	0.07 ± 0.01	0.99
3c (*cis*/*cis*)	C	0.07 ± 0.01	1
3d (*trans*/*cis*)	C	0.02 ± 0.002	1
MM36		0.05 ± 0.01[d]	0.70
Verapamil		1.60 ± 0.3	0.70

[a] Concentration of the inhibitor that causes a 50% increase in nuclear concentration of pirarubicin ($\alpha = 0.5$).
[b] Efficacy of MDR-modulator and maximum increase that can be obtained in the nuclear concentration of pirarubicin in resistant cells.
[c] See Ref. 25.
[d] See Ref. 33.

found in previous studies,[33] and verapamil used as reference compounds. These data show that reduction of the double bond in the isomers **1a–d** to give **2a–d** is detrimental for both potency and efficacy. The most potent isomers are in both sets the *trans*/*cis* ones, but **2d** is some 30 times less potent than **1d** and, as far as efficacy is concerned, the compounds of set **2** are definitely less efficacious than those of set **1**. On the contrary, the transformation of the double bond of set **1** into the triple bond of set **3** leave nearly unchanged the potency of the four isomers (the *trans*/*cis* still being the most potent one) but definitely increase the efficacy, so that all the isomers of set **3**, under the conditions of our essay, are able to completely restore the sensitivity of K562/DOX cells to pirarubicin.

Changing the double bond into a single or a triple one does not affect the conformational preference of the compounds, since in **1, 2**, and **3** sets the low energy conformers of matching isomers have a similar geometry, as shown in Figure 1 for the most potent isomers **1d, 2d**, and **3d**. Then it seems reasonable to hypothesize that the small variations in potency that smoothened the differences in activity among the isomers of set **3** with respect to the corresponding isomers of set **2** could be the consequence of the entropic variations due to the conformational restriction imposed by the triple bond. Accordingly, the entropic toll paid by the more flexible molecules obtained after reduction of the double bond would explain the drop in potency and efficacy of the isomers of set **2** with respect to that of set **3**.

The studied compounds possess chemical stability under the conditions of the biological tests. No information are available on their metabolic stability; however this problem will be taken into account for the most potent compounds in the near future. At the moment, research is in progress to synthesize metabolically stable ester bioisosteres.

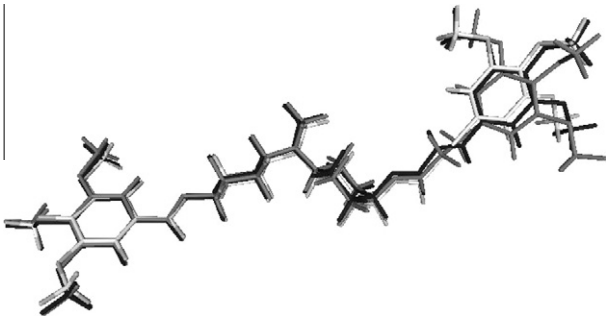


Figure 1. Overlay of **1d** (black) with **2d** (dark gray) and **3d** (light gray).

The compounds of set **3**, with their excellent efficacy are fairly interesting and, together with those of set **1**, have been selected for further pharmacological studies. Preliminary results show that the compounds of set **3** display very promising properties that will be reported in due time.

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Supplementary data

Supplementary data (the synthesis of the reported compounds; chemical and physical characteristics, IR and ^{1}H NMR spectra and elemental analyses of compounds **2a–d**, **3a–d**) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.11.059.

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