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N,*N*-Bis(cyclohexanol)amine aryl esters inhibit P-glycoprotein as transport substrates☆

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This work is dedicated
to the memory of
Prof. Giampaolo Pessina,
dear Colleague and Friend.

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ABSTRACT

P-Glycoprotein (Pgp) inhibition by three sets of four isomers of *N*,*N*-bis(cyclohexanol)amine aryl esters was assessed on rhodamine 123 (R123) efflux in human MDR1-gene transfected mouse T-lymphoma L5178 cells and on *Sf*9 ATPase activity. The most active compounds inhibited Pgp with IC_{50} values much lower than those of either cyclosporin A (CSA) or GF120918. As to R123 efflux inhibition, the role of the bond present in the second aryl moiety appeared important since the triple bond derivatives (3a–d) were the most powerful as compared to the double bond (2a–d) and the single bond (1a–d) counterparts. Concentration–inhibition curves of 2c and 3d exhibited a biphasic behaviour suggesting the existence of two binding sites in the recognition domain of Pgp. Persistence of inhibition by these compounds resulted to be intermediate between that caused by CSA and GF120918. R123 exhibited positive interaction with CSA, 1d, 1c, 2d, 2c and 3c, the concentration–inhibition curves being shifted leftward when R123 concentration was increased, while it exhibited negative interaction with 3d and no effect with GF120918. *Sf*9 ATPase activity was stimulated in an increasing order of potency by 2c, 3c, 2d, CSA, epirubicin and 3d. In a decreasing order of potency 3d, 2c, GF120918, CSA, 2d and 3c inhibited at sub-nanomolar concentrations epirubicin-stimulated ATPase activity. In conclusion, isomeric geometry and restriction of molecular flexibility of *N*,*N*-bis(cyclohexanol)amine aryl esters were crucial for their presentation to and inhibition of Pgp as transport substrates, R123 and epirubicin cooperating with them to this inhibition.

1. Introduction

Fighting cancer has ever since represented a formidable challenge. One of the most effective ways to treat disseminated cancer is still chemotherapy, often performed by administering various agents simultaneously. An important cause of failure of chemotherapy is commonly identified in the multidrug resistance (MDR) of cancer cells. MDR is an acquired drug resistance of cancer cells to chemotherapeutic drugs that usually are structurally and mechanistically unrelated. Classical MDR is due to a lowering of the intracellular concentration of cytotoxic drugs associated with accelerated efflux of the chemotherapeutic agent as a consequence of the overexpression of transporter proteins such as P-glycoprotein (Pgp), encoded by *ABCB1*, multidrug resistance protein 1 (MRP1), encoded by *ABCC1* and breast cancer resistance protein (BCRP), encoded by *ABCG2*. These belong to the ABC superfamily of transporters that use ATP as energy source and act as extrusion pumps [1]. To circumvent MDR the pharmacological inhibition of the functions of Pgp, MRP1 and sister proteins (“engage strategy”) has been and still is matter of intense investigation in many laboratories. Over the last years, however, other promising

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Abbreviations: CSA, cyclosporin A; DMSO, dimethylsulfoxide; GF120918, N-(4-(2-(1,2,3,4-Tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl)phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; MDR, multidrug resistance; MFI, mean fluorescence intensity; L5178 MDR1 cell line, L5178 mouse T-lymphoma MDR1 expressing cell line; Pgp, P-glycoprotein; R123, rhodamine123; *Sf9*, intestinal cell membranes of *Spodoptera frugiperda* enriched of human Pgp; Vi, sodium orthovanadate.

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approaches are emerging for combating multidrug resistant cancer cells. The "evade strategy" use antineoplastic drugs which are not substrates for MDR proteins such as cyclophosphamide, cisplatin, and epothilones [2] thus providing proof that new classes of antitumor drugs not interacting with MDR proteins can be developed to improve the response to therapy. Finally, the "exploit strategy" is based on the idea that drug efflux pumps can be exploited to selectively kill resistant cancer cells, while sparing sensitive normal cells [3,4]. In the field of "engage strategy" the main problems associated with the development of effective transporter-mediated MDR reverters seem due to poor specificity, low potency, interference with physiological functions and, as a consequence, interference with the pharmacokinetics of the chemotherapeutic drug used. These reasons may explain why none of the many MDR reverting agents so far developed has been approved for therapy [5].

After the seminal investigation by Zolnerciks et al. [6] which was the first experimental study to confirm that Sav1866 and Pgp share a common architecture, followed by the homology modeling studies by O'Mara and Tielemann [7] and by Globish et al. [8], it has been proposed the existence in the pump of a large, polymorphous drug recognition domain where a variety of molecules can be accommodated. From studies carried out on QacR transcriptional repressor protein – itself a multidrug-binding protein – the principles for simultaneous binding of two different drugs have emerged [9] and they likely apply to the multidrug efflux transporters [10]. Recently, Pgp structure described by Aller et al. [11] has revealed the molecular basis for polyspecific drug binding thus showing that the protein can accommodate a drug in multiple conformations. Compounds that mimic substrates of pumps can be envisaged to engage the drug binding domain of the pump, thus inhibiting its function in a competitive way.

Exploratory chemistry aimed at identifying novel MDR reverters gave rise to the synthesis of compounds inspired by pervilleine A and verapamil [12]. Structurally, these compounds are *N*,*N*-bis(cyclohexanol)amine aryl esters formed by a scaffold where a basic linker tethers two aromatic moieties. Some of them showed high potency and efficacy in inhibiting Pgp-dependent nuclear pirarubicin efflux in doxorubicin-resistant erythroleukemia K562 cells (K562/DOX) as well as rat intestinal mucosa ATPase activity mostly referable to MRP1; they also increased the cytotoxicity of doxorubicin towards doxorubicin-resistant erythroleukemia K562 cells [13,14].

We presently report the study of Pgp inhibition by three sets of *N*,*N*-bis(cyclohexanol)amine aryl esters sterically differing in the bond between C2 and C3 in the second aryl moiety. A highly flexible aryl ester analogue, compound 4, along with CSA and GF120918, were also studied for comparison. Pgp inhibition was assessed by R123 efflux measurements in human MDR1-gene transfected mouse T-lymphoma L5178 cells as well as by measuring ATPase activity of human Pgp-enriched intestinal *Spodoptera frugiperda* membranes (*Sf*9).

2. Materials and methods

2.1. Chemicals

McCoy's 5A medium, heat-inactivated horse serum, L-glutamine, sodium orthovanadate (Na_3VO_4, V_i), colchicine, rhodamine 123 (R123), trizma base, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), sodium azide (NaN_3), DL-dithiothreitol (DTT), pyruvate kinase from rabbit muscle (PK), L-lactic dehydrogenase from rabbit muscle (LDH), phospho(enol)pyruvic acid monopotassium salt (PEP), adenosine 5′-triphosphate magnesium salt (Mg-ATP), dimethylsulfoxide (DMSO) and epirubicin hydrochloride were purchased from Sigma Chemical Co. (Milan, Italy); penicillin (10,000 UI/ml) and streptomicin (10 mg/ml) mixture from Lonza (Basel, Switzerland) and cyclosporin A (CSA) from Alexis Biochemicals (San Diego, CA, USA). GF120918 was kindly supplied by GlaxoSmithKline (Stevenage, UK). Human PGP (MDR1)-enriched membranes (5 mg/mL) prepared from baculovirus-infected insect cells (*Sf*9) were purchased from Becton Dickinson and Company (Erembodegem, Belgium); NaCl and $MgCl_2$ from J.T. Baker (Phillipsburg, NJ, USA); KCl from Panreac

Compound	Structure	Ar_1	Ar_2
1a *(cis/trans)*	II	A	B
1b *(trans/trans)*	II	A	B
1c *(cis/cis)*	II	A	B
1d *(trans/cis)*	II	A	B
2a *(cis/trans)*	II	A	C
2b *(trans/trans)*	II	A	C
2c *(cis/cis)*	II	A	C
2d *(trans/cis)*	II	A	C
3a *(cis/trans)*	II	A	D
3b *(trans/trans)*	II	A	D
3c *(cis/cis)*	II	A	D
3d *(trans/cis)*	II	A	D
4	I	A	C

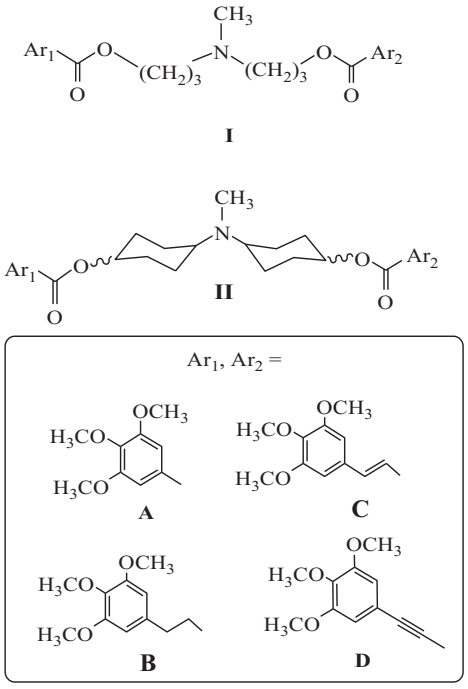

Fig. 1. Compounds investigated as inhibitors of Pgp-dependent R123 cell efflux. Three sets of drugs are represented, each composed of the four isomers (1a–d, 2a–d, 3a–d), where the *N*,*N*-bis(cyclohexanol)amine scaffold was esterified with 3,4,5-trimethoxybenzoic acid (A) at one end, and with 3-(3,4,5-trimethoxyphenyl)propionic acid (B, set 1), 3-(3,4,5-trimethoxyphenyl)acrylic acid (C, set 2) and 3-(3,4,5-trimethoxyphenyl)propynoic acid (D, set 3), respectively, at the other end.

Quimica (Barcelona, Spain); reduced β-nicotinamide adenine dinucleotide disodium salt (NADH) was purchased from Applichem (St. Louis, MI, USA). The three sets of *N*,*N*-bis(cyclohexanol)amine aryl esters (each composed of four geometrical isomers) – 1a–d, 2a–d, and 3a–d, see Fig. 1 – where the *N*,*N*-bis(cyclohexanol)amine scaffold was esterified with 3,4,5-trimethoxybenzoic acid at one end (Ar_1), and with 3-(3,4,5-trimethoxyphenyl)propionic acid (set 1), 3-(3,4,5-trimethoxyphenyl)acrylic acid (set 2) and 3-(3,4,5-trimethoxyphenyl)propynoic acid (set 3), at the other end (Ar_2) respectively, and compound 4 were synthesized following the literature [12–14]. V_i solution (0.1 M) was prepared by dissolving the salt in hot water and adjusting the pH to 7.4 with addition of 0.1 M HCl or 0.1 M NaOH.

2.2. Cell lines and cultures

The L5178Y mouse T-lymphoma parent cell line and the same cell line transfected with a recombinant MDR1/A retroviral vector (pHa MDR1/A) [15] were a generous gift from Dr. Michael M. Gottesman (National Cancer Institute, Bethesda, MD, USA). Human MDR1-expressing cells were selected by culturing the transfected cells with 60 ng ml^{-1} colchicine to maintain the expression of the MDR phenotype [16]. The L5178 MDR1 cell line was grown in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, 2 mM L-glutamine, 100 UI/ml penicillin and 0.01 mg/ml streptomycin. Cells were maintained in a humidified incubator with an atmosphere of 95% air and 5% CO_2 at 37 °C. When the cells reached confluence, they were harvested and plated for subsequent passages (up to 20) and for drug treatment. Cultures were initiated at a density of 2×10^5 cells/ml and grown exponentially to about 2×10^6 cells/ml in 48 h. Cells were counted in a Burker cytometer before use; their viability, tested by Trypan Blue exclusion, was always greater than 95%.

2.3. Cell loading with R123

R123 influx at 37 °C occurred rapidly into L5178 MDR1 cells where it reached a steady-state concentration in about 10 min. When cells were transferred to a medium not containing R123, their fluorescence decayed quite rapidly (apparent $t_{1/2} < 3$ min) indicating a fast efflux of R123. This efflux was mostly dependent on the capacity of the Pgp pump, as the wild type cells retained fluorescence for a long time. The same happened when Pgp in L5178 MDR1 cells was inhibited by V_i (see below). The 20 min incubation period was chosen to attain a steady-state concentration of R123 even in the presence of V_i; under these conditions the fluorescence values obtained were much higher and already maximal at an R123 concentration of about 5×10^{-6} M which was then selected as a standard concentration for all assays.

2.4. Inhibition of Pgp-mediated R123 efflux

L5178 MDR1 cells (2×10^6 ml^{-1}) were resuspended in serum-free McCoy's 5A medium and 0.5 ml aliquots of the cell suspension were distributed into Eppendorf centrifuge tubes. Compounds to be tested were added at different concentrations and samples were incubated for 10 min at room temperature. R123 was then added to the samples at a final concentration of 5×10^{-6} M and cells were incubated for 20 min at 37 °C. Thereafter, cells were washed twice by centrifugation for 5 min at 2000 × *g* and resuspended in 0.5 ml phosphate-buffered saline (PBS). R123 retained by cells was quantified by its fluorescence, using a FACS Calibur flow cytometer (Becton-Dickinson, San Josè, CA, USA) equipped with an ultraviolet argon laser (excitation at 488 nm, emission at 530/30 and 585/42 nm band-pass filters). FACS analysis was gated to include only individual, viable cells on the basis of forward and side light-scatter and was based on acquisition of data from 10,000 cells. Fluorescence signals were analyzed by the BDIS CellQuest software (Becton Dickinson, San Josè, CA, USA). The mean fluorescence intensity (MFI) was used for comparison among different conditions. V_i was selected as the positive control for a standard inhibitor since already at 5×10^{-3} M concentration it can maximally inactivate the Pgp efflux pump [17]. IC_{50} for V_i determined on L5178 MDR1 cells in the present study was about 7×10^{-4} M. This method is a slight modification of the previous protocol [18] which implies the use of the transfected/wild type cells [19] and compared favorably with it as assessed by assaying Pgp inhibition by GF120918.

2.5. Positive interaction of R123 with CSA, GF120918 and some N,N-bis(cyclohexanol)amine aryl esters for Pgp inhibition

L5178 MDR1 cells (2×10^6 ml^{-1}) were resuspended in serum-free McCoy's 5A medium and 0.5 ml aliquots of the cell suspension were distributed into Eppendorf centrifuge tubes. Compounds to be tested were added at different concentrations and samples were incubated for 10 min at room temperature. R123 was added to the samples at different concentrations (5×10^{-6} M, 1×10^{-5} M, 2×10^{-5} M and 5×10^{-5} M for 2c, 5×10^{-6} M and 5×10^{-5} M for the other compounds) in the presence or absence of V_i; in the latter case samples were also added with the compounds to be tested as Pgp inhibitors and cells were incubated for 20 min at 37 °C, washed twice and resuspended in 0.5 ml PBS containing 1% heat-inactivated horse serum. Cells were then subjected to flowcytometric measurements.

2.6. Reversion and persistence of inhibition of Pgp-mediated R123 efflux

L5178 MDR1 cells (2×10^6 ml^{-1}) were resuspended in serum free McCoy's 5A medium, and 0.5 ml aliquots of the cell suspension were distributed into Eppendorf centrifuge tubes. Compounds to be tested were added at different concentrations and samples were incubated for 10 min at room temperature. To assess the degree of reversibility of the tested compounds two cycles of centrifugation at 2000 × *g* for 5 min and resuspension in 0.5 ml of PBS were included in the procedure (+W samples) and compared with samples undergoing the standard procedure (−W samples).

For the study of the persistence of the inhibition of Pgp-mediated R123 efflux, L5178 MDR1 cells (2×10^6 ml^{-1}) were resuspended in serum-free McCoy's 5A medium and 0.5 ml aliquots of the cell suspension were distributed into Eppendorf centrifuge tubes. Compounds to be tested were added at different concentrations and samples were further incubated for 10 min at room temperature. R123 was added to the samples at a final concentration of 5×10^{-6} M and cells were incubated for 20 min at 37 °C, washed twice and resuspended in 0.5 ml PBS containing 1% heat-inactivated horse serum. Cells were maintained at 37 °C and analyzed by flowcytometry at the indicated times (0–180 min).

2.7. Effects on Sf9 Pgp-ATPase activity

Compounds were further investigated by evaluating their effects on the human Pgp-ATPase activity of membranes prepared from recombinant baculovirus-infected insect cells (*Sf9*). GF120918 and CSA were taken as reference Pgp inhibitors. ATPase activity was assayed by continuously monitoring spectrophotometrically NADH oxidation using an enzymatic ATP-regenerating system, as described by Vogel and Steinhart [20] and modified by Urbatsch et al. [21]. Briefly, 100 μg of membranes was added to 1 ml of Linked Enzyme (LE) buffer at 37 °C containing 1 mM phosphoenolpyruvate (PEP), 0.5 mM NADH, 0.1 mg/ml lactate

dehydrogenase (LDH), 0.1 mg/ml pyruvate kinase (PK), 0.1 mM Mg^{2+}ATP and 30 mM Tris–HCl, pH 7.5. Sodium azide (10 mM) and 1 mM EGTA into LE buffer were added to inhibit H^+- and Ca^{2+}-ATPases, respectively. ATP hydrolyzed was measured by the decrease in absorbance of NADH at 340 nm, using an UV-1601 spectrophotometer (Shimadzu, Milano, Italy) and expressed as nanomoles of ADP formed per minute per mg of *Sf9* protein. Effects on basal ATPase activity were determined by adding increasing amounts of the compounds to LE buffer. Inhibition of epirubicin-stimulated ATPase activity was determined in the same way using LE buffer supplemented with 0.1 μM epirubicin. Any effects of the compounds on the ancillary enzyme activities of the assay, i.e. pyruvate kinase and lactate dehydrogenase activities, were assessed on the ADP-driven test system.

2.8. Data analysis and statistics

Data are reported as mean ± SEM of at least three independent experiments run in triplicate. The fluorescence data are expressed as the mean of arbitrary fluorescence units derived from histogram plots of the 10,000 cells that were examined. The percent Pgp inhibition exerted by a single compound was calculated as described [17]. The relative fluorescence (i.e. percent inhibition of Pgp) was calculated as mean fluorescence intensity (MFI) of a discrete sample divided by the MFI in the presence of 5×10^{-3} M V_i, times 100:

$$\text{Relative fluorescence} = [\text{MFI of sample}/(\text{MFI of sample} + V_i)] \times 100$$

The denominator represents MFI of the sample when inactivation or complete preclusion of the function of Pgp active efflux is attained. The numerator is the resulting signal caused by test compound inhibiting the function of Pgp active efflux. Pgp blocking activity was described by α_{max}, which expresses the efficacy of the inhibitor and by IC_{50}, which measures its potency; α_{max} varies between 0 (in the absence of the inhibitor) and 1 (when the amount of R123 found in L5178 MDR1 cells was that determined in the presence of 5×10^{-3} M V_i). IC_{50} measures the potency of the inhibitor and represents the concentration that causes a half-maximal increase ($\alpha = 0.5$) in intracellular concentration of R123. IC_{50} values were obtained by best fitting the concentration/inhibition curves, according to the one-site or two-site models. In order to establish which equation was more appropriate, the two models were compared by using the F test (GraphPad Prism version 5, GraphPad Inc., San Diego, CA, USA). IC_{50} values were obtained by best fitting the concentration/inhibition data by plotting FAR values *vs* log [*M*] of Pgp inhibitor. EC_{50} and V_{max} values for substrates of *Sf9* ATPase activity were obtained by best fitting the concentration-dependent stimulation data of ATPase activity to a sigmoidal curve with variable slope by using GraphPad Prism version 5.

3. Results

3.1. Inhibition of Pgp-mediated R123cell efflux by CSA, GF120918, compound 4 and N,N-bis(cyclohexanol)amine aryl esters

The ability of compound 4, a *N*,*N*-bis(alkanol)amine aryl ester, to inhibit Pgp-mediated cell efflux of R123 was compared to that of the known Pgp inhibitors CSA and GF120918. The inhibition by two concentrations (out of the six–eight tested) for each inhibitor is shown by the histograms in Fig. 2. Due to the high level of Pgp expression in the L5178 MDR1 cell line, low R123 levels were

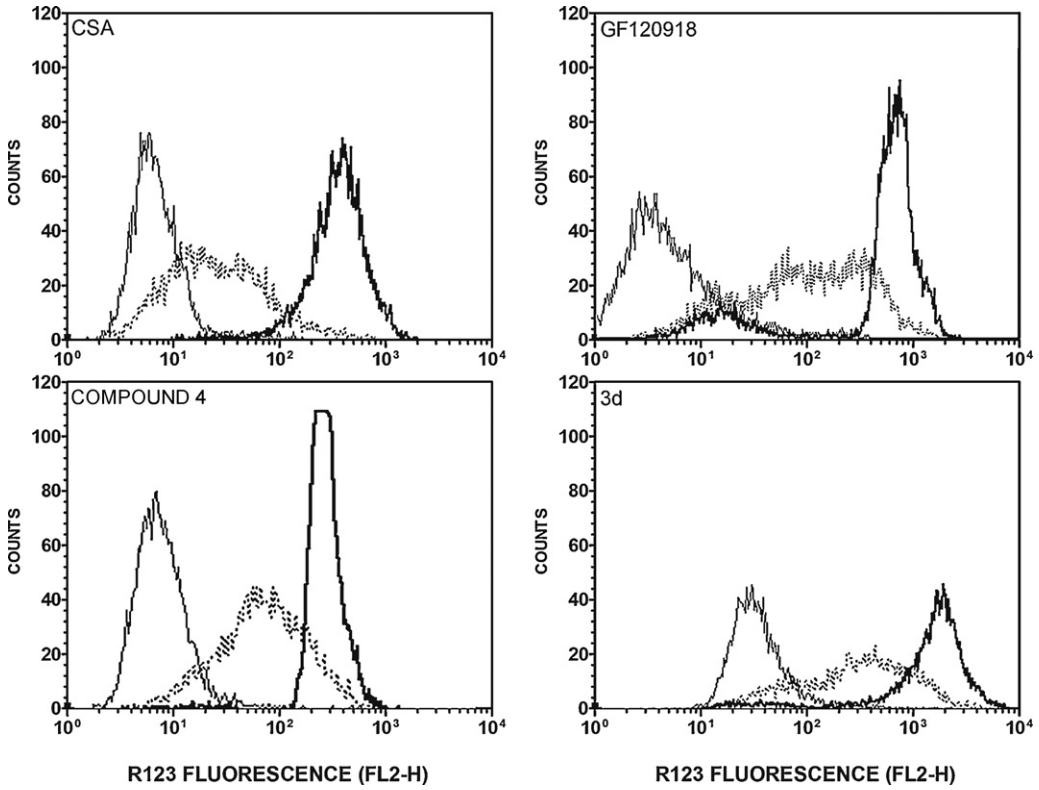

Fig. 2. Inhibition of Pgp-mediated R123 efflux from L5178 MDR1 cells by CSA, GF 120918, compound 4 and compound 3d. Cells were incubated at room temperature for 10 min in the presence of the aforementioned inhibitors at two different concentrations and then exposed at 37 °C for 20 min to 5×10^{-6} M R123. Cells were washed twice and resuspended at room temperature in 0.5 ml PBS containing 1% heat inactivated horse serum and processed for flow cytometry measurements. Histograms from one of at least three independent experiments are shown. Thin trace, no inhibitor; dotted line and thick trace, inhibitor at the following concentrations: CSA, 1×10^{-7} M and 1×10^{-6} M; GF120918, 1×10^{-8} M and 1×10^{-6} M; compound 4, 3×10^{-6} M and 1×10^{-5} M; 3d, 1×10^{-12} and 1×10^{-9} M, respectively.

Table 1
IC_{50} and α_{max} values of the *N*,*N*-bis(cyclohexanol)amine aryl esters and reference compounds investigated as inhibitors of Pgp-mediated R123 efflux from L5178 MDR1 cells.

Compound	IC_{50} (M)	α_{max}
4	5.3×10^{-6}	0.9
CSA	6.7×10^{-7}	0.9
GF120918	1.3×10^{-8}	1.0
1a	4.0×10^{-6}	1.0
1b	6.1×10^{-7}	1.0
1c	1.6×10^{-6}	1.0
1d	1.0×10^{-6}	1.0
2a	2.3×10^{-9}	1.0
2b	1.1×10^{-7}	0.9
2c	5.4×10^{-13}; 7.5×10^{-9}	0.9
2d	6.0×10^{-9}	1.0
3a	1.2×10^{-7}	1.0
3b	1.3×10^{-7}	0.9
3c	6.4×10^{-9}	1.0
3d	7.4×10^{-14}; 1.5×10^{-9}	1.0

IC_{50} values were obtained by the sigmoidal curve fitting of sets of data from at least three different experiments.

observed in the cells incubated with R123 in the absence of any inhibitor. On the contrary, in the presence of the various inhibitors tested there was a concentration-dependent increase of R123 fluorescence in the cells as shown by the rightward shift of the fluorescence peaks. To compare the potency of the inhibitors, the IC_{50} and α_{max} values from corresponding concentration–inhibition curves are reported in Table 1. GF120918 was very potent showing an IC_{50} value of 1.3×10^{-8} M, whereas compound 4 appeared less potent ($IC_{50} = 5.3 \times 10^{-6}$ M) by about one order of magnitude than CSA ($IC_{50} = 6.7 \times 10^{-7}$ M). Moreover, GF120918, CSA and compound 4 appeared to be efficacious Pgp inhibitors as their α_{max} values approached 1. Compounds 1a–d were effective inhibitors of Pgp-mediated R123 efflux, their α_{max} values approaching 1. However, they were weaker inhibitors as compared to both CSA and GF120918; their IC_{50} values were, in fact, all around 1×10^{-6} M. Interestingly, isomers 1a–d were slightly more potent than compound 4. Compounds 2a–d turned out to be also effective inhibitors of Pgp-mediated R123 cell efflux, their α_{max} values approaching 1. However, they were much more powerful as compared to the previous ones. In fact, while 2b (*trans*/*trans*) inhibited Pgp with an IC_{50} value of 1.1×10^{-7} M, 2d (*trans*/*cis*) and 2a (*cis*/*trans*) showed much lower IC_{50} values, averaging 4×10^{-9} M. Moreover, 2c (*cis*/*cis*) inhibition *vs.* concentration curve exhibited a biphasic behaviour. Analysis of the data according to either the one-site or the two-site models, gave the latter as the best model ($P < 0.02$), resulting into IC_{50} values of 5.4×10^{-13} M and 7.5×10^{-9} M. Remarkably, the former value turned out to be much lower than that exhibited by GF120918, which is recognized as one of the most powerful Pgp inhibitors so far investigated. An accurate kinetic analysis by Wang et al. [22] suggests that a form of "cross-talk" occurs between the identified binding sites in the transporter domains and the ATP binding site which could justify the observed complex kinetic behaviour.

The compounds 3a–d turned out to be effective inhibitors of Pgp-mediated R123 efflux, their α_{max} values approaching 1. However, while both 3a (*cis*/*trans*) and 3b (*trans*/*trans*) inhibited Pgp with IC_{50} values of about 1×10^{-7} M, the other two showed much lower IC_{50} values. Compound 3c (*cis*/*cis*), in fact, was characterized by an IC_{50} value of 6.4×10^{-9} M while 3d (*trans*/*cis*) inhibition *vs.* concentration curve exhibited a biphasic behaviour. Also in this case, analysis of the data according to either the one-site or the two-site models gave the latter as the best model ($P < 0.001$), with IC_{50} values of 7.4×10^{-14} M and 1.5×10^{-9} M. Overall, the activity of the 3a–d stereoisomers was similar or (as in the case of 3d) even higher than the 2a–d compounds.

3.2. Cooperation of R123 with CSA, GF120918 and N,N-bis(cyclohexanol)amine aryl esters for Pgp inhibition

GF120918 and CSA were taken as reference compounds for the non-substrate and substrate type of Pgp inhibitors, respectively. For this experiment two compounds were selected out of the four of each set of *N*,*N*-bis(cyclohexanol)amine aryl esters, namely *cis*/*cis* and *trans*/*cis* isomers, i.e. the ones exhibiting the greatest potency in inhibiting R123 cell efflux.

As shown in Fig. 3, the concentration–inhibition curve with GF120918 was not affected by increasing R123 concentration from 5 μM to 50 μM, the two curves being superimposed. Noticeably, compounds 1c, 1d, 2c, 2d, 3c and CSA, when tested in the presence of increasing R123 concentrations, showed a significant leftward shift of the concentration–inhibition curves without any changes in their efficacy. In particular, both 2c and 3d lost the biphasicity displayed at 5 μM R123 concentration. Only 3d caused a rightward shift of the concentration–inhibition curve when R123 concentration was raised to 50 μM. For a better comparison of how the increase in R123 concentration enhanced the potency of Pgp inhibitors under investigation, the relative IC_{50} values are reported in Table 2. Noticeably, with the only exception of 3d all compounds increased their potency, when R123 concentration was raised from 5 μM to 50 μM, from one up to several orders of magnitude, 2c and 3d IC_{50} values being the ones mostly affected by this change.

3.3. Reversion of inhibition by 2d of Pgp-mediated R123 cell efflux

The reversibility of the inhibition of Pgp-mediated R123 efflux was assessed by extensive washing of cells previously incubated with three different concentrations of 2d (i.e. 1×10^{-9}, 1×10^{-8} and 1×10^{-6} M). As reported in Fig. 4, panel B, despite the estimated 10^6-fold dilution of the drug, cells still exhibited a notable inhibition of R123 efflux as if upon washing, 2d had been diluted to a much lower extent. For example, in the case of 1×10^{-6} M inhibitor concentration, residual inhibition was about 80%, corresponding to that sustained by the inhibitor at 1×10^{-7} M concentration, instead of the expected residual inhibition of 15% produced by 2d at 1×10^{-12} M concentration. To test whether residual inhibition displayed by 2d could be due to the binding through the reactive enone function of its cynnamoyl moiety [23], we studied also 1a–d isomers, where the double bond had been

Table 2
Inhibition of Pgp-mediated R123 efflux from L5178 MDR1 cells by some *N*,*N*-bis(cyclohexanol)amine aryl esters and reference compounds, as a function of R123 concentration.

Compound	IC_{50} (M)			
	R123 5 μM	R123 10 μM	R123 20 μM	R123 50 μM
CSA	6.7×10^{-7}	N.D.	N.D.	1.2×10^{-7}***
GF120918	1.3×10^{-8}	N.D.	N.D.	9.8×10^{-9}
1c	1.6×10^{-6}	N.D.	N.D.	1.2×10^{-7}**
1d	1.0×10^{-6}	N.D.	N.D.	1.1×10^{-7}***
2c	5.4×10^{-13}; 7.5×10^{-9}	1.8×10^{-15}***	5.3×10^{-19}***	6.0×10^{-19}**
2d	6.0×10^{-9}	N.D.	N.D.	2.2×10^{-16}**
3c	6.4×10^{-9}	N.D.	N.D.	2.8×10^{-12}*
3d	7.4×10^{-14}; 1.5×10^{-9}	N.D.	N.D.	7.9×10^{-9}

IC_{50} values were obtained by the sigmoidal curve fitting of sets of data from at least three different experiments. N.D.: not determined.
* $P < 0.05$ *vs.* 5 μM R123.
** $P < 0.01$ *vs.* 5 μM R123.
*** $P < 0.001$ *vs.* 5 μM R123.

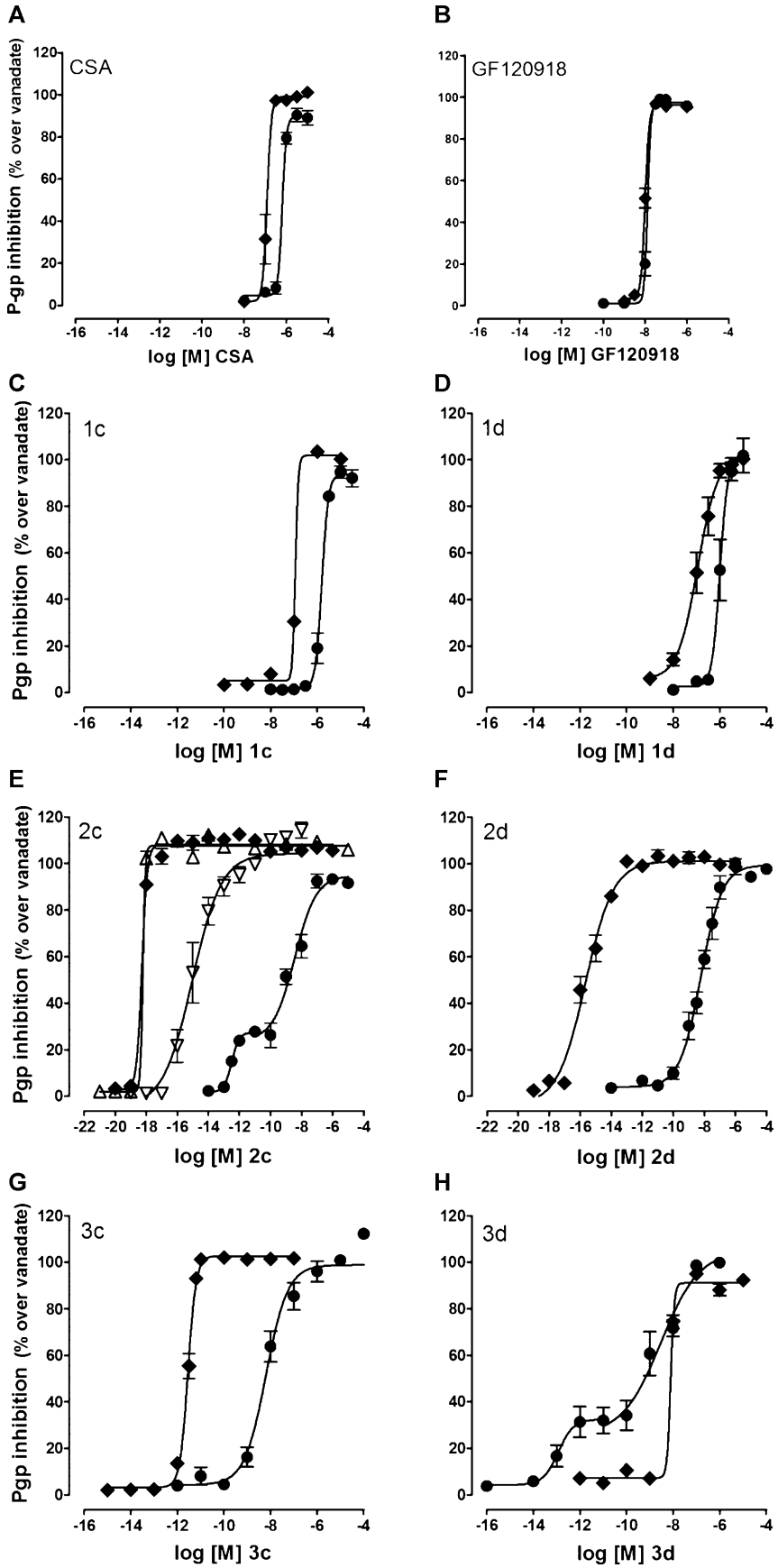

Fig. 3. Inhibition of Pgp-mediated R123 efflux from L5178 MDR1 cells by CSA (panel A), GF120918 (panel B), 1c (panel C), 1d (panel D), 2c (panel E), 2d (panel F), 3c (panel G) and 3d (panel H) as a function of R123 concentration (●, 5 μM; ▽,10 μM; △, 20 μM; ◆, 50 μM). Data are reported as the mean ± SEM of at least three independent experiments run in triplicate.

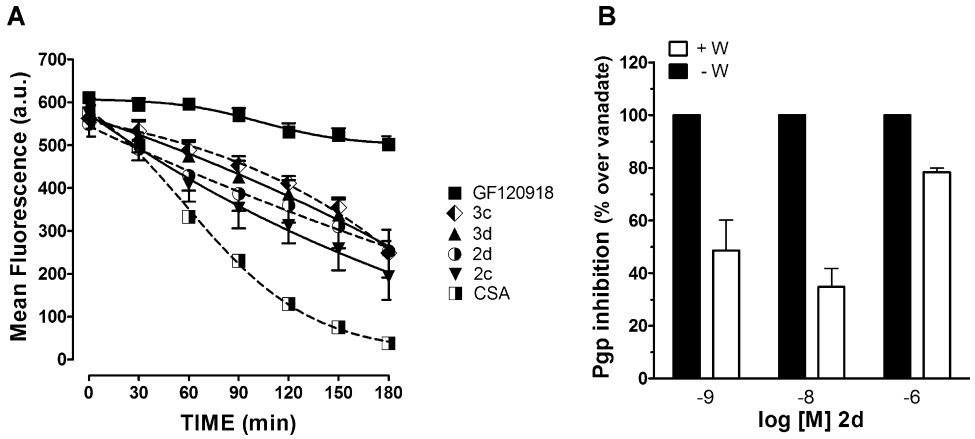

Fig. 4. (Panel A) Persistence of the inhibition of R123 efflux in L5178 MDR1 cells by CSA, GF120918, 3c, 3d, 2c and 2d. Cells were incubated at room temperature for 10 min with compounds at 1×10^{-6} M concentration and then exposed at 37 °C for 20 min to 5×10^{-6} M R123. Samples were washed twice and resuspended at room temperature in 0.5 ml PBS containing 1% heat-inactivated horse serum. Cells were subsequently exposed to 37 °C and processed at the indicated time points for flow cytometry measurements. Data are reported as the mean ± SEM of three independent experiments run in triplicate. (Panel B) Reversion of the inhibition by 2d of Pgp-mediated R123 cell efflux. The reversibility of the inhibition of Pgp-mediated R123 efflux was assessed by extensive washing of cells previously incubated with three different concentrations of 2d. Compound 2d was added to cells and samples were incubated for 10 min at room temperature. In not washed samples (−W) R123 was added at a final concentration of 5×10^{-6} M and cells were incubated for 20 min at 37 °C. In washed samples (+W) cells underwent two cycles of centrifugation at 2000 g for 5 min and resuspension in 0.5 ml of PBS, then R123 was added at a final concentration of 5×10^{-6} M and cells were incubated for further 20 min at 37 °C. Finally, both −W and +W samples were washed twice and then resuspended in 0.5 ml of PBS for flowcytometry measurements. Data are reported as the mean ± SEM of at least three independent experiments run in triplicate.

reduced. With this set of inhibitors, however, residual Pgp function after extensive washing was still present at comparable levels as for 2d (data not shown).

3.4. Persistence of inhibition of Pgp-mediated R123 cell efflux by various agents

To probe whether the most powerful among the four sets of inhibitors (2c, 2d, 3c and 3d) behaved or not as substrates of Pgp, we performed R123 retention experiments using GF120918 and CSA as positive and negative controls, respectively. In fact, GF120918 is known to be an inhibitor of Pgp but not a substrate [24] or is at least a very poor substrate [25], whereas CSA is known to be both an inhibitor and a substrate of Pgp [26]. We confirmed this under our conditions showing that R123 was retained in GF120918-treated cells (Fig. 4, panel A), indicating that it was not transported out of the cells during the course of the experiment. This is consistent with previously published findings [24,27]. The opposite trend is observed with CSA in which case R123 was poorly retained, presumably due to transport of CSA out of the cells along with R123 by Pgp. The persistence of the inhibition of Pgp-mediated R123 cell efflux by 2c, 2d, 3c and 3d resulted to be intermediate between that observed with CSA and GF120918. Noteworthy, the compound showing the highest persistence turned out to be 3c. Overall, this may imply that also these esters are substrates of Pgp.

3.5. Effects on Sf9 Pgp-ATPase activity

The compounds presently investigated were also evaluated for their effect on Pgp-ATPase activity using *Sf*9 cell membrane preparations. In this system epirubicin resulted to be a good substrate of the pump as it stimulated very potently the ATPase activity following a bell-shaped concentration/activation curve, the maximum activation being achieved at 1×10^{-7} M concentration, with an EC_{50} value of 2.6×10^{-8} M and a V_{max} of 55.1 ± 6.7 nmol/mg/min. Increasing concentrations of each of the isomers 2c, 2d, 3c and 3d were then tested on Pgp-ATPase activity in the absence (basal ATPase activity) or presence of 0.1 μM epirubicin (substrate-stimulated ATPase activity). Table 3 reports EC_{50}, V_{max} and IC_{50} values of the compounds tested. The effects on both ATPase activities – namely, basal ATPase activity and epirubicin-stimulated ATPase activity – of the two reference compounds, GF120918 and CSA, were also investigated (see Fig. 5, panels A and B, respectively). These last two compounds turned out to be potent inhibitors of the epirubicin-stimulated ATPase activity with IC_{50} of 1.3×10^{-15} M and 3.4×10^{-14} M, respectively. On the other hand, GF120918 did not affect basal ATPase activity, while CSA behaved like a substrate of the pump with an EC_{50} of 6.5×10^{-11} M and a V_{max} of 20.0 ± 0.6 nmol/mg/min. Remarkably, 3d was the most effective as a substrate as it stimulated ATPase activity up to a V_{max} of 76.6 ± 1.2 nmol/mg/min. 3d and 2c were found to be highly efficient inhibitors of the epirubicin-stimulated ATPase activity (Fig. 5, panel F and C). As for 2 c, fitting the data was consistent with the two-site model ($P < 0.05$), giving IC_{50} values of 2.5×10^{-18} M and 2.6×10^{-15} M. 3d and 2c were even more potent than CSA and GF120918 since their IC_{50} values were of several orders of magnitude lower than those relative to CSA and GF120918 averaging 4.9×10^{-19} M (3d) and 2.5×10^{-18} M (2c, lowest IC_{50}value). The compounds 3c and 2d were less efficient than the reference compounds (CSA and GF120918) in inhibiting epirubicin-activated ATPase activity.

Table 3
EC_{50} and V_{max} values of *Sf*9 ATPase activation and IC_{50} values of epirubicin-activated ATPase inhibition by selected Pgp inhibitors.

	ATPase activation		Epirubicin-activated ATPase inhibition
	EC_{50} (M)	V_{max} (nmol/mg/min)	IC_{50} (M)
CSA	6.5×10^{-11}	20.0 ± 0.6	3.4×10^{-14}
GF120918	N.D.	N.D.	1.3×10^{-15}
Epirubicin	2.6×10^{-8}	55.1 ± 6.7	N.D.
2c	7.9×10^{-11}	12.7 ± 0.3	2.5×10^{-18} 2.6×10^{-15}
2d	5.6×10^{-15}	17.1 ± 0.4	7.8×10^{-11}
3c	8.7×10^{-8}	12.5 ± 0.6	3.2×10^{-8}
3d	3.1×10^{-16}	76.6 ± 1.2	4.9×10^{-19}

Data represent the mean ± SEM of at least three different experiments. N.D.: not determined.

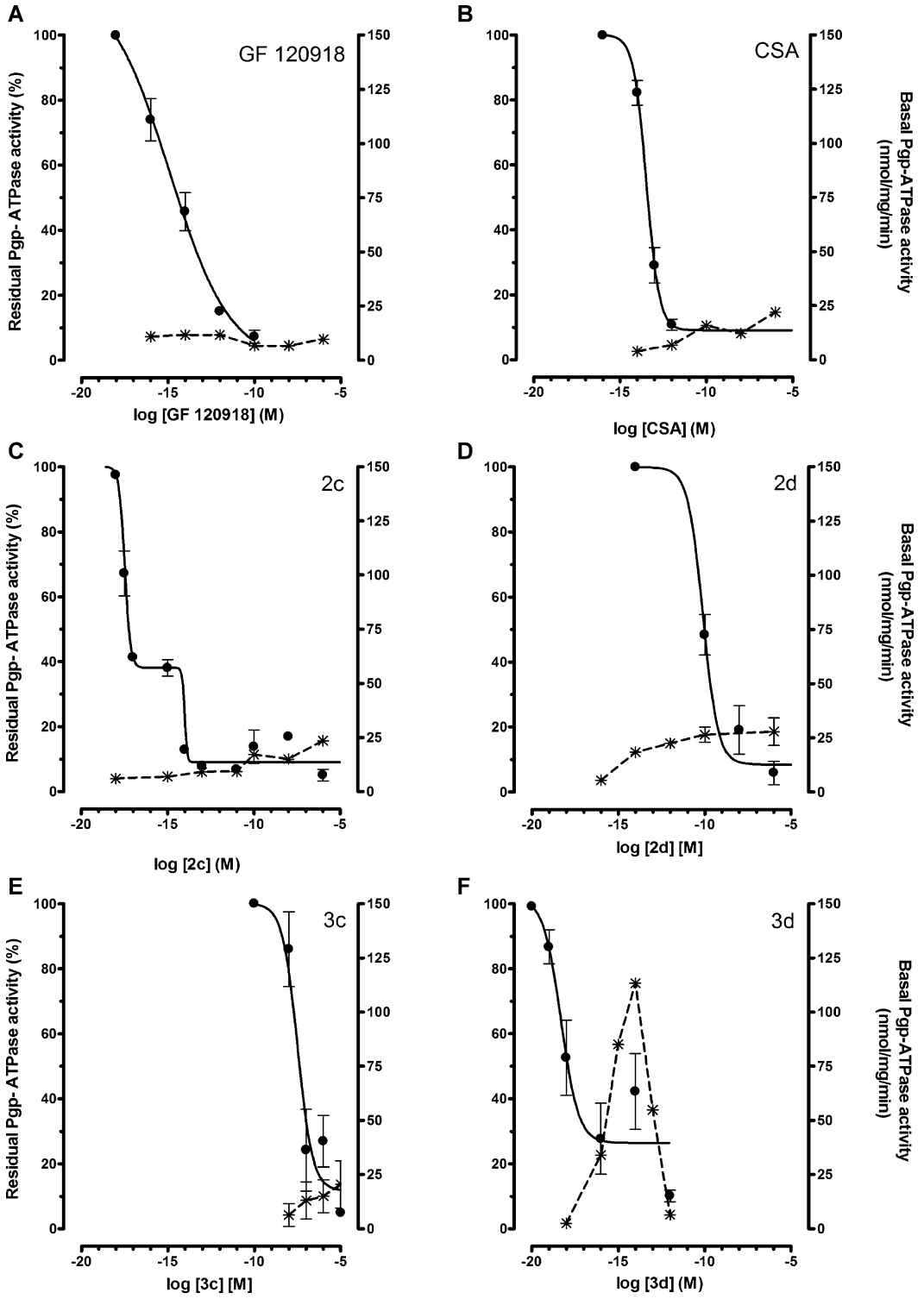

Fig. 5. Inhibition of *Sf*9 epirubicin-stimulated Pgp-ATPase activity by GF120918, CSA, 2c, 2d, 3c and 3d and stimulation of *Sf*9 Pgp ATPase activity by the same compounds. Epirubucin was present at 0.1 μM concentration. Dotted lines show the basal ATPase activity in the presence of increasing concentration of the Pgp inhibitors. Bars represent the SEM value of 3–6 assays per compound.

4. Discussion

The present investigation was aimed at defining the nature and extent of the inhibition by some *N*,*N*-bis(cyclohexanol)amine aryl esters of Pgp-mediated efflux of R123 on L5178 MDR1 cell line, as well as their effects on *Sf*9 ATPase activity. Insight into the structure-activity relationship has been achieved by comparing the inhibition parameters and by establishing the Pgp transport substrate feature of some of them. The fluorescent compound R123 has been shown to be a substrate of Pgp [28] and its concentration in the cell positively correlated to the degree of the inhibition of the outward transporter Pgp. Concentration–inhibition curves have been obtained for each of the twelve compounds under scrutiny (1a–d, 2a–d and 3a–d) and it was found that the corresponding IC_{50} values vary considerably from 4.0×10^{-6} M of 1a to 7.3×10^{-14} M of 3d isomer (see Table 1). The pronounced structural flexibility in

the 1a–d set resulted into a lower Pgp inhibition, the respective IC_{50} values ranging from 1.0×10^{-6} M to 6.1×10^{-7}. The increase of the structural rigidity along the C2–C3 bond of the Ar2 moiety was paralleled by the increase in activity of the compounds. The 2a–d set of compounds, characterized by a C2–C3 double bond which reduces the conformational space of the molecules, turned out to be very effective inhibitors. Within this set, 2c was the most powerful with IC_{50} values of 5.4×10^{-13} M and 7.5×10^{-9} M, followed by 2a and 2d with IC_{50} values of 2.3×10^{-9} M and 6.0×10^{-9} M, respectively. The introduction of a triple bond (set 3 compounds) and the consequent further restriction of the molecular flexibility resulted in an improved fitting to the hypothetical Pgp target site with respect to the set 2 counterparts, and this is particularly evident when comparing isomers 3d and 2c. It is noticeable that the most active compounds of the 2 and the 3 series in this study resulted to be more powerful than GF120918, so far considered as one of the most powerful Pgp inhibitors. Isomer 3d (*trans*/*cis*) resulted to be the most powerful among all of the compounds investigated with IC_{50} values of 7.3×10^{-14} M and 1.5×10^{-9} M, followed by 3c with an IC_{50} value of 6.4×10^{-9} M. Also in this set of compounds steric configuration played a major role as *cis*/*trans* (3a) and *trans*/*trans* (3b) isomers were characterized by an IC_{50} value of about 1×10^{-7} M, much greater, by more than two orders of magnitude, than the IC_{50} values of 3d (*trans*/*cis*) and 3c (*cis*/*cis*).

In previous studies [13,14], performed on doxorubicin-resistant erythroleukemia K562 cells, 2a–d and 3a–d isomers showed low-nanomolar potency and high efficacy in increasing nuclear pirarubicin content, whereas 1a–d isomers exhibited high nanomolar/micromolar potency. In particular, 2d, 3d, 2c and 3c were the most powerful with IC_{50} values of 1.2×10^{-8} M, 2.0×10^{-8} M, 3.0×10^{-8} M and 7.0×10^{-8} M, respectively; the IC_{50} value represents the concentration of the inhibitor that causes a half-maximal increase in nuclear concentration of pirarubicin. Overall, these compounds exhibited a lower potency towards doxorubicin-resistant K562 as compared to L5178 MDR1 cell lines, by at least one order of magnitude. The lower sensitivity of the former cell line may find an explanation on the different intracellular pharmacokinetics of the compounds, resulting into different concentrations at the target site(s), although the contribution of other, cell-specific ABC transporters cannot be excluded. Noticeably, data in the literature have confirmed the presence of Pgp, besides cell membrane, also in several organelles and/or the nuclear envelope [29–31]. Further studies, however, are needed to clarify whether the different intracellular distribution of the pump(s) is relevant to justify the observed different potency of the afore-mentioned compounds in the two cell lines. The present findings provide information on how these *N*,*N*-bis(cyclohexanol)amine aryl esters act on Pgp. The persistence of inhibition of Pgp-mediated R123 cell efflux by 2c, 2d, 3c and 3d resulted to be intermediate between that caused by CSA and GF120918 (Fig. 4, panel A). According to the paradigm introduced by other Authors [27], this finding suggests that these esters behave as transport substrates of Pgp and may compete for Pgp with R123. Further studies can shed light on the observed persistence of the inhibition of Pgp-mediated R123 cell efflux by 2c, 2d, 3c and 3d which, at first glance, may depend on their slow release from cell compartments where they distribute preferentially, as suggested also by the reversibility feature of the inhibition (Fig. 4, panel B). The concentration–inhibition curves of Pgp-mediated R123 efflux by either 2c (*cis*/*cis*) or 3d (*trans*/*cis*) gave for both compounds two IC_{50} values which differed by four orders of magnitude. This is consistent with data from several groups supporting the view that there are at least two, if not more, substrate binding sites in the drug-binding pocket of Pgp [32–35]. Murine Pgp (ABCB1) has a 87% sequence identity to human Pgp in a drug-binding competent state and its crystallographic structure has been described, although at a medium to low resolution level. It has been calculated that its 6000 Å^3 cavity can accommodate at once up to two identical or different LMW substrates/inhibitors [11,36]. In conclusion, some *N*,*N*-bis(cyclohexanol)amine aryl esters could well have two distinct binding sites in the drug-binding pocket of Pgp.

Of great interest appears to be the observation that these *N*,*N*-bis(cyclohexanol)amine aryl esters interact positively with R123 in the inhibition of Pgp. In fact, the concentration–inhibition curves with five (1c, 1d, 2c, 2d and 3c) out of the six *N*,*N*-bis(cyclohexanol)amine aryl esters investigated in the presence of increasing concentrations of R123 showed a clear shift of the curves towards lower concentrations without any change in their efficacy (see Fig. 3). If *N*,*N*-bis(cyclohexanol)amine aryl esters were substrates for Pgp and were acting on Pgp at the same recognition site as for R123, it would have been expected a reduced potency of the inhibitors upon increase of R123 concentration which would have caused a rightward shift of the concentration–inhibition curve. Moreover, the fact that the concentration–inhibition curve of the substrate CSA showed the same positive interaction with R123, while that of the non-substrate inhibitor GF120918 was not affected, strengthens the hypothesis that *N*,*N*-bis(cyclohexanol)amine esters behave like transport substrates inhibiting the Pgp-mediated R123 efflux mechanism. Each element of the couple of agents employed in this experiment, namely R123 and the tested *N*,*N*-bis(cyclohexanol)amine aryl esters, displayed this positive interaction at very different concentrations (i.e. micromolar and subnanomolar, respectively), thus suggesting that they were acting on distinct sites characterized by very different affinities. Many Pgp modulators exhibit an allosteric mode of action on Pgp. A kinetic study of the Pgp-mediated efflux of daunorubicin in A2780 cell line, has shown a positive cooperativity of daunorubicin for its binding to P-glycoprotein, at least two binding sites for daunorubicin being involved in the active transport of daunorubicin itself [37,38]. Furthermore, Pgp has been shown to contain at least two transport-competent drug-binding sites that exhibit (positive) cooperativity for drug transport, as well as distinct substrate specificities. These distinct sites may interact as demonstrated by the R123 stimulation of Hoechst 33342 transport [39,40]. The multiple – i.e. transport and regulatory – sites on Pgp display complex allosteric interactions by which the interaction of a drug at one site switches the other site between high- and low-affinity conformations [41]. This is the case of the Pgp modulator xupentixol and its closely related analogs which interact with Pgp at a site functionally distinct from the substrate recognition site and modulate substrate binding and ATP hydrolysis in an allosteric fashion [42]. The positive interaction of R123 with some *N*,*N*-bis(cyclohexanol)amine aryl esters in the present study further suggests the existence of several binding sites within the multifaceted drug-binding pocket of Pgp, the site pertaining to R123 – once it is bound to R123 – being capable of switching Pgp binding site of *N*,*N*-bis(cyclohexanol)amine aryl esters from a low- to a high-affinity conformation. The most potent inhibitors, i.e. 2c, 2d, 3c and 3d, were also tested for their effect on either basal or epirubicin-stimulated *Sf9* Pgp-ATPase activity and were found to behave like either substrates or inhibitors of the enzyme. In fact, they were found to stimulate ATPase activity – as it was also observed with CSA – with EC_{50} values ranging from 8.7×10^{-8} M (3c) to 3.1×10^{-16} M (3d). On the other hand, they potently inhibited epirubicin-stimulated ATPase activity with IC_{50} values ranging from 3.2×10^{-8} M (3c) to 4.9×10^{-19} M (3d). It is likely, therefore, that these inhibitors behave as transport substrates and that they exert their effect by competing with drug-substrates at the drug-binding pocket of Pgp. The data obtained by the ATPase modulation show that for compound 3d the inhibition and activation curves were clearly distinguishable pointing towards a dual-site effect. The fact that the range of effective inhibitory concentrations resulting from R123 efflux experiment were close

to those found in the kinetically distinct ATPase inhibition experiment further supports this hypothesis. It is remarkable, when comparing the IC_{50} values of ATPase assay with those exhibited by the same compounds towards R123 cell efflux as a function of R123 concentration, to see how the former values are close to those obtained with the highest R123 concentration. This suggests a positive interaction of epirubicin with 3d and 2c towards Pgp-ATPase activity. Whatever be the mechanism by which epirubicin besides R123 and some *N*,*N*-bis(cyclohexanol)amine aryl esters cooperate in the inhibition of Pgp, these findings open the way to using these inhibitors for combating MDR in cancer taking advantage of their synergizing effect when administered together with anthracyclines. In conclusion, among the *N*,*N*-bis(cyclohexanol)amine aryl esters investigated, 2c and 3d exhibit the highest potency as inhibitors of Pgp function and may represent leads for the design of effective MDR reverters in cancer cells under the "engage strategy" approach, also in view of the recent observation that they are devoid of vascular effects [43].

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