Biochemical Pharmacology, Vol. 39, No. 4, pp. 723–728, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

ANTHRACYCLINE RESISTANCE IN MURINE LEUKEMIC P388 CELLS

ROLE OF DRUG EFFLUX AND GLUTATHIONE RELATED ENZYMES*

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(*Received* 8 *May* 1989; *accepted* 3 *August* 1989)

Abstract—Energy-dependent drug efflux is a major factor in cellular resistance of P388/R84 mouse leukemic cells to anthracyclines such as doxorubicin (DOX), and blocking of efflux increases sensitivity. However, efflux does not play a significant role in resistance to *N*-trifluoroacetyladriamycin-14-valerate (AD 32), a DOX analog. Since drug efflux alone cannot account for resistance to anthracyclines, we have, in the present study, measured cellular glutathione (GSH) content and activity of GSH cycle related enzymes to determine their role in resistance. Cellular GSH content was similar in DOX-sensitive and -resistant mouse leukemic cells (P388 and P388/R84). GSH peroxidase, glucose-6-phosphate dehydrogenase and glutathione reductase activities were 1.36-, 1.58- and 1.14-fold higher in P388/R84 cells. Incubation of P388/R84 cells with 100 μM buthionine-*S*,*R*-sulfoximine (BSO) for 24 hr reduced cellular GSH content to 6% of control and reduced their resistance to DOX [dose modification factor (DMF) 3.9]. GSH depletion had no significant effect on the cytotoxicity of AD 32 (DMF 1.5). Exposure of P388/R84 cells to BSO (for GSH depletion) and trifluoperazine (for efflux blocking) further reduced their resistance to DOX (DMF 14). These results indicate that DOX resistance in P388/R84 cells is multifactorial and that changes in GSH cycle related enzymes such as GSH peroxidase may also contribute to their resistance.

Cellular resistance to the anthracycline doxorubicin (DOX‡) is multifactorial, and rapid drug efflux is a major factor in resistance of several *in vitro* cell lines to DOX [1, 2]. Efflux blocking by unrelated agents such as verapamil or phenothiazines enhances chemosensitivity of resistant cells in culture by increasing cellular DOX retention [3, 4].

Anthracyclines are postulated to cause cellular damage by the generation of highly reactive oxygen free radicals [5–7] as well as by DNA intercalation and possible binding to topoisomerase-II [8–10]. Glutathione (GSH), a tripeptide thiol, has been shown to protect cells from the cytotoxicity of several drugs [11], and a positive correlation between cellular GSH content and chemosensitivity to melphalan, cisplatin and DOX has been reported [12]. Recent studies suggest that depletion of cellular GSH by buthionine sulfoximine (BSO) can also enhance chemosensitivity of resistant tumor cells against several unrelated drugs [13–15].

In our earlier studies, we compared cellular retention of DOX in P388 (parental drug-sensitive line) and its doxorubicin-resistant P388/R84 subline. The resistant cell line has 4- to 6-fold less cellular DOX content, and blocking of efflux with a phenothiazine (e.g. chlorpromazine) or verapamil enhances DOX retention and reduces resistance [4, 16]. However, efflux blocking does not completely overcome resistance, and P388/R84 cells after efflux blocking are still 60-fold more resistant than P388 drug-sensitive cells. In contrast to DOX, cellular retention of a DOX analog, *N*-trifluoroacetyladriamycin-14-valerate (AD 32), in P388/R84 cells is not significantly lower than that of P388 cells and efflux blocking does not alter resistance [16, 17]. These observations would suggest that anthracycline resistance in P388/R84 cells is not due solely to DOX efflux but must involve other cellular mechanisms.

To understand further the cellular basis for anthracycline resistance in P388/R84, we have, in the present study, analyzed the effect of BSO-induced GSH depletion (with and without efflux blocking) on the cellular resistance of P388/R84 cells to DOX and AD 32. We have also compared the activities of GSH regeneration cycle enzymes (GSH peroxidase, GSH reductase, and glucose-6-phosphate dehydrogenase) in the drug-sensitive and -resistant cell lines.

MATERIALS AND METHODS

Doxorubicin (DOX, Adriamycin hydrochloride, NSC-123127) was purchased from Adria Laboratories (Columbus, OH). Stock solutions (1 mg/mL) of DOX were made in distilled water and diluted

* A preliminary report on this study was presented at the American Association for Cancer Research meeting, 1987.

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‡ Abbreviations: DOX, doxorubicin (NSC-123127); AD 32, *N*-trifluoroacetyladriamycin-14-valerate; P388, murine leukemic doxorubicin-sensitive cell line; P388/R84, 150-fold doxorubicin-resistant cell line; TFP, trifluoperazine; PBS, phosphate-buffered saline; GSH, glutathione; GSSG, oxidized glutathione; BSO, buthionine-*S*,*R*-sulfoximine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); and DMF, dose modification factor.

appropriately in RPMI 1640 medium. AD 32 was a gift from Dr Mervyn Israel, Memphis, TN, and stock solutions were made in 100% ethanol. L-Buthionine-*S*,*R*-sulfoximine (BSO), NADPH, NADP, cumene hydroperoxide, glucose-6-phosphate and DTNB were purchased from the Sigma Chemical Co. (St Louis, MO). Glutathione reductase, oxidized glutathione (GSSG) and reduced glutathione (GSH) were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Trifluoperazine (TFP) was obtained from Smith, Kline & French Laboratories (Carolina, PR).

Mouse leukemic cell lines (P388 and Adriamycin-resistant P388/ADR) were obtained in 1978 from Dr Randall K. Johnson and Arthur D. Little, Inc., Cambridge, MA, and maintained in DBA/2 mice by weekly i.p. transplantation. A DOX-resistant cell line (P388/R84) was isolated by serial cultivation of the P388/ADR cells in methylcellulose containing gradually increasing DOX concentrations. This subline has been maintained in drug-free medium for the last several years. The IC_{50} values for DOX in P388 and P388/R84 cell lines (in soft agar after a 1-hr incubation with DOX) were 0.062 and 9.3 μM respectively. The IC_{50} values for AD 32 in P388 and P388/R84 cells (after continuous incubation) were 0.03 and 0.99 μM respectively. Log phase suspension cultures were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 I.U./mL), streptomycin (100 μg/mL), and 2-mercaptoethanol (10 μM).

For determination of total GSH content, cells were washed two times with phosphate-buffered saline (PBS), lysed in 3% sulfosalicyclic acid and sonicated for 1 min. After centrifugation at 15,000 *g*, total GSH content in the supernatant fraction was measured by the enzymatic recycling method of Tietze [18] as modified by Akerboom and Sies [19].

GSH peroxidase activity towards cumene hydroperoxide was determined according to the method of Awasthi *et al.* [20]. GSH reductase and glucose-6-phosphate dehydrogenase activities were determined by the method of Beutler [21].

Cytotoxicity was determined by growth inhibition assay after incubating the cells with DOX for 24 hr, whereas soft agar clonogenic assays were done after a 1-hr incubation with DOX and continuous incubation with AD 32. Colonies larger than 50 μm in soft agar were counted from triplicate plates for determination of the mean number of colonies ± SD per plate. Details of our soft agar method have been described before [22].

BSO-treated and control P388 and P388/R84 cells were incubated with DOX (3.5 μM) at 37° for 2 hr. Cellular DOX fluorescence, after excitation with a 488 nm argon laser line, was monitored in a Coulter Electronics Epics V cell sorter interfaced with a MDADS (multiple data acquisition display system). Fluorescence emission (above 530 nm) and forward angle light scatter from a minimum of 10,000 cells were collected, amplified, and scaled to generate a multiparameter histogram. Details of our instrumentation and procedures for flow cytometric determination of cellular anthracycline content have been published earlier [4, 23].

For fluorometric determination of cellular DOX content, BSO-treated and control cells, after incubation with DOX (3.5 μM for 2 hr), were washed three times with cold saline solution and resuspended in an acid–alcohol mixture containing 95% ethanol, 1 N hydrochloric acid, and distilled water in a 5:2.8:1.6 volume ratio. The supernatant solution, recovered after sonication and centrifugation of the cell suspension, was analyzed in a spectrofluorometer using excitation and emission wavelengths of 485 and 574 nm respectively.

Cellular DNA content and cell cycle distribution were determined by our propidium iodide-hypotonic citrate method [24].

RESULTS

In the present study, the total GSH contents of P388 and P388/R84 cells, as determined by the enzymatic recycling method, were similar, confirming our earlier reported data [25]. Incubation with different concentrations of BSO (25–100 μM) for 24 hr reduced the total GSH content in P388 and P388/R84 cells by 75–95% (data not shown). Approximately 70% of GSH depletion occurred within the first 6 hr of incubation with 100 μM BSO. For all subsequent experiments, cells were incubated with BSO (100 μM) for 24 hr which resulted in 95% depletion of GSH (Table 1). In P388 and P388/R84 cells reincubated in fresh medium after GSH depletion (100 μM BSO for 24 hr), the rates of GSH resynthesis were similar, and approximately 60% of the GSH was replaced within 8 hr in both cell lines (data not shown).

Data in Table 1 show the effect of BSO-induced GSH depletion on DOX cytotoxicity by growth inhibition assay. In P388/R84 cells pretreated with BSO for 24 hr, and then grown in the presence of DOX and BSO for another 24 hr, the IC_{50} for DOX was reduced from 7.4 to 1.48 μM with a resulting dose modification factor (ratio of IC_{50} for DOX:DOX + BSO) of 5.0. In P388 cells similarly treated, GSH depletion had no major effect on the IC_{50} value and the dose modification factor was about 1.26. P388/R84 cells, which were approximately 100-fold resistant to DOX (as compared to P388), were approximately 26-fold resistant after BSO-induced GSH depletion in this experiment. Similarly, in clonogenic assay, GSH depleted P388/R84 cells showed increased DOX cytotoxicity and the dose modification factor was 3.9 (Table 2).

Earlier we reported that blocking of DOX efflux with trifluoperazine (TFP) increases sensitivity of P388/R84 cells to DOX [16]. In the present study, we sought to determine whether co-incubation of GSH-depleted P388/R84 cells with TFP would further reduce the resistance to DOX in soft agar assays. Table 2 shows data from BSO-treated and control P388/R84 cells exposed to DOX or TFP alone or to a combination of DOX + TFP for 1 hr. Exposure of cells to BSO or TFP alone had little growth inhibitory effects on P388/R84 cells (10–15%). Exposure to a combination of DOX with TFP or BSO enhanced DOX cytotoxicity as compared to cells treated with DOX alone and IC_{50} values were 4.0 and 2.4 μM respectively. Thus, neither GSH depletion by BSO nor efflux blocking by TFP alone

Table 1. Effect of BSO on cellular GSH content and DOX toxicity in P388 and P388/R84 cells

Cell line	GSH content (nmol/mg protein)		IC_{50} (μM)	
	−BSO	+BSO	−BSO	+BSO
P388	14.8 ± 0.14*	0.88 ± 0.01	0.072 ± 0.01†	0.057 ± 0.01
P388/R84	14.4 ± 0.02	0.78 ± 0.02	7.4 ± 0.31	1.48 ± 0.31

Cells were treated with 100 μM BSO for 24 hr and then grown in the presence of DOX and BSO for 24 hr. Control cultures received no BSO.

* Mean ± SD of four experiments.

† Mean ± SD of three experiments.

Table 2. Effect of GSH depletion on DOX and AD 32 cytotoxicity in clonogenic assays

Treatment*	IC_{50}, μM (DMF)
P388/R84 + DOX	9.3†
P388/R84 + DOX + BSO	2.4 (3.9)
P388/R84 + DOX + TFP	4.0 (2.3)
P388/R84 + DOX + BSO + TFP	0.67 (14)
P388/R84 + AD 32	0.99
P388/R84 + AD 32 + BSO	0.66 (1.5)

* BSO-treated and control cells were exposed to different concentrations of DOX and TFP for 1 hr, washed, and plated in soft agar. In the case of AD 32, BSO-treated and control cells were incubated continuously with different concentrations of AD 32 and cells were counted after 4–5 days.

† IC_{50} values are means of three experiments with SD of 10–15% of the reported value. DMF = dose modification factor.

could completely overcome the resistance of P388/R84 cells to DOX. However, cells exposed to BSO for 24 hr (for GSH depletion) and later incubated with TFP for 1 hr (to block DOX efflux) had markedly increased sensitivity to DOX and the IC_{50} was only 0.67 μM. In contrast, GSH depletion did not have a major effect on the resistance of P388/R84 to AD 32 and the IC_{50} for AD 32 after GSH depletion was 0.66 μM compared to the control IC_{50} of 0.99 μM (Table 2).

The enhanced chemosensitivity of P388/R84 cells after GSH depletion and efflux blocking to DOX was further confirmed by flow cytometric analysis of DOX effects on cell cycle traverse. Earlier we reported a pronounced accumulation of cells with G_2/M DNA content in log phase cultures exposed to DOX [26]. DNA distribution histograms in Fig. 1 (A–D) show the effect of different DOX concentrations on cell cycle traverse of P388/R84 cells. Exposure to 9.0 μM DOX caused a pronounced G_2 block (D, 41% cells in G_2/M as compared to 19% in control). Exposure of cells to TFP (E), BSO (I) or BSO + TFP (M) had no major effect on cell cycle traverse. Histograms (F, G and H) in Fig. 1 are of P388/R84 cells co-incubated with three different DOX concentrations (0.9, 1.8 and 9.0 μM) and TFP (15 μM) for 1 hr, washed, and incubated in drug-free medium for 24 hr. A pronounced accumulation of cells with G_2/M DNA content was seen in cells exposed to 1.8 μM DOX after efflux blocking by TFP. This enhanced cell cycle traverse effect was presumably due to increased retention of DOX and the resulting cytotoxicity. In cells exposed to BSO (100 μM) for 24 hr, followed by exposure to DOX for 1 hr and reincubation in drug-free medium for 24 hr (J, K and L), a similar effect on accumulation of cells with G_2/M DNA content (Fig. 1L) was evident. This effect could probably be due to reduced GSH content which, in turn, may not be enough to overcome the cytotoxic effect of DOX. These flow cytometric observations on DOX-induced G_2 block confirm that a combination of GSH depletion (by BSO) and efflux blocking (by TFP) has far more pronounced effects on cell cycle traverse and cytotoxicity than exposure to BSO or TFP alone. In cultures exposed to 9.0 μM DOX in the presence of TFP and after GSH depletion, a pronounced accumulation of cells with less than G_1 DNA content (arrow) was also evident (P), indicating the presence of dead cells.

To rule out the possibility that BSO was acting by blocking efflux of DOX, we compared the cellular retention of DOX fluorescence in control and BSO-pretreated (100 μM for 24 hr) P388 and P388/R84 cells. No major differences in retention of cellular DOX fluorescence between BSO-treated and control cells were seen by flow cytometric or fluorometric measurements. In BSO-treated and control P388 cells incubated with 3.5 μM DOX for 2 hr, 1.7 μM DOX per 5×10^6 cells was recovered, whereas P388/R84 cells, incubated similarly, had 0.34 μM DOX per 5×10^6 cells in both BSO-treated and control cells. These observations would indicate that BSO effects on DOX toxicity were not due to enhanced retention of DOX.

To determine the protective role of GSH related enzymes in DOX cytotoxicity, we compared the activities of GSH peroxidase, glucose-6-phosphate dehydrogenase and glutathione reductase. Data in Table 3 show that whereas GSH reductase activity was only slightly higher, GSH peroxidase and glucose-6-phosphate dehydrogenase activities were 1.36- and 1.58-fold higher, respectively, in P388/R84 cells. These observations would suggest that P388/R84 cells may have greater efficiency for free radical detoxification through the GSH peroxidase activity than the P388 cells.

DISCUSSION

Energy-dependent drug efflux is a major factor for

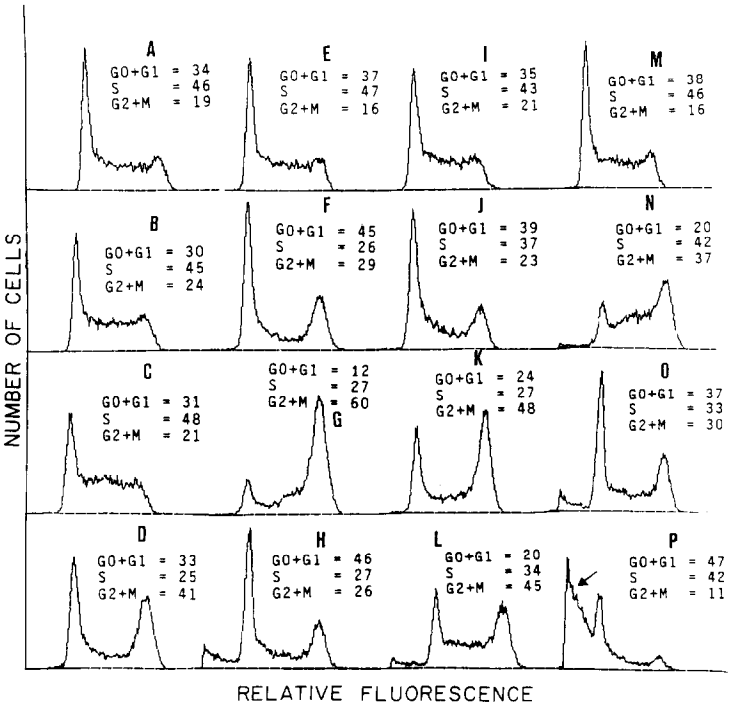

Fig. 1. Effects of TFP, BSO and/or DOX on cell cycle distribution of P388/R84 cells. Control cultures (A–D) received no BSO but were treated with DOX at a concentration of 0.9 μM (B), 1.8 μM (C) and 9.0 μM (D). Cultures E, I and M were treated with 15 μM TFP (for 1 hr followed by reincubation in fresh medium for 24 hr), 100 μM BSO for 24 hr, and 100 μM BSO for 24 hr followed by TFP for 1 hr respectively. Cultures F, G and H show the effects of DOX and TFP; cultures J, K and L show the effects of DOX and BSO; and cultures N, O and P show the combined effect of DOX, BSO, and TFP.

Table 3. GSH peroxidase, GSH reductase, and glucose-6-phosphate dehydrogenase activities in P388 and P388/R84 cells

	GSH peroxidase*	GSH reductase† (units/mg protein)	Glucose-6-phosphate dehydrogenase‡
P388	88.2 ± 9.6	73.0 ± 5.1	58.3 ± 5.5
P388/R84	120.6 ± 4.6	83.6 ± 8.0	92.3 ± 6.6

Values are means ± SD of three separate determinations.
* One unit of enzyme converts 1 nmol GSH to GSSG/min at 37°.
† One unit of enzyme utilizes 1 nmol NADPH/min at 37°.
‡ One unit of enzyme catalyzes formation of 1 nmol NADPH/min at 37°.

reduced cellular DOX retention, and blocking of efflux has been shown to increase chemosensitivity [1–4]. Several recent studies suggest that other protective mechanisms besides that of rapid drug efflux may contribute to anthracycline resistance [27–32].

Free radical mediated cellular damage has been suggested as one of the major mechanisms for DOX toxicity [5–7, 33]. The protective role of GSH in cells exposed to xenobiotic compounds and depletion of cellular GSH has been shown to increase DOX toxicity in tumor cells [12–15]. Yet no direct correlation between free radical production and the role of GSH in cellular drug resistance to DOX has been established. In addition, the mechanism(s) associated with the protective role of GSH against DOX cytotoxicity has not been investigated.

Elevated GSH level has been reported in a variety of drug-resistant cells [13–15]. In the present study, however, we did not find any difference in GSH content of our murine leukemic P388 and P388/R84 cells. Batist *et al.* [30] have also reported similar GSH content in their DOX-sensitive and -resistant MCF-7 cell lines.

Incubation of cells with BSO, a γ-glutamylcysteine synthetase inhibitor, reduced the cellular GSH content in both P388 and P388/R84 cell lines. In the resistant cells, GSH depletion was accompanied by a significant increase in chemosensitivity to DOX

with a dose modification factor of 3.9 in clonogenic assay. Similar results were reported by Hamilton *et al.* [12] who found a significant potentiation of DOX cytotoxicity by BSO (DMF 1.5 to 5) in a human ovarian cancer cell line. Compared to DOX, we could not find a significant increase in AD 32 cytotoxicity (DMF 1.5) in spite of a quinone moiety in AD 32 which generates free radicals.

Our earlier studies established the role of rapid drug efflux in resistance of P388/R84 to DOX. These studies clearly show that efflux blocking alone by phenothiazines or verapamil cannot reverse completely the resistance of P388/R84 cells, and even after enhanced drug retention (drug content similar to that of P388 cells) the P388/R84 cells are about 64-fold resistant. These observations would support the hypothesis that cellular resistance of P388/R84 cells to DOX is multifactorial, and drug efflux may be only one of the major mechanisms involved. In the present study and as reported by us earlier [25], we attempted to combine efflux blocking with GSH depletion for reducing more than 100-fold resistance of P388/R84 cells to DOX. Our data clearly showed that this combination treatment does further reduce the resistance of P388/R84 cells to DOX. The remaining equivalent 10-fold resistance (after efflux blocking and GSH depletion) may possibly be related to other factors such as differences in cell membrane structure, lipid peroxidation, and drug metabolism.

DOX is known to exert cytotoxicity by free radical production which, in turn, may lead to DNA damage and lipid peroxidation [5–7, 33]. GSH is a key factor in the regulation of several related enzyme activities, and it affects the efficacy and interaction of a variety of antineoplastic drugs mainly through nucleophilic thioether formation [34]. GSH is present in both oxidized and reduced forms, and the cellular levels and ratio of these two forms are of critical importance in maintaining the reducing environment as well as in glutathione-dependent enzyme activities [11].

The mechanism of protection afforded by GSH against DOX toxicity is not understood completely. In the present study, we observed that the activities of GSH peroxidase, an enzyme which protects against oxidants, were increased in P388/R84 cells. This increased activity in resistant cells may thus contribute to cellular resistance by enhanced free radical scavenging. However, a lack of correlation between overexpression of GSH peroxidase activity (1.34-fold) and degree of resistance suggests that this factor may be contributing only in part to DOX resistance.

Acknowledgement—We are thankful to Ms Kristie Gordon for help with the laser flow cytometry.

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