Biochemical Pharmacology 82 (2011) 1822–1831

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

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

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ARTICLE INFO

Article history:
Received 6 July 2011
Accepted 30 August 2011
Available online 6 September 2011

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

Keywords:
MDR reverters
Mouse T-lymphoma MDR1 cells
N,*N*-Bis(cyclohexanol)amine aryl esters
Epirubicin
Sf 9-ATPase activity
Pgp inhibitors
Rhodamine123 cell efflux

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

☆ These data have been presented before the 15th ISCB International Conference (4–7 February 2011) at Rajkot, Gujarat, India.

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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0006-2952/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2011.08.025

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

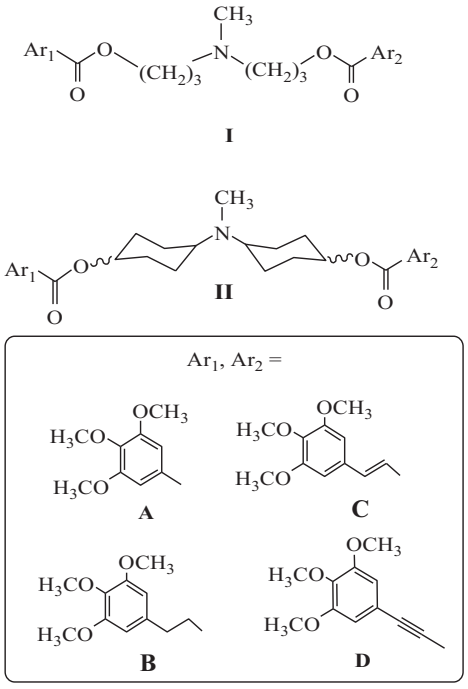

Fig. 1. Compounds investigated as inhibitors of Pgp-dependent R123 cell efflux. Three sets of drugs are represented, each composed of the four isomers (1a–d, 2a–d, 3a–d), where the *N*,*N*-bis(cyclohexanol)amine scaffold was esterified with 3,4,5-trimethoxybenzoic acid (A) at one end, and with 3-(3,4,5-trimethoxyphenyl)propionic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