Anti-Cancer Drugs 1998, 9, pp. 825–832

Research paper

Reversal of resistance against doxorubicin by a newly developed compound, oxalyl bis(*N*-phenyl)hydroxamic acid *in vitro*

SK Choudhuri and Amitava Chatterjee

Department of Biochemistry, Chittaranjan National Cancer Institute (Research Wing), 37 SP Mukherjee Road, Calcutta 700 026, India. Fax: (+91) 33 475 7606.

A drug-resistant cell line (EAC/Dox) was developed by repeated exposure of Ehrlich ascites carcinoma cells to Doxorubicin (Dox) *in vivo* in male albino Swiss mice (6–8 weeks old). The weekly i.p. injections of Dox to mice (2 or 4 mg/kg/week for 4 months) gave rise to Dox-resistant cell line EAC/Dox, which displayed typical multidrug resistant (MDR) features of cross-resistance to a number of structurally and functionally unrelated drugs like doxorubicin, vinblastine and cisplatin. Moreover, the EAC/Dox cell line had lower drug accumulation than drug-sensitive (EAC/S) cells. Study of Western blots and immunofluorescence revealed that P-glycoprotein 170 kDa (P-gp) was absent in EAC/Dox cells. The drug resistance appeared to be due to the presence of a higher level of reduced glutathione (GSH) and glutathione S-transferase (GST) in EAC/Dox cells than in drug-sensitive (EAC/S) cells. The two structurally similar hydroxamic acid derivatives, i.e. oxalyl bis(*N*-phenyl)hydroxamic acid (X1) and succinyl bis(*N*-phenyl)hydroxamic acid (X2), having very low *in vitro* toxicity (IC_{50} value 250 μg/ml), were investigated for their efficacy to reverse MDR. The compound X1 was able to reverse the effect of MDR and reduce GST in EAC/Dox cells. The compound X2 had no ability to reverse the effect of MDR. Further study on the mechanism of glutathione depletion and the resistance modifying property of X1 on other cell lines is warranted.
[© 1998 Lippincott Williams & Wilkins.]

Key words: Ehrlich ascites carcinoma, glutathione S-transferase, multidrug resistance (MDR), oxalyl bis(*N*-phenyl)hydroxamic acid, reduced glutathione, resistance modifying agent.

Introduction

In some cell systems multidrug resistance (MDR) is accompanied by changes in biochemical parameters, e.g. changes in glutathione levels, or changes in the enzymes which synthesize or use glutathione.[1] It has been documented that elevated levels of glutathione together with increased activity of glutathione S-transferase (GST) may protect the cells from cytotoxic drugs, e.g. anthracyclines, platinum compounds.[2] Another important mechanism of MDR is attributed to the reduced accumulation of antitumor agents in resistant cells.[3–6] P-glycoprotein 170 kDa (P-gp) is considered to cause the active efflux of antitumor agents from the cells.[7–11]

Various modulators have been used so far for circumventing MDR. For P-gp-expressing cells the clinically significant modulators are calcium channel blockers like verapamil, calmodulin inhibitors like phenothiazine and trifluoroperazine, and immunosuppressive agents like cyclosporine A and cyclosporine G.[12] Clinically important modulators of glutathione levels and glutathione enzyme activity are buthionine sulfoxamine (BSO),[13–17] sulfasalazine[18] and ethacrynic acid.[19–21]

Verapamil and other calcium channel blockers overcome the effect of MDR by inhibiting the efflux of antitumor agents from the cells.[7–11] Clinical studies have also been carried out combining calcium channel blockers and anticancer drugs. Such combination chemotherapy is useful against a number of tumor systems, but has several adverse effects on the host.[22–24] The modulators of glutathione and glutathione enzyme activity also have dose-limiting toxicity.[25]

In the present study, two non-toxic and chemically similar hydroxamic acids, i.e. oxalyl bis(*N*-phenyl)hydroxamic acid (X1)[26] and succinyl bis(*N*-phenyl)hydroxamic acid (X2),[27] were investigated for their efficacy to reverse MDR. The effective hydroxamic acid derivative (X1) was compared with verapamil, with regard to its ability to overcome MDR *in vitro*. X1 almost completely reversed the resistance of doxorubicin (Dox) in Dox-resistant Ehrlich ascites carcinoma (EAC) cells *in vitro*. X2 had no ability to reverse the effect of MDR, rather X2 decreased Dox toxicity in both drug-sensitive (EAC/S) and drug-resistant (EAC/

Correspondence to SK Choudhuri

© *1998 Lippincott Williams & Wilkins*

Dox) cells. P-gp was not detected in EAC/Dox cells. EAC/Dox cells had a higher level of reduced glutathione (GSH) and GST than EAC/S cells. X1 reduced GST in the EAC/Dox cell line.

Materials and methods

Cell line

EAC was maintained as an ascitic tumor in male Swiss albino mice weighing 18-20 g (6-8 weeks old), collected from our own animal colony. A Dox (Meiji Keika Keisha, Tokyo, Japan)-resistant subline was developed following the method of Ellen Friche *et al.*[28] by sequential transfer of EAC cells to a subsequent generation of host mice with continuous Dox treatment. The treatment regime consisted of 2.0 mg/kg/week Dox i.p. The daily treatment dose was 0.4 mg/kg for 5 days. The drug was started 24 h after inoculation of 1×10^6 ascites tumor cells i.p. to mice. The mean survival time (MST) $\pm$ SEM of untreated male Swiss mice bearing EAC cells was 22.4 ± 1.5 days (n=20). The MST $\pm$ SEM of the host mice bearing this tumor after 4 months treatment with Dox was 35.4 ± 3.06 days (n=20), whereas MST of the 12th transfer generation of host mice was 19.8 ± 3.71 days (n=20). After this degree of resistance had been developed, the dose of Dox was increased to 4 mg/kg/week (daily treatment dose was 0.8 mg/kg for 5 days), which resulted in 25.5 ± 1.7 days MST (n=20). When this tumor subline was re-treated with Dox after the 17th transfer, MST was 21.09 ± 1.4 days (n=20). After the 20th transfer, MST was noted to be 19.2 ± 2.87 days (n=20). The survivability of Dox-treated (2 mg/kg/week for 4 months) mice (35.4 ± 1.5 days) was statistically significant ($p < 0.05$) in comparison to the untreated mice group (22.4 ± 1.5 days). The animals with drug-resistant cells (up to the 20th generation) had the survivability (19.2 ± 2.87 days) close to that of animals with drug-sensitive cells (22.4 ± 1.5 days).

Ascites fluid was extracted from the mice after 12-15 days of inoculation of tumor cells. No treatment was given during the last passage before an *in vitro* experiment. HeLa cells were supplied by the National Facility for Cell and Tissue Culture (Pune, India) and maintained in RPMI 1640 with 10% fetal calf serum (FCS) (Gibco/BRL, New York, NY).

Compounds under investigation

MDR reverting agent, oxalyl bis(*N*-phenyl)hydroxamic acid (X1), was prepared according to the method of Choudhuri *et al.*[26] In brief, oxalyl chloride (8 g) was added to a solution of freshly prepared β-phenyl hydroxylamine (16 g) in diethyl ether containing pyridine (8 g) at -10 to -15°C. The gummy mass so obtained was repeatedly extracted with 5 N ammonia, washed with diethyl ether and acidified to pH 3 by addition of HCl when the compound precipitated. On decoloration with active charcoal and recrystallization from rectified spirit, an ash colored compound (2.5 g) was obtained, melting point 180°C.

Succinyl bis(*N*-phenyl)hydroxamic acid (X2) was prepared according to the method of Ghosh *et al.*[27]

Concentration and dose of the drugs

For *in vitro* experiments, an ethanol solution of X1 and an aqueous solution of X2 were used. Stock solutions of X1 (10 mg/ml) and X2 (20 mg/ml) were prepared in ethanol and deionized water, respectively. The concentration of ethanol in the culture medium was maintained within non-toxic limits (1-3%). Stock solutions of Dox (5 mg/ml), vinblastine (Sigma, St Louis, MO) (10 mg/ml), cisplatin (Sigma) (5 mg/ml) and verapamil (Sigma) (10 mg/ml) were prepared in deionized water. For all assays involving verapamil, verapamil was added 15 min prior to addition of drugs. The effective concentration (EC) of verapamil in the culture medium was maintained within non-toxic limits (6 μg/ml).

In vitro assay for cell cytotoxicity

One-dimensional titrations were performed to determine the IC_{50} of individual drugs in drug-sensitive (EAC/S) and drug-resistant (EAC/Dox) cells. Cells were collected from the mice after killing by cervical dislocation and washed in PBS three times. Cells (2×10^5) were plated in each well in RPMI 1640 medium, containing HEPES (Sigma), penicillin-streptomycin (PEN-STREP) and 10% FCS (Gibco/BRL). Cells were incubated without drug for 24 h to permit attachment. Different concentrations of Dox (EC 0.1-4 μg/ml), cisplatin (EC 0.05-10 μg/ml), vinblastine (Sigma) (EC 0.05-10 μg/ml), X1 (EC 10-400 μg/ml) or X2 (EC 25-400 μg/ml) were added. The cells were then incubated for an additional 4 days. Wells were washed with PBS and adherent cells were stained with crystal violet. Cell-associated dye was then solubilized with 10% acetic acid and the viable cell number was assayed by measuring the concentration of stain in the well with an ELISA reader at

570 nm.[29] The accuracy of this method was verified by counting the trypsinized cells in a hemocytometer in duplicate experiments.

To assess the effect of X1, X2 and verapamil on modulation of drug resistance, two-dimensional titrations in 96 wells in plastic plates were performed with increasing concentrations of Dox along the *y*-axis and increasing concentrations of resistance modification agent (RMA) along the *x*-axis. EAC/S and EAC/Dox cells were plated in RPMI 1640 medium, containing HEPES, PEN-STREP and 10% FCS. After 24 h of cell addition, increasing concentrations of Dox (EC 0.1-2.5 μg/ml), X1 (EC 5-100 μg/ml), X2 (EC 10-200 μg/ml), verapamil (EC 1-6 μg/ml) or Dox+X1, Dox+X2 and Dox+verapamil were added in 96-well plates and incubated for an additional 4 days. The adherent cells were stained with crystal violet and the viable cell number was assayed according to the method adopted for one-dimensional titration.[29]

Cellular pharmacokinetics

Steady-state drug accumulation in drug-resistant (EAC/Dox) and drug-sensitive (EAC/S) cells was reached at 70 min, as observed in preliminary experiments. Cells (1×10^6) in 1 ml of RPMI 1640 with HEPES (15 mM, pH 7.0), FCS (10%) and PEN-STREP in triplicates were brought to 37°C, and Dox (EC 5 μg/ml), verapamil (EC 6 μg/ml) and X1 (EC 10 μg/ml) were added. Verapamil and X1 were added 15 min prior to addition of drugs. After incubation for 70 min to 8 h, the tubes were centrifuged rapidly at 10°C (5000 r.p.m. for 2 min) and the cells were washed twice in ice-cold PBS. Cell pellets were suspended and lysed with 0.3 ml of HCl 0.3 mol/l/50% EtOH, followed by 1 h shaking at 37°C. At the end of this time period, 0.15 ml of 10% sulfosalicylic acid was added and mixed thoroughly, followed by centrifugation at 10°C (9000 *g* for 10 min). A volume of 0.3 ml of supernatant was mixed with 3 ml of distilled water. The concentration of Dox was measured in a fluorometer with an excitation wavelength of 495 nm and an emission wavelength of 590 nm.[30]

Western blot analysis

For the determination of P-gp expression, immunoblotting was carried out on EAC/Dox and EAC/S cell extracts, collected from male Swiss mice after killing the animals by cervical dislocation. Cells were washed in PBS twice and homogenized with a Dounce homogenizer, and then sonicated on ice for 10-15 s × 3, using a micro sonicator and centrifuged at 5000 r.p.m. Protein determination was done according to Lowry.[31] Protein (30 μg) was used for SDS-PAGE analysis, using 7.5% acrylamide gel.[32] Gels were transferred to nitrocellulose in Towbin Buffer[33] at 300 mA for 3 h.

The blots were probed according to the standard method with monoclonal antibody C219 (Centocor, Malvern, PA).[34]

The blots were also probed with polyclonal antibody in the following manner. For getting positive expression of P-gp, HeLa cells cultured in RPMI 1640 medium followed by trypsinization were taken as control. The nitrocellulose paper was incubated with 1 μg/ml *mdr* (Ab1) polyclonal antibody (Oncogene Science, NY) for 2 h with shaking at room temperature. The excess antibody was removed by repeated washing in Tris-buffered saline-Tween, pH 8 (TBST) and incubated with goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) for 2 h. The paper was washed and incubated for 15 min with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (NBT/BCIP) substrate (Genni, Bangalore, India). The antibody *mdr* (Ab1) used was a polyclonal rabbit antiserum raised against a sequence common to all *mdr* genes,[35] amino acids 1205-1224 of the human *mdr* proteins, the peptide (SALDTESEKVVQEALDKAREG) in the P-gp C-terminal cytoplasmic domain. The antibody reacts with P-gp in Western blot as a broad band running at about 200 000 MW in 7.5% SDS-PAGE.[36]

Immunofluorescence assay of P-gp

After cytospinning on clean glass slides, HeLa (control), EAC/S and EAC/Dox cells were air dried and fixed in ice-cold acetone for 5 min. Immunostaining of the cells was done by a standard technique.[37] Briefly, cells were incubated with anti-P-gp *mdr* (Ab1) (diluted in PBS, 1:200) at 37°C for 1.5 h, then washed in TBST three times. FITC-coupled rabbit antiserum (Sigma) was added, incubated for 1 h and then washed with TBST twice. The slides were mounted over glycerol and examined under a fluorescence microscope.

GSH and GST

EAC/S and EAC/Dox cells were collected from mice after cervical dislocation and washed twice in PBS. GSH was measured following the method of Sedlack

and Lindsay.[38] Briefly, to 2×10^5 cells in 0.2 ml PBS, 4.8 ml EDTA (0.2 M) was added and kept on ice for 10 min. Then 4 ml deionized water and 1 ml of 5% trichloroacetic acid (TCA) were added. The mixture was kept on ice for 10–15 min and then centrifuged at 3000 r.p.m. for 15 min. Then 2 ml of supernatant was taken and 4 ml of 0.4 M Tris buffer was added. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) (0.1 ml) solution was added and vortexed thoroughly. Optical density (OD) was read (within 2–3 min after addition of DTNB) at 412 nM against a reagent blank. Appropriate standards were run simultaneously.

The result found by this method was compared with the method of GSH measurement by Cohn and Lyle.[39] Briefly, cells were washed in PSA solution (0.4 g KCl, 8 g NaCl, 0.35 g $NaHCO_3$, 1 g D-glucose per liter). Cell pellets were resuspended in 2 ml PSA solution and divided into two tubes, one for measuring GSH content and the other for protein content. For GSH determination, cell pellets were resuspended in 0.2 ml ice-cold water and vortexed for 5 min, and 50 μl 25% metaphosphoric acid was then added slowly. After centrifugation at 12000 r.p.m. for 15 min, 20 μl of clear supernatant was transferred into a small tube containing 2 ml water, then 0.5 M sodium phosphate (pH 8) and 0.1 ml of 1% phthalaldehyde (dissolved in methanol) were added and mixed well. The mixture was kept at room temperature for 15 min. Fluorescence was measured with a Hitachi 650-40 fluorescence spectrophotometer.

No significant difference was observed in GSH content between these two[38,39] methods. Cellular GSH was expressed as ng GSH/μg protein.

Glutathione S-transferase (GST) enzyme activity was assayed according to the method of Habig *et al.*,[40] with the use of 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. Briefly, the EAC/S and EAC/Dox cells were cultured in RPMI 1640 medium supplemented with FCS (10%), HEPES and PEN-STREP. The drug X1 (EC 5–20 μg/ml) and verapamil (EC 4–6 μg/ml) were added in different concentrations after 24 h of the addition of cells and cultured for 48 h. Cells were detached by trypsinization from culture flasks and washed twice in PBS. To 0.1 ml cell suspension (1×10^5 cells) in PBS, 500 μl sodium phosphate buffer (0.2 M, pH 6.5) and 50 μl GSH (20 mM) were added. The final volume was made up to 1 ml. The reaction was carried out at 30°C by the addition of CDNB. The reaction was monitored spectrophotometrically by increase in absorbance. A correction was made by measuring and subtracting the rate in the absence of enzyme.

Results

The structure of X1 is shown in Figure 1. X1 contains two hydroxamic acid units (-CO-N-OH).

The profile of drug resistance of EAC/Dox cells against various drugs is listed in Table 1. The resistance index values, i.e. the ratio of IC_{50} values of various drugs in drug-resistant and drug-sensitive cells, have been found to be quite different. Drug-resistant cells (EAC/Dox) are more resistant to Dox (10 times), vinblastine (20 times) and cisplatin (48 times) in comparison to drug-sensitive (EAC/S) cells.

Reversal of resistance to Dox in the EAC/Dox cell line by X1

The IC_{50} of Dox versus the concentration of X1 for EAC/S and EAC/Dox cells is shown in Figure 2. Increasing concentrations of X1 progressively reverse the resistance of EAC/Dox cells. At higher concentra-

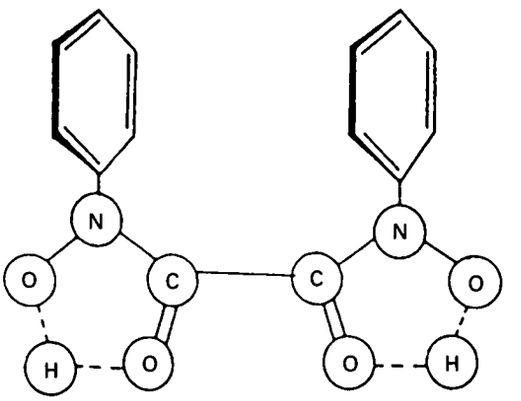

Figure 1. Structure of oxalyl bis-(*N*-phenyl)hydroxamic acid (X1).

Table 1. Drug sensitivity of EAC/Dox and EAC/S cells

Drugs	IC_{50} (μg/ml)		RI
	EAC/Dox	EAC/S	
Dox	2±0.2	0.21±0.3	10
Cisplatin	4.8±0.6	0.10±0.02	48
Vinblastine	2.5±0.3	0.12±0.08	20
X1	250±30	242±25	1
X2	260±30	253±24	1

RI (resistance index) means the relative resistance of EAC/Dox cells in comparison to EAC/S cells, for different antitumor drugs. Data are means±SD of four independent experiments.

tions of X1 (50 μg/ml), the IC_{50} value of drug-resistant cells (EAC/Dox) coincides with the IC_{50} of drug-sensitive (EAC/S) cells (Figure 2). X2 and verapamil, even at higher concentrations of 60 and 10 μg/ml, respectively, produce no effect on the IC_{50} of Dox (data not shown).

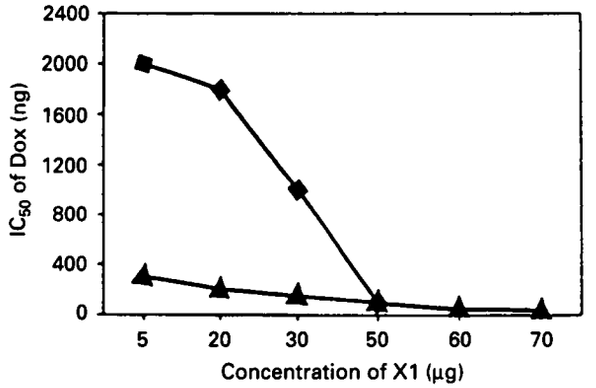

Figure 2. Reversal of resistance to Dox in EAC/Dox cells (■) by X1. At higher concentrations, the IC_{50} of EAC/Dox coincides with the IC_{50} of EAC/S cells (▲).

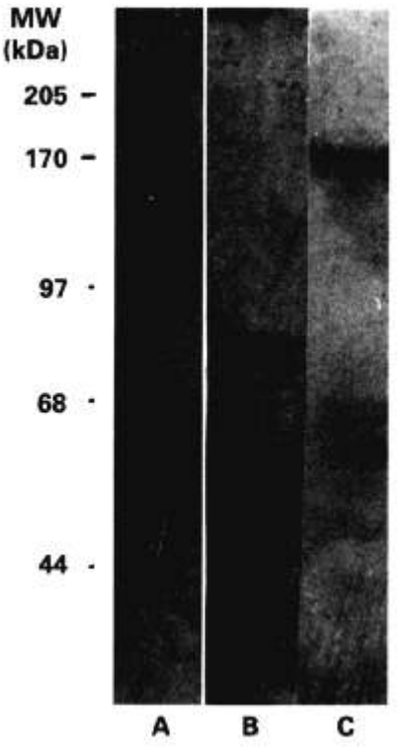

Figure 3. Immunostaining of Western blot of HeLa cells (lane C), drug-resistant (EAC/Dox) cells (lane A) and drug-sensitive (EAC/S) cells (lane B). A strong band at 170 kDa indicates P-gp in control HeLa cells (lane C). P-gp is absent in drug-resistant EAC/Dox cells (lane A) and in drug-sensitive EAC/S cells (lane B).

In Western blot analysis, P-gp is not found in drug-resistant (EAC/Dox) cells by the monoclonal antibody C219, which recognizes mouse protein (data not shown). With polyclonal antibody *mdr* (Ab1), P-gp remains undetected in drug-resistant cells (EAC/Dox). A band at 170 kDa demonstrates P-gp in control HeLa cells (Figure 3, lane C) but not in EAC/Dox (Figure 3, lane A) and EAC/S cells (Figure 3, lane B).

The absence of immunofluorescence in EAC/Dox and EAC/S cells indicates the non-expression of P-gp in the drug-resistant cells (data not shown).

Drug accumulation

The effect of verapamil and X1 on drug (Dox) accumulation in EAC/S and EAC/Dox cells is shown in Figure 4. Steady-state drug accumulation tests show that the concentration of Dox in drug-resistant EAC/Dox cells (60 ng/ml) is much less (Figure 4, C and 3) in comparison to the concentration of Dox (180 ng/ml) in EAC/S cells after 70 min of drug addition. Verapamil (6 μg/ml) partially restores (20% of control) drug accumulation in EAC/Dox cells within 70 min of drug addition. In EAC/S cells, verapamil and X1 have no such effect of drug accumulation (Figure 4, B and 2). X1 (EC 10 μg/ml) restores drug accumulation in EAC/Dox cells considerably (above 70% of control).

GSH and GST

The levels of GSH and GST are higher in drug-resistant cells (EAC/Dox) than in drug-sensitive (EAC/S) cells (Table 2). X1 reduces the level of GST (Table 3) in EAC/Dox cells at EC 15 μg/ml significantly ($p < 0.05$). The inhibition of GST by verapamil (EC 6 μg/ml) in the EAC/Dox cell line is not statistically significant ($p > 0.05$).

Discussion

The important aspect of the present work is the finding of (i) a drug-resistant cell line (EAC/Dox) from drug-sensitive cells (EAC/S) and (ii) a hydroxamic acid derivative, i.e. X1, which can modify the drug resistance *in vitro*. The chemical compound similar to X1, i.e. X2, cannot modify drug resistance. The EAC/Dox cells, though derived from repeated exposure to Dox, demonstrate much more cross-resistance

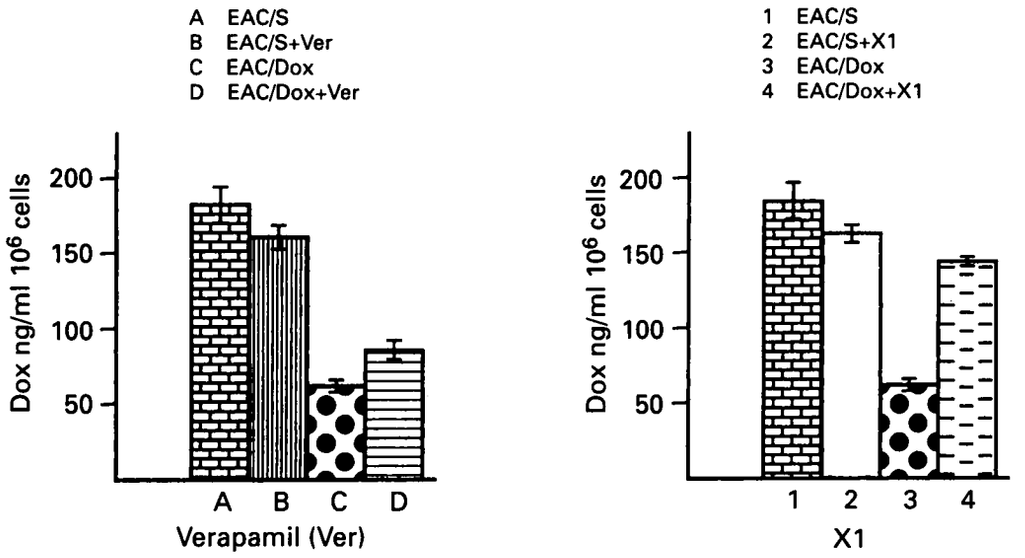

Figure 4. Effect of verapamil (6 μg/ml) and X1 on Dox accumulation after 70 min. Verapamil or X1 does not alter drug (Dox) accumulation in drug-sensitive (EAC/S) cells (B or 2) in comparison to control (A or 1). Drug-resistant (EAC/Dox) cells (C and 3) have lower accumulation of drugs (60 ng) than control (180 ng). In EAC/Dox cells, verapamil restores (D) drug accumulation slightly (from 60 to 90 ng). X1 restores (4) drug accumulation in drug-resistant cells appreciably (from 60 to 145 ng).

Table 2. GSH and GST in drug-sensitive (EAC/S) and drug-resistant (EAC/Dox) cell lines

Cell line	GSH (μg/mg protein)	GST (nmol/mg protein/min)
EAC/S	1.3±0.19	95.5±22.3
EAC/Dox	2.8±0.43	355±90.3

The levels of GSH and GST are higher in EAC/Dox cells than in EAC/S cells. The data are means±SD of four independent experiments. The values of GSH and GST in EAC/Dox cells are significant ($p<0.05$) in comparison to control (EAC/S).

Table 3. Effect of X1 and verapamil (V) on GST in drug-sensitive (EAC/S) and drug-resistant (EAC/Dox) cell lines

Cell line	GST (nmol/mg protein/min)
EAC/Dox	355±90.3
EAC/Dox+X1	128±18.8*
EAC/Dox+V	302±61.7**

The data are means±SD of four independent experiments. X1 reduces GST in EAC/Dox cells much higher in comparison to verapamil. *GST depletion by X1 is significant ($p<0.05$), whereas **GST depletion by verapamil is not significant ($p<0.05$) when compared with control.

to vinblastine and cisplatin than Dox. This phenomenon, that MDR cells display more resistance to compounds other than the inducing drug, was rarely reported and its underlying mechanistic basis is as yet unknown.[30] We had studied the effect of verapamil on the modulation of drug resistance as we supposed in the beginning that P-gp might be involved in the EAC/Dox cells. The non-involvement of P-gp in the present investigation is indicated, as verapamil has no significant effect in the modulation of drug resistance of EAC/Dox cells.

We had injected a lower dose of Dox (2 and 4 mg/kg/week) and could not find P-gp in the drug-resistant cell line (EAC/Dox), up to the 20th generation mice. We injected a lower dose of Dox as higher doses caused toxic death to animals. This observation is similar to the findings of Ellen Friche *et al.* who reported that a doxorubicin derivative, 4-deoxy-4-iodo doxorubicin (di-dox)-resistant EAC cells express P-gp when the higher dose of di-dox (12 mg/kg/week) was injected to the animals but at low dose (4 mg/kg/week), the resistance developed without any detectable range of P-gp.[28]

The resistant cell line (EAC/Dox) exhibits a significantly higher concentration of GSH and higher activity of GST than the sensitive cells. Glutathione, an

important intracellular antioxidant, is the most abundant non-protein thiol present in the cells.[41] Glutathione reduces the Dox-derived free radical species and hence decreases the cytotoxicity of the drug.[42] The increased GSH level is paralleled with the development of resistance to doxorubicin in tumor cells.[43]

Although, Dox has not been shown to be a substrate for GST or to form conjugates with GSH, resistance may be a consequence of protection from indirect doxorubicin damage.[41] For example, lipid peroxidation is a frequent cellular result of adriamycin exposure. Such peroxides may further break down to yield hydroxy alkenals, which have been shown to be substrates for GST isozymes.[44,45] It has been reported that BSO enhances drug transport in a number of cell lines by cellular GSH depletion. Cellular glutathione depletion, following BSO exposure, inhibiting the enhanced drug efflux then suggests that the drugs are transported in the form of glutathione S conjugates.[46] Most investigators accept that GSH has a role in drug resistance; the involvement of GSH-associated enzymes (especially GSTs) has been most contentious.[2]

The role of over-expression of MRP has also been reported in several non-P-gp MDR cell lines. GSH levels can regulate the transport of drugs in MRP over-expressing, but not in P-gp over-expressing cells.[46,47] The MRP transporter may be a good candidate to explain the phenotype of the drug-resistance cells.

X1 causes the depletion of GST significantly. The effect of X1 on the GST/GSH system is, perhaps, due to the formation of conjugates between X1 and glutathione. It has been reported that hydroxylamine (R-NH-OH), a precursor of hydroxamic acid (-CO-N-OH), forms stable conjugates with glutathione.[48] Takanuki *et al.* have reported that the hydroxamic acid group (in imidazole skeleton) is most important for GSH depletion.[49] The present compound, X1, having two hydroxamic acid units (OH-N-CO-CO-N-OH) may be a better glutathione-depleting agent than the compound having one hydroxamic acid unit. X1 is non-toxic and may be utilized as a RMA in the cases where MDR is due to elevated levels of the GSH/GST system. Further studies to explain the phenotype of drug-resistant cells are warranted.

Acknowledgments

The authors acknowledge the help of Professor Dr KN Thimmaiah, Department of Studies in Chemistry, University of Mysore, India.

References

1. Crown KH, Batist G, Tulpul A, Sinha BK, Myers CE. Similar biochemical changes associated with multidrug resistance in human breast cancer cells and carcinogen-induced resistance to xenobiotics in rats. *Proc Natl Acad Sci USA* 1986; **83**: 9328-32.
2. Tew KD. Glutathione associated enzyme in anticancer drug resistance. *Cancer Res* 1994; **54**: 4313-20.
3. Dano K. Active outward transport of daunomycin in resistant Ehrlich ascites tumor cells. *Biochem Biophys Acta* 1973; **323**: 466-83.
4. Incaba M, Jhonson RK. Uptake and retention of adriamycin and daunorubicin by sensitive and anthracycline-resistant sublines of P 388 leukemia. *Biochem Pharmacol* 1978; **27**: 2123-30.
5. Fojo A, Akiama S, Gottesman MM, Patsan I. Reduced drug accumulation in multiple drug-resistant human KB carcinoma cell lines. *Cancer Res* 1985; **45**: 3002-7.
6. Tsuoro T, Satio HI, Kawabata H, Oh-hara T, Hamada H, Utakoji T. Characteristics of resistance to adriamycin in human myelogenous leukemia K562 resistant to adriamycin and in isolated clones. *Jpn J Cancer Res* 1986; **77**: 682-92.
7. Shen DW, Cardarelli C, Hawang J, *et al.* Multiple drug-resistant human KB carcinoma cells independently selected for high level resistance to colchicine, adriamycin or vinblastine show changes in expression of specific proteins. *J Biol Chem* 1986; **261**: 7762-70.
8. Cornwell MM, Safa AR, Felsted RL, Gottesman MM, Pastan I. Membrane vesicles from multidrug resistant cancer cells contain a 150 to 170 kDa protein detected by photoaffinity labeling. *Proc Natl Acad Sci USA* 1986; **83**: 3847-50.
9. Safa AR, Glover CJ, Mayers MB, Biedler JL, Felsted RL. Vinblastine photoaffinity labeling of a high molecular weight surface membrane glycoprotein specific for multidrug-resistant cells. *J Biol Chem* 1986; **261**: 6137-40.
10. Edincott JA, Ling V. The biochemistry of P-glycoprotein mediated multidrug resistance. *Annu Rev Biochem* 1989; **58**: 137-71.
11. Gottesman MM, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* 1993; **62**: 385-427.
12. Ford JM, Hait WN. Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol Rev* 1990; **42**: 155-98.
13. Suzukake K, Petro BJ, Vistica DT. Reduction in glutathione content of L-PAM resistant L1210 cells confers drug sensitivity. *Biochem Pharmacol* 1982; **31**: 121-4.
14. Suzuka K, Vistica BP, Vistica DT. Dechlorination of L-phenylalanine mustard by sensitive and resistant tumor cells and its relationship to intracellular glutathione content. *Biochem Pharmacol* 1983; **32**: 165-7.
15. Prezioso JA, Fitzgerald GB, Wick MM. Melanoma cytotoxicity of buthionine sulphoxamine (BSO) alone and in combination with 3,4,-dihydroxybenzylamine and melphalan. *J Invest Dermatol* 1992; **99**: 289-93.
16. La Creta FP, Brennam JM, Hamilton TC, Ozols RF, O'Dwyer PJ. Streoselective pharmacokinetics of L-buthionine *SR*-sulphoxamine in patients with cancer. *Drug Metab Disp* 1994; **22**: 835-42.

17. Ciaccio PJ, Tew KD, La Creta FP. The spontaneous and glutathione S-transferase mediated reaction of chlorambucil with glutathione. *Cancer Commun* 1990; **2**: 279-86.
18. Ahamad H, Singhal SS, Awasthi S. The inhibition of the α, μ and π class isozymes of glutathione S-transferases by sulphasalazine, 5-aminosalicylic acid and sulphapyridine. *Biochem Arch* 1992; **8**: 355-61.
19. Pillante SL, Lisek CA, Dulik DM, Fenselau C. Glutathione conjugate. Immobilized enzyme synthesis and characterisation by fast atom bombardment mass spectroscopy. *Drug Metab Disp* 1986; **14**: 313-8.
20. Ploemen JHTM, Van Ommen B, Van Bladeren PJ. Inhibition of rat and human glutathione S-transferase isoenzymes by ethacrynic acid and its glutathione conjugate. *Biochem Pharmacol* 1990; **40**: 1631-5.
21. Philips MF, Mantle TJ. The initial rate-kinetics of mouse glutathione S-transferase YfYYf. Evidence for an allosteric site for ethacrynic acid. *Biochem J* 1991; **275**: 703-9.
22. Tsuoro T, Iada H, Tsukagoshi S, Sakurai Y. Overcoming of vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res* 1981; **41**: 1967-72.
23. Kartner N, Shales M, Riordan JR, Ling V. Daunorubicin resistant Chinese hamster ovary cells expressing multidrug resistance and cell surface P-glycoprotein. *Cancer Res* 1983; **43**: 4413-9.
24. Ling V, Kartner N, Sudo T, Siminovitch L, Riordan JR. Multidrug resistance phenotype in Chinese hamster ovary cells. *Cancer Treat Rep* 1983; **67**: 869-74.
25. O'Dwyer PJ, La Creta F, Nash S, *et al.* Phase 1 study of thio TEPA in combination with glutathione transferase inhibitor ethacrynic acid. *Cancer Res* 1991; **51**: 6059-65.
26. Choudhuri SK, Roy PS, Ghose AK. Oxalyl-bis(*N*-phenyl/*N*-*p*-tolyl) hydroxamic acids as coordinating ligands: coordination compounds with organotins. *Indian J Chem* 1984; **23**: 917-9.
27. Ghose NN, Sarkar DK. Succinyl bis(*N*-phenyl) hydroxamic acid. *J Indian Chem Soc* 1968; **45**: 550-2.
28. Friche E, Danks MK, Beck WT. Characterization of tumor cell resistance to 4-deoxy-4-iododoxorubicin developed in Ehrlich ascites cells *in vivo*. *Cancer Res* 1992; **52**: 5701-6.
29. Kueng W, Silber E, Eppenberger U. Quantification of cells cultured on 96 well plates. *Anal Biochem* 1989; **182**: 16-9.
30. Hu X, Yang H, Pan QR, Zhen S. Does *p*-glycoprotein play a pivotal role in the drug resistance of an MDR variant, K562/Dox? *Chemotherapy* 1995; **41**: 296-305.
31. Lowry OH, Rosenberg NJ, Farr AL, Randal RJ. Protein measurement with folin phenol reagent. *J Biol Chem* 1951; **196**: 265-71.
32. Laemmli, UK. Cleavage of structural proteins during the assembly of the head of bacteriofage T4. *Nature* 1970; **227**: 680-5.
33. Towbin H, Stachelin T, Gorden J. Electrophoretic transfer of proteins form polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979; **76**: 4350-4.
34. Kartner N, Evernden-Porelle D, Bradley G, Ling V. Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies. *Nature* 1985; **316**: 1985.
35. Chen CJ, Chin JE, Ueda K, *et al.* Internal duplication and homology with bacterial transport proteins in the *mdr-1* (P-glycoprotein) gene from multidrug-resistant human cells. *Cell* 1986; **47**: 381-9.
36. Redmond MS Shelagh, Joncourt F, *et al.* Assessment of P-glycoprotein, glutathione-based detoxifying enzymes and O^6-alkylguanine-DNA alkyltransferase as potential indicators of constitutive drug resistance in human colorectal tumors. *Cancer Res* 1991; **51**: 2092-7.
37. Freeman JW, Chatterjee A, Ross BE, Busch H. Epitope distribution and immunochemical characterisation of nuclear phosphoprotein C23 using ten monoclonal antibodies. *Mol Cell Biochem* 1985; **68**: 87-96.
38. Sedlack J, Lindsay RN. Estimation of total protein-bound and non protein sulphohydral groups in tissues with Ellmans reagent. *Anal Biochem* 1968; **25**: 192-205.
39. Cohn VH, Lyle J. A fluorometric assay for glutathione. *Anal Biochem* 1966; **14**: 434-90.
40. Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferase. The first enzymatic step in marcapturic acid formation. *J Biol Chem* 1974; **249**: 7130-9.
41. O'Brien ML, Tew KD. Glutathione and related enzymes in multidrug resistance. *Br J Cancer* 1966; **32**: 967-78.
42. O'Brien PJ. Molecular mechanism of quinone cytotoxicity. *Chem-Biol Interact* 1991; **80**: 1-41.
43. Al-Kabban M, Stewart MJ, Watson ID, Reglinski J. The effect of doxorubicin on the glutathione content and viability of cultured human lung cancer cell lines A549 and GLC4 210. *Clin Chem Acta* 1990; **194**: 121-9.
44. Meijer C, Mulder NH, Timmer-Bosscha H, Linter WJ, Meersma GJ, de Vries EG. Relationship of cellular glutathione to cytotoxicity and resistance of seven platinum compounds. *Cancer Res* 1993; **52**: 6885-9.
45. Sellin S, Holmquist B, Mannervik B, Vallee BL. Oxidation and reduction of 4-hydroxyalkenals catalysed by isozymes of human alcohol dehydrogenase. *Biochemistry* 1991; **30**: 2514-8.
46. Versantvoort CHM, Broxterman HJ, Bagrich T, Scheper RJ, Tweentyman PR. Regulation by glutathione of drug transport in multidrug-resistant human lung tumor cell lines overexpressing multidrug resistance-associated protein. *Br J Cancer* 1995; **72**: 82-9.
47. Cole SPC, Bhardawaj G, Gerlach JH, *et al.* Overexpression of a novel transport gene in a multidrug resistant human lung cancer cell line. *Science* 1992; **258**: 1650-4.
48. Vargese AJ. Glutathione conjugates of Misonidazole. *Biochem Biophys Res Commun* 1983; **112**: 1013-20.
49. Takanuki K, Igarashi I, Hata K, *et al.* Glutathione depletion by 2-nitromidazole-1-acetohydroxamic acid as a radiosensitizer in hypoxic rat hepatocytes. *Biochem Int* 1988; **17**: 155-62.

(Received 16 April 1998; revised form accepted 21 July 1998)