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A proposed mechanism of resistance to cyclophosphamide and phosphoramide mustard in a Yoshida cell line in vitro*

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Summary. A Yoshida sarcoma cell line (Y_R/cyclo) showing decreased sensitivity to metabolically activated cyclophosphamide in vitro has been shown to be cross-resistant to phosphoramide mustard, the ultimate alkylating agent formed from cyclophosphamide. Resistance to these alkylating agents has been shown to be associated with increased activity of the glutathione *S*-transferase group of enzymes, and with elevated levels of glutathione, the co-substrate of the enzyme. The resistant cell line shows lower levels of cellular damage, as measured by alkaline elution following treatment with phosphoramide mustard, than the parental (Ys) line. The mechanism of resistance is ascribed to increased deactivation of potentially damaging metabolites of cyclophosphamide by the glutathione *S*-transferase enzymes, resulting in decreased cellular damage in the resistant cell line.

Introduction

The development of resistance to antitumour agents is widely recognised as a major problem in cancer chemotherapy [16]. The mechanisms by which this resistance arises have been the subject of many investigations.

Cyclophosphamide, one of the most widely used anticancer drugs, is unusual amongst clinically useful alkylating agents in that it requires metabolic activation before showing any antitumour effect. The number of active, or potentially active, metabolites formed complicates the study of the drug. Considering the importance of this drug surprisingly little information has been reported on the mechanisms by which tumour cells develop resistance to this agent. A mechanism of resistance to cyclophosphamide involving deactivation of aldophosphamide by aldehyde dehydrogenase has been described in L1210 cells [12]. However, this mechanism cannot account for the cross-resistance observed towards phosphoramide mustard, a final product of cyclophosphamide metabolism, in the Yoshida cell line used in this work. In this study elevation of the level of activity of the glutathione *S*-transferase class of enzymes (EC 2.5.1.18) is shown to be associated with acquired cellular resistance to cyclophosphamide. This enzyme has recently been shown to be involved in resistance to other alkylating agents [15]. It is proposed that resistance arises because of increased deactivation of potentially damaging species within the cell by this enzyme, and that this mechanism would be effective on both the parent drug and its metabolites.

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Materials and methods

Chemicals. Cyclophosphamide was used as the commercial preparation (Farmitalia Carlo Erba Ltd., Barnet, Herts., UK). Phosphoramide mustard was synthesised by the method of Friedman and Seligman [7]. Glutathione and glutathione *S*-transferase (rat) were obtained through Sigma (Poole, Dorset, UK). 1-Chloro-2,4-dinitrobenzene was obtained from Pfaltz and Bauer Inc. (Stamford, Conn, USA).

Enzyme kinetics. Glutathione *S*-transferase activities of cell extracts were determined spectrophotometrically using glutathione (1 m*M*), and 1-chloro-, 2,4-dinitrobenzene (1 m*M*) as co-substrates [11] in potassium phosphate buffer (100 m*M*, pH 6.5, 25 °C). Absorption changes at 340 nm were measured on a Beckman DU8 spectrophotometer with a kinetics II accessory. The uncatalysed reaction between the co-substrates was monitored in the absence of protein extract. Enzyme inhibition studies were carried out under identical conditions but with the addition of phosphoramide mustard.

Cell culture. A Yoshida sarcoma cell line resistant to cyclophosphamide (Y_R/cyclo) was developed from the parental tumour (Y_S) by incremental challenge with the drug in vivo in female Wistar rats. Both cell lines have been established in culture in vitro and are routinely maintained in Fischer's medium supplemented with 20% horse serum (Gibco, UK). Both cell lines are regularly screened and shown to be mycoplasma-free. The Y_R/cyclo cell line has been shown to maintain its resistance in culture for periods of more than 3 months. Cell stocks are routinely replaced from freezer stock after 90 days' continuous culture.

Growth inhibition studies were performed by back extrapolation of growth curves. Cell counts were estimated using an electronic cell counter (Coulter Electronics) for 10 days following drug treatment. Drugs were incubated with cells for 2 h prior to resuspension of the cells in fresh medium and serum. Phosphoramide mustard solutions

were preapred immediately prior to use. Cyclophosphamide was activated using a liver microsomal preparation [6]. Microsomes were filter sterilised prior to addition to cell cultures. Cell growth was determined in the absence of drug but in the presence of the microsomal preparation to obtain a control growth rate.

Preparation of cell homogenates. Cells were pelleted by centrifugation (150 *g*, 5 min) and washed three times in phosphate-buffered saline, followed by homogenisation using a Teflon-glass homogeniser in double-distilled water. The resulting homogenate was centrifuged (2000 *g*, 15 min) and total protein was estimated (Bio-Rad Protein Assay, Munich FDR) in the supernatant. Protein concentrations were equalised by dilution.

Estimation of nonprotein sulphydryl and glutathione. Tumours were excised from aged-matched rats (200–250 g). Total nonprotein sulphydryl levels were determined using Ellman's reagent [5,5'-dithiobis-(2-nitro)benzoic acid] according to the method Ball [2]. Glutathione levels in the Ys and YR/cyclo cell lines were edetermined enzymatically using glyoxalase I according to the method of Akerboom and Sies [1].

Alkaline elution. Cellular cross-linking (both DNA-DNA and DNA-protein) following drug treatment was estimated by the technique of alkaline elution [14], as modified by Bedford and Fox [3]. Cells are prelabelled with either ^{14}C-thymidine (60 mCi/mmol, 0.05 μCi/ml) or ^{3}H-thymidine (4 Ci/mmol, 1 μCi/ml) for 16 h. The ^{14}C-labelled cells were then treated with phosphoramide mustard (1 h, 37 °C). The ^{3}H-labelled cells are mixed with the drug-treated cells and act as an internal measure of the flow rate of each filter. A radiation dose of 3000 rad was delivered to all cells prior to lysis on the filter, to minimise the effect of DNA single-strand breakage caused by the drug.

Results

The growth inhibitory effect of activated cyclophosphamide and phosphoramide mustard on the parental (Y_S) and resistant (Y_R/cyclo) cell lines are summarised in Table 1. It can be seen that resistance to cyclophosphamide in these cell lines is accompanied by cross-resistance to the ultimate alkylating agent, phosphoramide mustard. The ratios of ID_{50} values between the Y_R/cyclo and Y_S cell lines for these agents are similar, 19 and 22 for cyclophosphamide and phosphoramide mustard, respectively.

The glutathione *S*-transferase activities of both cell lines, utilising glutathione and 1-chloro-2,4-dinitrobenzene as co-substrates, can be seen to be directly proportional to the quantity of soluble cellular protein used in the enzyme assay (fig. 1). However, from the dual scale on the abscissa, it can be seen that a six-fold excess of crude protein from the parental cell line must be used to obtain equivalent enzyme activity with that observed in the cyclophosphamide-resistant line. All enzyme activities are expressed as the number of micromoles of glutathione conjugate formed per minute. The concentration of protein extract which is used in the assay is limited by the turbidity of the assay solution (~1800 μg/ml).

Table 1. Growth inhibition studies on the parental (Y_S) and cyclophosphamide-resistant (Y_R/cyclo) cell lines in vitro

Cell line	Drug	ID_{50} (μ*M*)
Y_S	Activated cyclophosphamide	1.9
Y_R/cyclo	Activated cyclophosphamide	36.4
Y_S	Phosphoramide mustard	2.1
Y_R/cyclo	Phosphoramide mustard	47

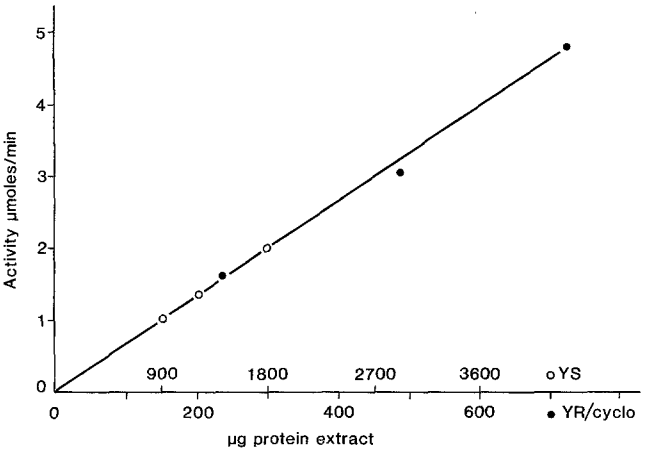

Fig. 1. Glutathione *S*-transferase activities of soluble protein extracts from parental (○) and cyclophosphamide-resistant (●) cell lines. The *upper abscissa scale* refers to the total quantity of protein used in the enzyme assay for the parental cell line, while the *lower* one refers to the quantity used from the YR/cyclo cell line. All activities were determined using 1-chloro,-2,4-dinitro-benzene (1 m*M*) and glutathione (reduced form, 1 m*M*) as co-substrates in potassium phosphate buffer (0.1 *M*, pH 6.5, 25 °C). All enzyme activities are corrected for the uncatalysed reaction between the co-substrates

Cyclophosphamide can be shown to inhibit the activity of commercial glutathione *S*-transferase towards 1-chloro-2,4-dinitrobenzene. The rate of coupling of glutathione to this agent, measured by the absorption increase at 340 nm, can be seen to be decreased on addition of increasing concentrations of cyclophosphamide (A–F in Fig. 2). Phosphoramide mustard also inhibits the reaction. The concentrations of cyclophosphamide and phosphoramide mustard required to inhibit 50% of the enzyme activity against 1-chloro,-2,4-dinitrobenzene (1 m*M*) are shown in Table 2. Both the parent drug and its metabolite have similar inhibitory effects on glutathione *S*-transferase from rat liver and in the parental and resistant cell lines.

The levels of nonprotein sulphydryl in the parental and resistant tumours and the level of glutathione in the Y_S and Y_R/cyclo cell lines are shown in Table 3. A 2.2-fold increase in nonprotein sulphydryl is observed in the resistant tumour compared with the parental one. A similar elevation (~3-fold) in glutathione is seen in the Y_R/cyclo cell line.

Alkaline elution studies on the Y_S and Y_R/cyclo cell lines show markedly lower levels of cross-links (both DNA-DNA and DNA-protein) in the resistant cell line following treatment with phosphoramide mustard than in the parental line (Fig. 3). A post-drug incubation time of 4 h was chosen as this gave maximal cross-linking in both cell lines. It can be seen that there is an approximately 20-fold elevation of cross-links in the parental cell line

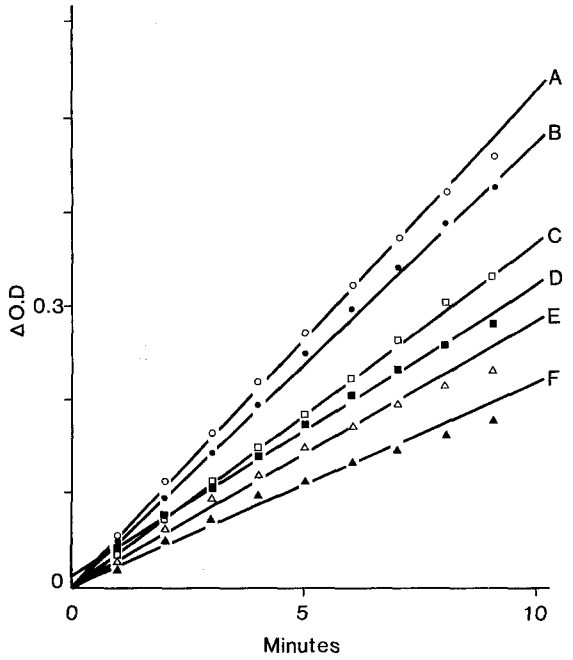

Fig. 2. Effect of various concentrations of cyclophosphamide on the rate of enzymatic conjugation of 1-chloro,-2,4-dinitrobenzene (1 m*M*) to glutathione (1 m*M*) by commercial rat liver glutathione *S*-transferase. The concentrations of cyclophosphamide used were: *A*, 0 m*M* (control); *B*, 0.96 m*M*; *C*, 1.91 m*M*; *D*, 3.83 m*M*; *E*, 5.75 m*M*; *F*, 9.6 m*M*. The uncatalysed reaction between substrates was used to correct the enzyme catalysed rate.

Table 2. Inhibition of glutathione S-transferase activity by cyclophosphamide and phosphoramide mustard

Enzyme source	Drug	ID_{50} (μ*M*)
Commercial (rat liver)	Cyclophosphamide,	5.7
	phosphoramide mustard	5.5
Y_S	Cyclophosphamide	5.8
Y_R/cyclo	Cyclophosphamide	5.3

ID_{50} represents the drug concentration required to reduce enzyme activity by 50% against 1-chloro,-2,4-dinitrobenzene

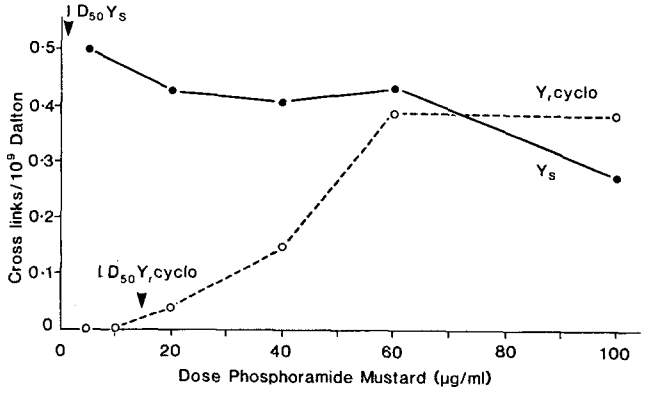

Fig. 3. Dose-response curve showing the effect of increasing concentrations of phosphoramide mustard on the number of cross-links formed (both DNA-DNA and DNA-protein) in the parental (●) and cyclophosphamide-resistent (○) Yoshida cell lines following drug treatment. The ID_{50} values indicate the drug concentrations necessary to cause 50% growth inhibition for these cell lines

Table 3. Nonprotein sulphydryl levels in the parental (Y_S) and cyclophosphamide resistant (Y_R/cyclo) tumours, and glutathione levels in the derived cell lines

Tumour	(Non-protein-SH)/g wet weight
Y_S	0.48×10^{-6} mol
Y_R/cyclo	1.07×10^{-6} mol
Cell line	**Glutathione level**
Y_S	1.4×10^{-6} mol/10^8 cells
Y_R/cyclo	4.2×10^{-6} mol/10^8 cells

compared with the resistant line when both are treated with a level of phosphoramide mustard equivalent to the ID_{50} dose of the Y_R/cyclo cells.

Discussion

The known mechanisms of resistance to the alkylating agents include decreased drug accumulation [8], increased repair of drug-induced lesions [3] and cellular inactivation of drug [5, 12]. It is unclear which are the important cyclophosphamide metabolites accumulated by tumour cells. Since cyclophosphamide itself is inactive it may be argued that resistance to the drug is unlikely to arise owing to decreased cyclophosphamide uptake [9], but more likely to arise as a result of modified uptake of potentially active metabolites formed in the liver (e.g. 4-hydroxycyclophosphamide, 4-hydroperoxy-cyclophosphamide, aldophosphamide and phosphoramide mustard). However, these agents are known not to share a common uptake mechanism [5], and therefore a resistance mechanism involving transport alterations is unlikely owing to the number of pathways which would have to be altered. The antitumour effects of the alkylating agents is due to their interactions with DNA, notably cross-linking of the DNA strands within the helix.

Cyclophosphamide has been shown to crosslink DNA [12]. The mechanism of this reaction relies on the bifunctional nature of these agents, the analogous monofunctional drugs showing little or no antitumour action [5]. Hence the enzymatic conjugation of only one of the alkylating groups on the mustard to glutathione would decrease the cytotoxic action of the drug.

The decreased level of cross-linking observed in the resistant cell line (Fig. 3) following treatment with phosphoramide mustard can be explained if a process which deactivates the reactive species is assumed. The glutathione *S*-transferase enyzmes are known to deactivate electrophilic alkylating agents [4]. These enzymes have broad and overlapping substrate specificities, and are highly active against organo-halide substrates. The inhibition of enzyme activity by both cyclophosphamide and phosphoramide mustard can be explained by direct competition of these organo-halides with 1-chloro,-2,4-dinitrobenzene for the enzyme.

The range of substrate specificities of the isoenzymes complicates the quantitation of enzyme activity. 1-Chloro-2,4-dinitrobenzene has been used in this study because of its ability to act as a substrate for most of the isoenzymes [10]. Hence, although a sixfold increase in enzyme activity is observed in the resistant cell line this value may be al-

tered if another substrate was used. If the elevated level of glutathione *S*-transferase observed in the Y_R/cyclo cell line was induced in response to cellular insult by the drug, then it is possible that the activity of this protein against the drug would be greater than that observed for 1-chloro,--2,4-dinitrobenzene.

The increased level of glutathione *S*-transferase activity is accompanied by a twofold elevation in nonprotein sulphydryl between the tumours and by threefold increase in glutathione between the resistant and sensitive cell lines derived from the solid tumours.

Surprisingly little is known about the mechanisms of resistance to cyclophosphamide and its metabolites. An increased level of aldehyde dehydrogenase has been observed in L1210 cells with acquired resistance to the drug [12]. This enzyme deactivates aldophosphamide (a precursor of phosphoramide mustard), resulting in a loss of production of phosphoramide mustard, the ultimate alkylating species. This mechanism of resistance explains the lack of cross-resistance to phosphoramide mustard (which is not a substrate for the enzyme) observed in these L1210 cells. However, the cyclophosphamide-resistant Yoshida cell line used in this study shows complete cross-resistance to phosphoramide mustard, and increased aldehyde dehydrogenase therefore cannot be the resistance mechanism operative in these cells.

The deactivation of aldophosphamide by aldehyde dehydrogenase in cyclophosphamide resistant L1210 cells [12] can only be effective in tumours in which the drug is accumulated either as the aldo form or as one of its precursors. An increase in glutathione *S*-transferase activity, however, would result in the deactivation of all potentially cytotoxic metabolites of cyclophosphamide, leading to decreased cellular damage by the drug. Thus an elevation of this enzyme represents a more general mechanism of resistance to cyclophosphamide. The biochemical processes which give rise to the elevated enzyme levels are at present unknown, but investigations into whether gene amplification or some other mechanism is involved, and the ratio of the individual isoenzymes produced by this process, may give information on the sequence of events involved in the development of resistance.

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