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Melphalan Resistance and Photoaffinity Labelling of P-glycoprotein in Multidrug-Resistant Chinese Hamster Ovary Cells

REVERSAL OF RESISTANCE BY CYCLOSPORIN A AND HYPERTHERMIA

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ABSTRACT. The multidrug resistance phenotype is often associated with overexpression of P-glycoprotein, an energy-dependent efflux pump responsible for decreased intracellular accumulation of chemotherapeutic agents. The role of P-glycoprotein in the mechanism of cross-resistance to melphalan in multidrug-resistant Chinese hamster ovary cells (CH^RC5) was investigated by photoaffinity labelling of P-glycoprotein using [^{3}H]azidopine. We investigated whether the chemosensitiser cyclosporin A and hyperthermia, either used alone or combined, could reverse melphalan resistance and alter transport processes for [^{14}C]melphalan in CH^RC5 cells. Melphalan inhibited azidopine photolabelling of P-glycoprotein, implicating drug efflux mediated by P-glycoprotein in the mechanism of melphalan resistance in CH^RC5 cells. Azidopine photolabelling also was inhibited by the chemosensitiser cyclosporin A, which binds to P-glycoprotein. Cyclosporin A alone reversed melphalan resistance in CH^RC5 cells, but had no effect in drug-sensitive AuxB1 cells. Hyperthermia (40–45°) alone increased melphalan cytotoxicity in both cell lines. When hyperthermia was combined with cyclosporin A, a large increase in melphalan cytotoxicity occurred, but only in CH^RC5 cells. This effect increased with temperature and exposure time. Sensitisation to melphalan cytotoxicity by heat and cyclosporin A in CH^RC5 cells appeared to be explained by altered drug transport processes. Lower accumulation of melphalan occurred in CH^RC5 cells than in drug-sensitive cells. At 37°, cyclosporin A increased drug accumulation in CH^RC5 cells, but not in AuxB1 cells, by slowing drug efflux from cells. Heat alone increased both melphalan uptake and drug efflux for both cell lines. Our findings suggest that the combination of cyclosporin A and hyperthermia could be very useful in overcoming melphalan resistance by increasing intracellular drug accumulation in multidrug-resistant cells. BIOCHEM PHARMACOL **58**;2:291–302, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. photoaffinity labelling; melphalan resistance; cyclosporin A; P-glycoprotein; hyperthermia; drug efflux

Resistance to antineoplastic agents constitutes a major obstacle in the treatment of many types of cancer. The development of resistance to one particular drug often confers cross-resistance to a wide range of structurally unrelated drugs [1, 2]. The mechanisms underlying MDR‡ are not understood completely. It has been shown that some resistant cell lines accumulate less drug due to overexpression of a 170-kDa energy-dependent membrane glycoprotein (P-glycoprotein) [3–6]. P-glycoprotein exhibits a wide range of substrate specificity, including *Vinca* alkaloids, actinomycin D, and anthracyclines such as doxorubicin and daunorubicin [1, 2]. Chemosensitisers such as verapamil [7–9] and cyclosporin A [10–13] were shown to reverse partially the MDR phenotype associated with overexpression of P-glycoprotein, in several different cell lines *in vitro*. These chemosensitisers appear to act by reducing the active efflux of anticancer drugs from cells, mediated by P-glycoprotein, thus restoring intracellular drug accumulation. However, the efficacy of chemosensitisers such as cyclosporin A *in vivo* in solid tumors needs further clarification [14–17]. Therefore, several different approaches should be considered in order to effectively overcome multidrug resistance.

In recent years, much attention has been focused on the effects of heat in biological systems. In the treatment of cancer, elevated temperatures (40–45°) have potential for increasing the anticancer effects of radiation and certain chemotherapeutic agents [18]. The combination of hyperthermia and radiotherapy is very promising, both from cellular and clinical studies [19]. The combination of chemotherapeutic agents and hyperthermia also has poten-

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‡ *Abbreviations*: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CHO, Chinese hamster ovary; FBS, fetal bovine serum; GST, glutathione *S*-transferase; HSP, heat shock protein; MDR, multidrug resistance; and MEM, minimum essential medium.

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tial for improving antitumor efficacy, based on cellular and animal studies [20], although there have been fewer clinical studies. Hyperthermia has been shown to increase the cytotoxic effect of many anticancer drugs such as Adriamycin®, bleomycin, BCNU, cisplatin, and the alkylating agent melphalan, from both *in vitro* and *in vivo* studies [20–26]. The mechanisms by which hyperthermia enhances cytotoxicity of anticancer drugs are complex, and they are not well understood. Possible mechanisms of interaction include increased drug uptake, DNA effects, protein damage, and pharmacokinetics [20]. Increased uptake has been shown for most, but not all, anticancer agents at elevated temperatures. This could be explained by membrane damage [27] or by altered membrane permeability [28] caused by heat, therefore allowing larger quantities of drugs to enter the cell.

Hyperthermia has been used successfully as an adjuvant to melphalan chemotherapy for the treatment of melanoma, acute leukemia, and non-Hodgkin's lymphoma [29–35]. Several *in vitro* and *in vivo* studies have shown enhancement of melphalan cytotoxicity by hyperthermia [36]. Hyperthermia allows larger quantities of melphalan to enter the cell, and this could account, at least in part, for the increased cytotoxicity of the drug at elevated temperatures [22, 23, 37]. We previously reported that heat sensitises pleiotropic MDR Chinese hamster ovary cells (CH^RC5) with overexpression of P-glycoprotein to melphalan [24]. Melphalan is not generally considered to be a substrate for P-glycoprotein, and thus the mechanism of resistance to melphalan in CH^RC5 cells is unclear. The effect of chemosensitisers that bind to P-glycoprotein, such as verapamil and cyclosporin A, on resistance to melphalan in MDR cells has received little attention.

We have investigated the possible role of drug efflux via P-glycoprotein in the mechanism of resistance to melphalan in CH^RC5 cells. We show here, for the first time, that melphalan inhibits photoaffinity labelling of P-glycoprotein with [^{3}H]azidopine, confirming that melphalan is a substrate for P-glycoprotein. Knowing that cyclosporin A can increase the intracellular accumulation of certain anticancer drugs in several MDR cell lines [8, 10, 11, 13, 38], we would expect the chemosensitiser to sensitise the MDR cell line CH^RC5 to melphalan by increasing intracellular drug accumulation. When cyclosporin A is combined with hyperthermia, enhanced drug cytotoxicity should occur in resistant cells, due to the action of heat on the increased level of intracellular drug. Our findings show that cyclosporin A sensitises CH^RC5 cells to melphalan and increases intracellular drug accumulation. When hyperthermia was combined with cyclosporin A, a large increase in drug cytotoxicity occurred in MDR cells. Our findings suggest that the combination of cyclosporin A and hyperthermia could be very useful in overcoming melphalan resistance by increasing intracellular drug concentrations in MDR cells.

MATERIALS AND METHODS

Tissue Culture

The MDR cell line CH^RC5 was selected for resistance to colchicine from the drug-sensitive AuxB1 parent cell line [3]. The resistance factor to colchicine is about 300-fold, but CH^RC5 cells are also cross-resistant to other anticancer agents including *Vinca* alkaloids, Adriamycin, and melphalan [39]. We have obtained a resistance factor to melphalan of about 2- to 3-fold. The CHO cell lines AuxB1 and CH^RC5 were grown in monolayers in 75-cm^2 tissue culture flasks at 37° under 5% CO_2 in MEM Alpha (Gibco Canada), supplemented with 10% FBS (Gibco Canada) and 1% penicillin (50 U/mL)–streptomycin (50 μg/mL) (Gibco Canada). The culture medium for the drug-resistant cell line CH^RC5 contained colchicine (5 μg/mL). Colchicine was removed for the final passage before experiments. Studies were carried out using cells grown to confluence and incubated for 24 hr at 37° with fresh culture medium prior to each experiment. Cells were harvested with PBS containing sodium citrate (0.015 M), washed by centrifugation, and resuspended in PBS containing 1% BSA and 10 mM glucose.

Drugs

Melphalan (Sigma Chemical Co.) was freshly prepared before each experiment and was dissolved in a minimum volume of ethanol (92%) containing 2% HCl. Cyclosporin A (Sigma) was dissolved in a minimum amount of ethanol (100%). Solutions of melphalan and cyclosporin A were diluted to their appropriate final concentrations in PBS containing 1% BSA and 10 mM glucose (pH 7.3). The final concentrations of ethanol and HCl were not toxic to cells and did not affect photoaffinity labelling experiments. To prevent alkylation reactions by melphalan, the drug was hydrolysed by heating at 50° for 3 hr in PBS [40].

Photoaffinity Labelling of P-glycoprotein

CHO cells (5×10^5 per assay), in a final volume of 50 μL in 7 mM potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.5 μM [^{3}H]azidopine (specific activity: 3.11 GBq/mg) (Amersham Canada), were photolabelled after incubation for 60 min at room temperature in the dark, either with or without the nonradioactive competing ligand (melphalan or cyclosporin A). Cell suspensions were then irradiated for 5 min at 254 nm. Photolabelled samples (20 μg of proteins) were solubilized in sample buffer [4% SDS, 0.08 M Tris–HCl (pH 6.8), 20% glycerol, 4.5 M urea, 0.1 M mercaptoethanol, and 0.04% bromophenol blue] and then processed for electrophoresis on SDS/8% polyacrylamide gels containing 4.5 M urea. After electrophoresis, the gels were stained with Coomassie blue, soaked in Amplify (Amersham Life Science), dried under vacuum at 70°, and exposed to X-ray film (Fuji Medical) for 6 days at −70°. Proteins were quantitated by the Bradford assay, and

staining of gels with Coomassie blue confirmed the equal protein loading of 20 μg for each lane (data not shown). Photoaffinity labelling of the 170-kDa band for P-glycoprotein was quantitated using a laser densitometer (Personal Densitometer SI, Molecular Dynamics).

Cytotoxicity Experiments

Aliquots of 0.1 mL of cells (10^6/mL) were preincubated with or without cyclosporin A (5 μM) for 10 min at 37°. The cells were then incubated with various concentrations of melphalan in temperature-controlled water baths (Haake D8, Fisher Scientific), at temperatures ranging from 37° to 45°. After the incubation, the cells were washed twice by centrifugation (2 min, 1000 *g*) with cold PBS–1% BSA and then resuspended in culture medium. The cells were diluted to the appropriate concentrations in MEM Alpha containing 10% FBS and plated in tissue culture-coated dishes. The culture dishes were incubated in a water-jacketed incubator at 37° with 5% CO_2 for 8 days (AuxB1) or 10 days (CH^RC5). The dishes were washed with PBS, fixed with 95% ethanol, and stained with methylene blue before counting the colonies (>50 cells). Percentage survival was expressed as the mean number of colonies obtained relative to the mean number of colonies obtained in the control. Two hundred cells were seeded in the control plates, but where there was a loss of cell survival, cells were plated at several different densities to ensure that countable colonies would be obtained, and the results were corrected accordingly. We have demonstrated previously that, in this system, there is linearity between the number of cells plated and colonies formed over the range of 10 to 10^4 [41].

Melphalan Uptake

^{14}C-Labelled melphalan (L-*p*-(di-2-chloroethyl[^{14}C]amino)-phenylalanine) with a specific activity of 1.62 Bq/mg was a gift from Mr. Maurice Leaffer, SRI International. The radiochemical purity was 97% as determined by thin-layer chromatography on silica gel 60 (Merck) in *n*-butanol: acetic acid:water (7:2:1). A solution of [^{14}C]melphalan was prepared as described above for cytotoxicity experiments.

Aliquots of 0.1 mL of freshly harvested CHO cells (10^7/mL) in PBS–1% BSA–10 mM glucose (pH 7.3) were preheated for 2 min in a circulating water bath to allow them to reach the incubation temperature. At time zero, 0.1-mL aliquots of melphalan solution (6.5 μM), with or without cyclosporin A, were added to the cells, and the suspension was incubated for 15 min at 37° or 42°. Uptake was then stopped by the addition of 4 mL of ice-cold PBS–1% BSA, followed by centrifugation (1000 *g*, 1 min). The cells were washed three times with PBS–1% BSA to remove extracellular melphalan. The final pellet of cells was resuspended in 0.5 mL of 1% SDS to which 4 mL of liquid scintillation fluid (Scintiverse II; Fisher Scientific Co.) was added. The radioactivity was determined using a liquid scintillation analyzer (Packard 2200 CA, Canberra-Packard).

Melphalan Efflux

Freshly harvested CHO cells (10^7/mL) were preloaded with [^{14}C]melphalan (3.25 μM) for 20 min at 37° in an incubation medium consisting of PBS–1% BSA–10 mM glucose. The cells were centrifuged (2 min, 1000 *g*) and washed three times with PBS–1% BSA at 0° to remove extracellular melphalan. For efflux measurements, cells were resuspended in ice-cold PBS–1% BSA–10 mM glucose and divided in aliquots of 0.1 mL (10^6 cells) in cold glass tubes. A volume of 0.4 mL of incubation medium, prewarmed at the incubation temperature, with or without 5 μM cyclosporin A, was added to the cells to start the efflux process. Efflux experiments were carried out at 37° or 42°, without melphalan in the extracellular medium. To stop efflux, the cells were centrifuged (1000 *g*, 2 min) after the addition of 4 mL of ice-cold PBS–1% BSA. The radioactivity was determined in the cell pellet as for the melphalan uptake assay. The data points at time zero represent the absolute value for melphalan content in each of the cell lines prior to efflux.

Analysis of Data for Cytotoxicity of Melphalan and Heat

In combination experiments, the type of interaction between melphalan, cyclosporin A, and heat was determined by the method of Drewinko and coworkers [42]. For this evaluation, the two cytotoxic agents, A (heat) and B (melphalan), were assumed to cause independent effects. The third agent, cyclosporin A, did not cause cytotoxicity under these conditions. For a given temperature, either with or without cyclosporin A treatment, the experimental data value for percent cell survival is E_A. For the independent effects of the drug treatment (E_B), E_{B1} and E_{B2} represent the experimental data values for percent cell survival in cells exposed to melphalan, either with or without treatment with cyclosporin A, respectively. The results obtained were defined according to the following criteria:

Experimental $E_{A+B} = \Sigma\, E_A \times E_B$ indicates an additive effect;

Experimental $E_{A+B} < \Sigma\, E_A \times E_B$ indicates a potentiation effect;

where Experimental E_{A+B} represents the experimental data values for the effects of A and B in combination, and

$\Sigma\, E_A \times E_B$ represents the theoretical values, which are the sum of the individual effects of the drug and heat.

Analysis of Data for Melphalan Efflux

We previously showed that melphalan efflux from cells is described by the following equation, where c is the melphalan concentration in the cell pellet as a function of time (t) [37]:

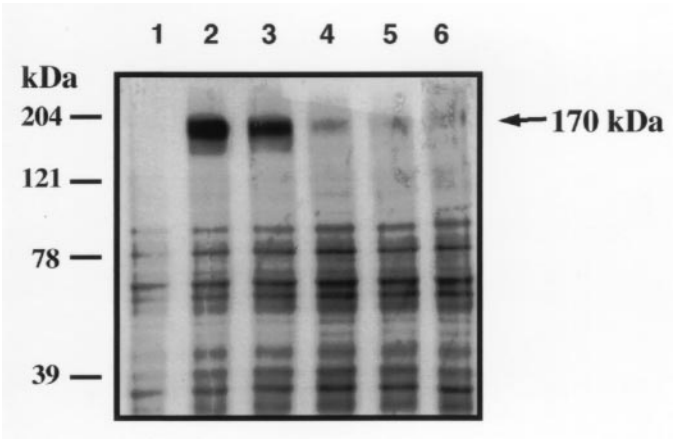

FIG. 1. SDS–PAGE fluorography of AuxB1 and CH^RC5 cells after photoaffinity labelling with [^{3}H]azidopine: effect of cyclosporin A. AuxB1 cells (lane 1) and CH^RC5 cells (lanes 2–6) were photolabelled with 0.5 μM [^{3}H]azidopine. Lanes 3–6 represent CH^RC5 cells with 0.5, 5, 50, and 500 μM cyclosporin A, respectively. Each lane displays the migration of 20 μg of protein. Results are shown from one gel, which was reproduced on two occasions.

$$c(t) = A + B \exp(-\kappa t) \quad (1)$$

The parameter A represents the final steady-state melphalan concentration, B is the value of the total change in melphalan concentration from time zero until steady-state conditions are attained, and κ is the rate constant for the efflux process. The best fit of the experimental efflux data points to Equation 1 was obtained by an iterative procedure using the Marquardt algorithm (KaleidaGraph Software). The rate constants and the parameters A and B of Equation 1 were determined by the best fit of the nonlinear regression of the efflux data points using an iterative procedure based on the Marquardt algorithm. The SD values of the rate constants and parameters were based on the SD values of the original data set.

RESULTS

We determined whether P-glycoprotein is involved in resistance to melphalan in CH^RC5 cells. To achieve this, MDR cells were labelled with the photoreactive agent [^{3}H]azidopine, which has a high binding affinity for P-glycoprotein [43–45]. Several studies showed that agents with a binding affinity for P-glycoprotein can inhibit photoaffinity labelling of this glycoprotein by azidopine. In drug-sensitive AuxB1 cells, no photolabelling of protein at 170 kDa was seen (Fig. 1), indicating that P-glycoprotein is not expressed at sufficient levels to be detected by this method. CH^RC5 cells were photolabelled with azidopine, either with or without increasing concentrations of cyclosporin A. The relative values for inhibition of photolabelling of the 170-kDa band are given in Table 1. Photoaffinity labelling of the 170-kDa protein was inhibited by 40% by a relatively low concentration of cyclosporin A (0.5 μM), and by 90% with higher concentrations of 5 μM and above (Fig. 1, Table 1). CH^RC5 cells were then photolabelled with azidopine, either with or without increasing concentrations of melphalan (Fig. 2). In MDR cells, photoaffinity labelling of the 170-kDa protein was inhibited by melphalan (Fig. 2, Table 1). A concentration of 50 μM melphalan inhibited photoaffinity labelling by 40%, whereas higher concentrations of drug allowed further inhibition of labelling. In addition to P-glycoprotein, other proteins were labelled by [^{3}H]azidopine, and this was inhibited by cyclosporin A. This result has also been shown for cyclosporin A and its analogues in other systems [44]. Melphalan also inhibited the azidopine labelling of several minor bands in addition to the 170-kDa band.

TABLE 1. Effect of cyclosporin A and melphalan on photoaffinity labelling of P-glycoprotein with [^{3}H]azidopine in CH^RC5 cells

Cyclosporin A (μM)	Labelling (relative)*	Melphalan (μM)	Labelling (relative)†
0	1.00	0	1.00
0.5	0.47–0.73	0.5	0.94 ± 0.19
5	0.03–0.13	5	1.03 ± 0.23
50	0.05–0.11	50	0.59 ± 0.13
100	0.03–0.19	500	0.43 ± 0.08
500	0.08	1000	0.15 ± 0.01

*The range of values for photoaffinity labelling of P-glycoprotein was obtained by densitometric analysis of gels from two independent experiments and are relative to the control value obtained without cyclosporin A.

†The values (mean ± SEM) for photoaffinity labelling of P-glycoprotein were obtained by densitometric analysis of gels from four independent experiments and are relative to the control value obtained without melphalan.

Alkylating agents such as melphalan are highly reactive and have the potential to undergo a wide variety of reactions, including reactions with macromolecules such as proteins, DNA, and RNA [46]. We determined whether inhibition of photoaffinity labelling by melphalan could be

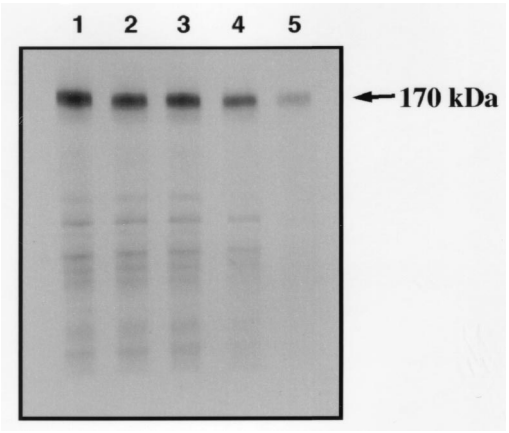

FIG. 2. SDS–PAGE fluorography of CH^RC5 cells after photoaffinity labelling with [^{3}H]azidopine: effect of melphalan. Cells were photolabelled with 0.5 μM [^{3}H]azidopine in the absence of melphalan (lane 1), or with 0.5, 5, 500, and 1000 μM melphalan, respectively (lanes 2–5). Each lane displays the migration of 20 μg of protein. Results are shown from one gel, which was reproduced on five occasions.

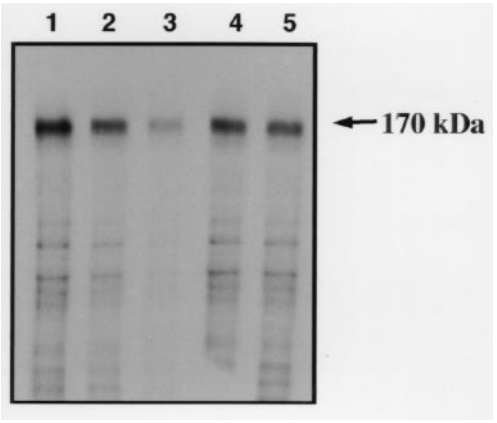

FIG. 3. SDS–PAGE fluorography of CH^{R}C5 cells after photoaffinity labelling with [^{3}H]azidopine: effect of preincubation with melphalan. Cells were photolabelled with 0.5 μM [^{3}H]azidopine in the absence of melphalan (control, lane 1), or with 500 and 1000 μM melphalan, respectively (lanes 2 and 3). Cells were preincubated for 20 min with 500 or 1000 μM melphalan, respectively (lanes 4 and 5), and then were washed three times to remove extracellular drug before photolabelling with 0.5 μM [^{3}H]azidopine alone. Each lane displays the migration of 20 μg of protein. Results are shown from one gel, which was reproduced on two occasions.

attributed to alkylation reactions of melphalan with P-glycoprotein. We determined whether preincubation of cells with the parent drug, thus allowing the possibility of alkylation of sites on P-glycoprotein, could affect photoaffinity labelling. [^{3}H]Azidopine labelling in cells that had been pretreated with melphalan was not different from that in untreated controls (Fig. 3). When hydrolysed, melphalan is inactive as an alkylating agent [46]. We found that the parent compound and the hydrolysed form of melphalan were both able to inhibit photoaffinity labelling of P-glycoprotein (Fig. 4). We also investigated whether another alkylating anticancer agent, BCNU, which is not a known substrate for P-glycoprotein, could affect [^{3}H]azidopine labelling. BCNU did not inhibit photoaffinity labelling (Fig. 4), and although data are shown for only one drug concentration (1000 μM), this was verified over a wide concentration range (0.5, 5, 50, and 500 μM; data not shown). These findings demonstrate clearly that melphalan interacts directly with P-glycoprotein and shares the same binding site(s) as cyclosporin A. Alkylating reactions do not appear to be involved.

Given that melphalan transport may depend on the phenylalanine component of the drug, we determined whether this amino acid could affect [^{3}H]azidopine labelling. However, phenylalanine (1000 μM) did not affect photolabelling of P-glycoprotein (Fig. 4). Although data are shown for only one phenylalanine concentration, this was also the case for concentrations of 0.5, 5, 50, and 500 μM (data not shown).

We determined whether resistance to melphalan could be reversed by the chemosensitiser cyclosporin A and by hyperthermia. We investigated the cytotoxic responses of CHO cells to melphalan, either with hyperthermia alone, or with cyclosporin A alone, or with these two modalities used together in combination. Concentration–response curves for cytotoxicity of melphalan compare the responses between the drug-sensitive AuxB1 cells (Fig. 5A) and the MDR CH^{R}C5 cells (Fig. 5B). In both cell lines, hyperthermia (42°) alone increased the cytotoxicity of melphalan, relative to 37°. Cyclosporin A alone had no effect on melphalan cytotoxicity at either 37° or 42° in drug-sensitive cells (Fig. 5A). In contrast, cyclosporin A alone caused reversal of resistance to melphalan in MDR cells at 37° (Fig. 5B). When hyperthermia was combined with cyclosporin A, there was a large increase in melphalan cytotoxicity in MDR cells, which exceeded the level of enhancement obtained when either heat or chemosensitiser was used separately.

Sensitisation of CH^{R}C5 cells to melphalan cytotoxicity by heat or by the chemosensitiser, used separately or in combination, was determined as a function of time (Fig. 6). For each of these conditions, cytotoxicity of melphalan increased gradually with time up to 60 min. Relative to melphalan cytotoxicity alone at 37°, the order of magnitude for the increase in cytotoxicity in MDR cells was as follows: heat and cyclosporin A combined > heat alone > chemosensitiser alone. Co-incubation of MDR cells with melphalan and cyclosporin A for 30 min at 42° caused a large reduction in cell survival to about 0.03%, whereas incubation with melphalan alone at 37° reduced cell survival to about 30%. Heat and cyclosporin A exerted their effects very rapidly, as differences in cytotoxic response were detected after only 10 min.

The temperature dependence for cyclosporin A-induced sensitisation of CH^{R}C5 cells to melphalan cytotoxicity was determined (Table 2). Cyclosporin A alone did not cause cytotoxicity in cells at any of the temperatures tested (data

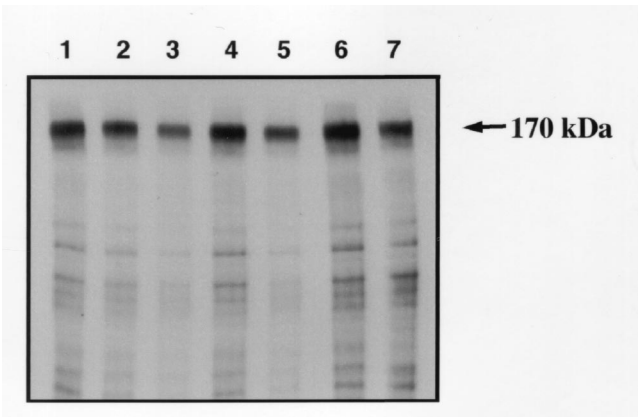

FIG. 4. SDS–PAGE fluorography of CH^{R}C5 cells after photoaffinity labelling with [^{3}H]azidopine: effect of melphalan and alkylation reactions. Cells were photolabelled with 0.5 μM [^{3}H]azidopine (control, lane 1). Cells were photolabelled with azidopine in the presence of 500 μM melphalan (lane 2), 1000 μM melphalan (lane 3), 500 μM hydrolysed melphalan (3 hr at 50°) (lane 4), 1000 μM hydrolysed melphalan (lane 5), 1000 μM phenylalanine (lane 6), or 1000 μM BCNU (lane 7). Each lane displays the migration of 20 μg of protein. Results are shown from one gel, which was reproduced on three occasions.

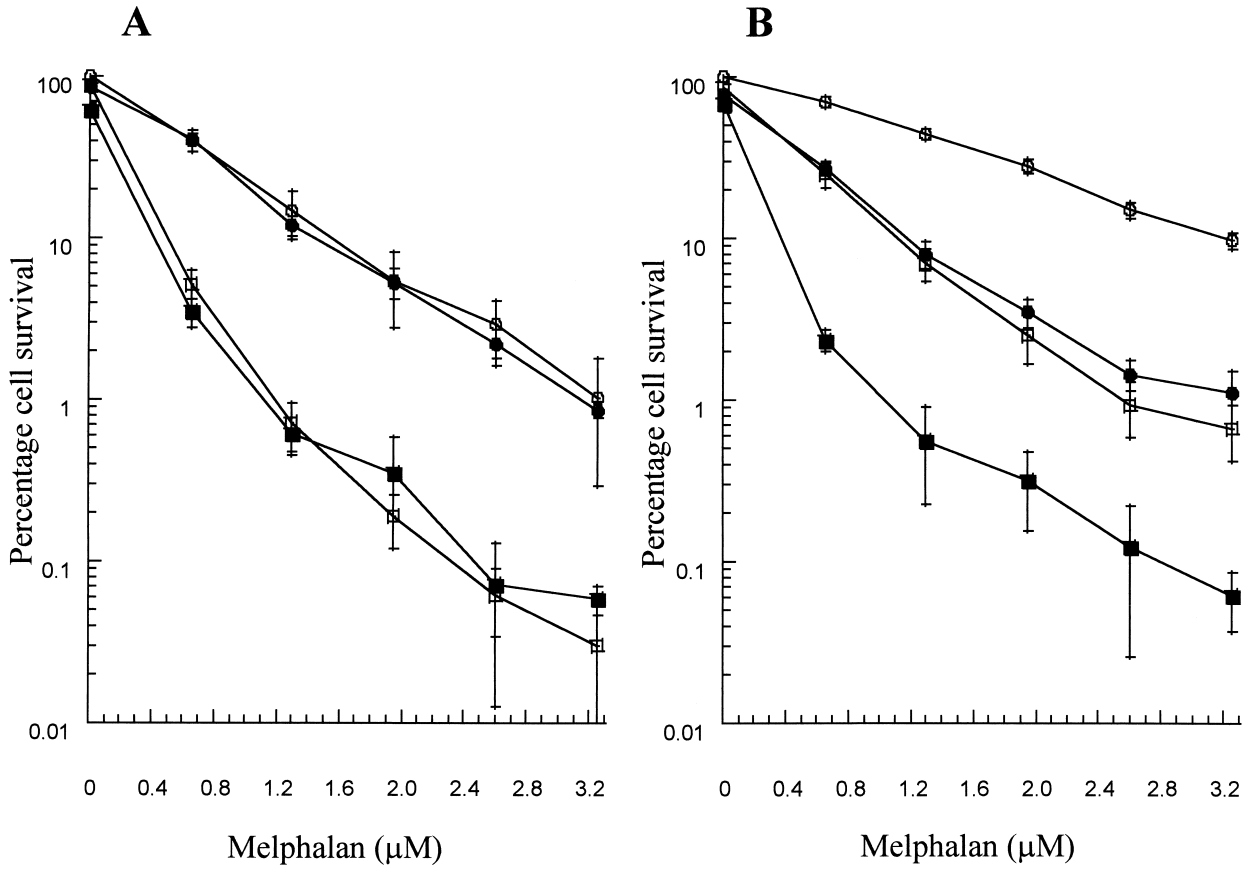

FIG. 5. Concentration dependence of melphalan cytotoxicity in AuxB1 cells (panel A) and CH^{R}C5 cells (panel B). Cells (10^5/mL) were incubated with melphalan during 20 min in PBS–1% BSA–10 mM glucose, at 37° with (●) or without (○) 5 μM cyclosporin A, or at 42° with (■) or without (□) 5 μM cyclosporin A. Controls were incubated without melphalan. Cyclosporin A (5 μM) did not cause cytotoxicity to cells during 60 min (data not shown). Means ± SEM are shown from six independent experiments.

not shown). Heat alone was nontoxic to cells up to 41°, but higher temperatures caused some cytotoxicity. Experimental data values show that melphalan became increasingly cytotoxic as the temperature was increased from 37° to 43°, both with and without the chemosensitiser. Melphalan was more cytotoxic at all temperatures when cyclosporin A was present.

The experimental data values were analysed to determine whether the interactions between the two cytotoxic agents, heat and melphalan, were a potentiation or additive effect (Table 2). Theoretical values represent the added effect of the two cytotoxic agents when used individually. A potentiation effect occurred at temperatures from 41° to 43°, since experimental data values for heat and melphalan in combination were much lower than theoretical values (additive effect). The evaluation was carried out for two conditions, either with or without cyclosporin A. A potentiation effect occurred between heat and melphalan in both conditions.

We determined whether enhancement of melphalan cytotoxicity by heat or by the chemosensitiser, used separately or in combination, could be explained by changes in drug transport processes in MDR cells. Melphalan transport was studied at the levels of intracellular drug accumulation (Fig. 7) and drug efflux from cells (Fig. 8). In CH^{R}C5 cells, melphalan uptake was lower than in AuxB1 cells, at 37° (Fig. 7A). However, addition of cyclosporin A re-established melphalan accumulation in CH^{R}C5 cells to a level similar to that observed in AuxB1 cells. Cyclosporin A alone did not affect drug uptake in AuxB1 cells (Fig. 7A). Hyperthermia (42°) alone caused greater melphalan accumulation in both cell lines, relative to 37° (Fig. 7B). When heat was combined with cyclosporin A, the level of drug uptake in CH^{R}C5 cells was similar to that obtained with the chemosensitiser alone at 37°.

Studies of melphalan efflux were then carried out in CHO cells at 37° (Fig. 8A) or 42° (Fig. 8B), with or without cyclosporin A. Cells were preloaded with melphalan prior to efflux experiments, which were subsequently performed without drug in the extracellular medium. Data values at the time point 0 min represent the higher drug accumulation into AuxB1 cells relative to CH^{R}C5 cells. The data values at other time points show the concentration of melphalan remaining in cells at various times during the efflux process. The rate constants (κ) for melphalan efflux (Table 3) and plateau levels of drug (Equation 1, parameter A) (Table 4) were evaluated from the data in Fig. 8. The intracellular concentration of melphalan de-

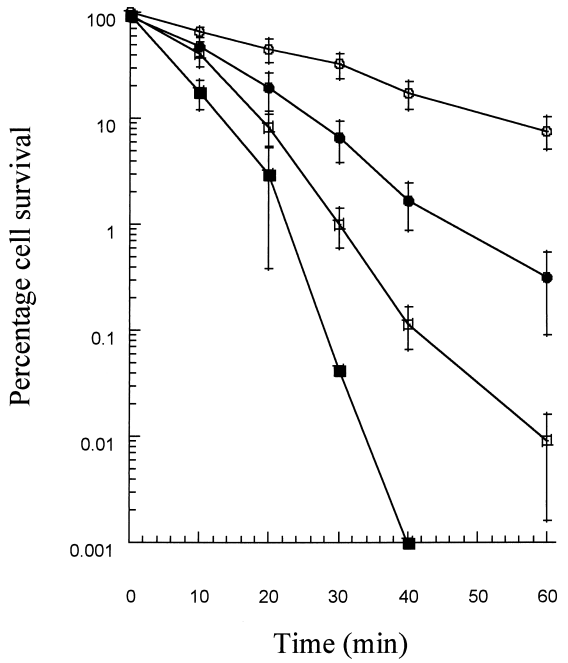

FIG. 6. Time course of melphalan cytotoxicity in CH^{R}C5 cells. Cells (10^5/mL) were incubated with melphalan (0.62 μM) in PBS–1% BSA–10 mM glucose, at 37° with (●) or without (○) 5 μM cyclosporin A, or at 42° with (■) or without (□) cyclosporin A. Means ± SEM are shown from three independent experiments.

creased with time and tended toward a steady-state level, which was attained much faster (2.5-fold increase in rate) in the drug-resistant cells than in the sensitive cells (Fig. 8A, Table 3). Cyclosporin A diminished the rate of melphalan efflux by about 40% in MDR cells at 37° (Fig. 8A, Table 3). The chemosensitiser had no effect in AuxB1 cells. However, even with cyclosporin A, the efflux rate from CH^{R}C5 cells remained higher than that from AuxB1 cells, by a factor of 1.55 (Table 3). The efflux rate was higher at 42° than at 37°, in both cell lines (Fig. 8B, Table 3). Cyclosporin A also slowed melphalan efflux from CH^{R}C5 cells at 42°, but not from AuxB1 cells.

The effect of cyclosporin A and heat on the plateau levels of drug remaining in cells after the efflux process (Equation 1, parameter A) also was determined (Table 4). In AuxB1 cells, a plateau level of melphalan is achieved after about 100 min of efflux at 37° [37]. At 37° and 42°, almost 30% of the initial quantity of drug remained inside the cells, both with or without the chemosensitiser (Table 4). In CH^{R}C5 cells, a similar plateau level of about 30% of initial melphalan was reached after about 30–40 min at 37° (Table 4, Fig. 8A). Cyclosporin A alone altered the plateau level of drug, which increased from 30 to 50%, in MDR cells. At 42°, the plateau level was higher than at 37° and increased again when cyclosporin A was present (Table 4, Fig. 8B). The plateau values achieved after efflux in CH^{R}C5 cells also were compared with those obtained in drug-sensitive cells. Despite the presence of cyclosporin A, the plateau level of melphalan in CH^{R}C5 cells after efflux (in terms of picomoles) was still lower than that in AuxB1 cells (Table 4, Fig. 8A).

DISCUSSION

Drug resistance in the well-characterised CH^{R}C5 cell line has been associated with enhanced efflux of structurally unrelated drugs by an energy-dependent process [3, 39]. The mechanism of resistance to melphalan in CH^{R}C5 cells has been attributed mainly to enhanced drug efflux, although the precise mechanism involved is unclear [24, 46, 47]. Melphalan is not usually considered to be a substrate for P-glycoprotein, although this is also unclear. We previously showed that verapamil, a calcium channel blocker that binds to P-glycoprotein, is able to restore partially the sensitivity to melphalan in CH^{R}C5 cells [48]. The findings from the present study strongly support a mechanism involving melphalan efflux mediated by P-glycoprotein. We showed that melphalan binds to P-glycoprotein and inhibits photoaffinity labelling of P-glycoprotein with azidopine. We ruled out the possibility that alkylation reactions of melphalan could be involved in this interaction

TABLE 2. Effect of heat on the cytotoxicity of melphalan in drug-resistant CHO cells

	Percentage cell survival				
	Heat only	Melphalan		Melphalan, with cyclosporin A	
Temperature (°)	Experimental*† (E_A)	Theoretical‡ ($E_A \times E_{B1}$)	Experimental*	Theoretical‡ ($E_A \times E_{B2}$)	Experimental*
37	100.0 ± 0	52.3	52.3 ± 4.8§	16.83	16.83 ± 4.92‖
40	90.9 ± 0.91	47.54	41.1 ± 8.25	15.30	10.64 ± 7.75
41	72.30 ± 2.93	37.81	8.47 ± 3.03	12.17	0.62 ± 0.16
43	43.1 ± 5.75	22.54	0.79 ± 0.17	7.25	0.043 ± 0.034

*Experimental data values in CH^{R}C5 cells for the effects of the two cytotoxic agents A (heat) and B (melphalan, 0.62 μM), used either separately or together. Cyclosporin A (5 μM) alone did not cause cytotoxicity at any of the temperatures tested (data not shown). Values (mean ± SEM) are from three independent experiments.

†Experimental values of E_A which represent the effects of a 20-min heat exposure alone.

‡Expected levels of percent cell survival calculated from the independent effects of a 20-min heat exposure (E_A) and a 20-min drug exposure at 37°, E_{B1} or E_{B2}, without or with cyclosporin A, respectively.

§Experimental value of E_{B1} which represents the effect of a 20-min drug exposure alone at 37°.

‖Experimental value of E_{B2} which represents the effect of a 20-min drug exposure at 37°, with 5μM cyclosporin A.

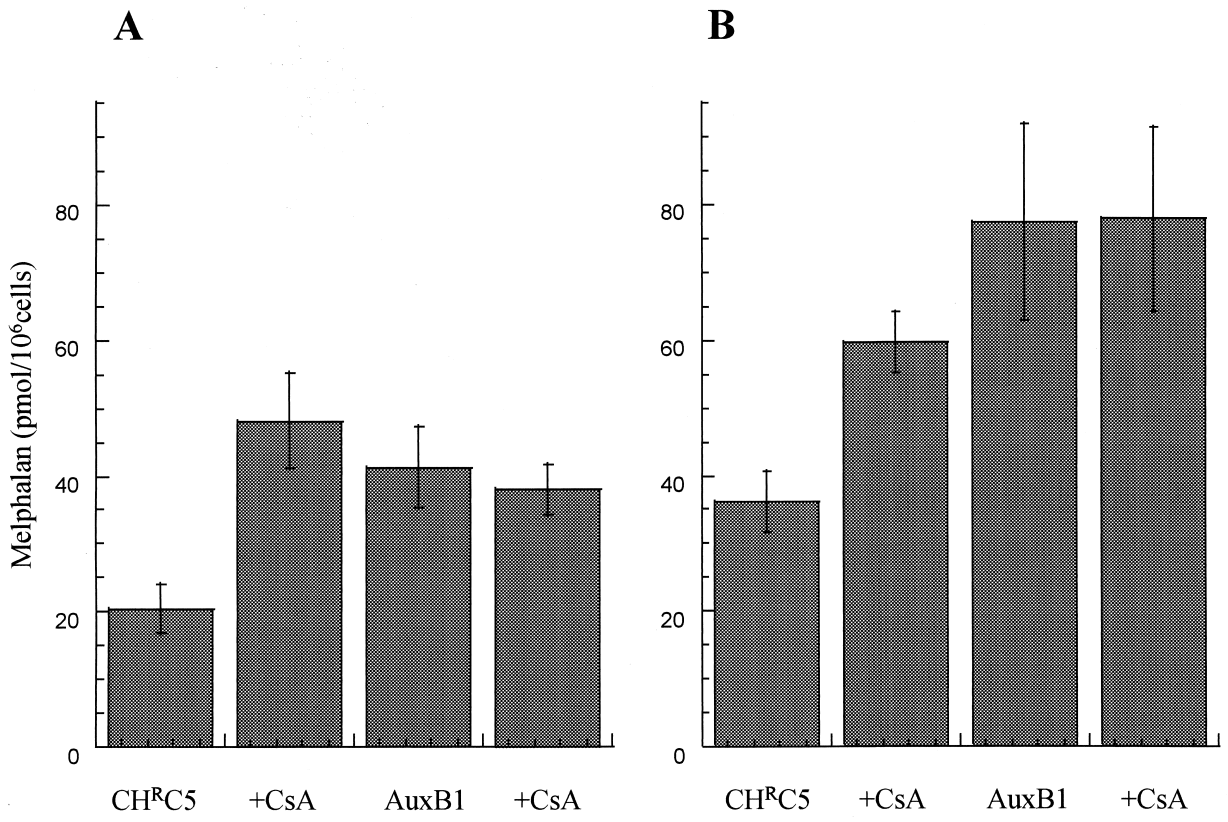

FIG. 7. Effect of cyclosporin A on melphalan uptake in AuxB1 and CH^{R}C5 cells at 37° (panel A) and 42° (panel B). Cells (10^6) were incubated with [^{14}C]melphalan (3.25 μM) for 15 min in PBS–1% BSA–10 mM glucose at 37° or at 42°, either with or without 5 μM cyclosporin A. Means ± SEM are shown from seven independent experiments.

with P-glycoprotein. Furthermore, cyclosporin A, which is also able to reverse MDR associated with P-glycoprotein, sensitised CH^{R}C5 cells to melphalan and decreased drug efflux from cells. However, our findings do not rule out the possible existence of other mechanisms of resistance, in addition to P-glycoprotein.

The implication of P-glycoprotein in resistance to melphalan in CH^{R}C5 cells was confirmed by photolabelling of P-glycoprotein using the arylazide 1,4-dihydropyridine calcium antagonist [^{3}H]azidopine. Azidopine is known to specifically photolabel the 170-kDa glycoprotein [49]. It was shown that azidopine photoaffinity labelling of P-glycoprotein can be inhibited by many agents including vinblastine, actinomycin D, Adriamycin, and MDR modulators such as cyclosporin A. We confirmed here that cyclosporin A, at low concentrations, also inhibits azidopine photoaffinity labelling of P-glycoprotein in CH^{R}C5 cells. A 200-fold molar excess of melphalan reduced the photolabelling of P-glycoprotein by about 40%. This interaction strongly suggests that melphalan is a substrate for drug efflux by P-glycoprotein and that this may be the reason for resistance to melphalan in CH^{R}C5 cells. However, high concentrations of melphalan are required for inhibition of photolabelling, thus indicating that the drug binds to P-glycoprotein with a low affinity. This could explain why cell lines that do not overexpress P-glycoprotein at sufficient levels may not show detectable resistance to melphalan. Therefore, only cell lines that express P-glycoprotein at high levels, such as is the case for CH^{R}C5 cells, display resistance to melphalan.

Resistance to alkylating agents such as melphalan is often associated with alterations in levels of glutathione [50, 51] and related enzymes such as GST [52–56], by mechanisms that do not involve P-glycoprotein. We found no differences in glutathione levels or in GST activity (data not shown) between CH^{R}C5 cells and AuxB1 cells, which could explain this resistance. Several other mechanisms have been reported for MDR, where overexpression of P-glycoprotein is not involved. These mechanisms include overexpression of multiple drug resistance-associated protein [57] and changes in the nuclear enzyme DNA topoisomerase II [58, 59]. Furthermore, an ATP-dependent GSH-xenobiotic (GSH-X) efflux pump, with wide tissue distribution, has been described [60]. This transporter is also sensitive to verapamil. The synthesis of a family of proteins known as HSP is induced by stresses such as heat shock and by several cytotoxic agents [61]. A small 27-kDa protein, HSP27, afforded cellular protection against several anticancer agents, suggesting yet another form of chemoresistance [62]. It is possible that such mechanisms could be involved in melphalan resistance in CH^{R}C5 cells, but this is not presently known.

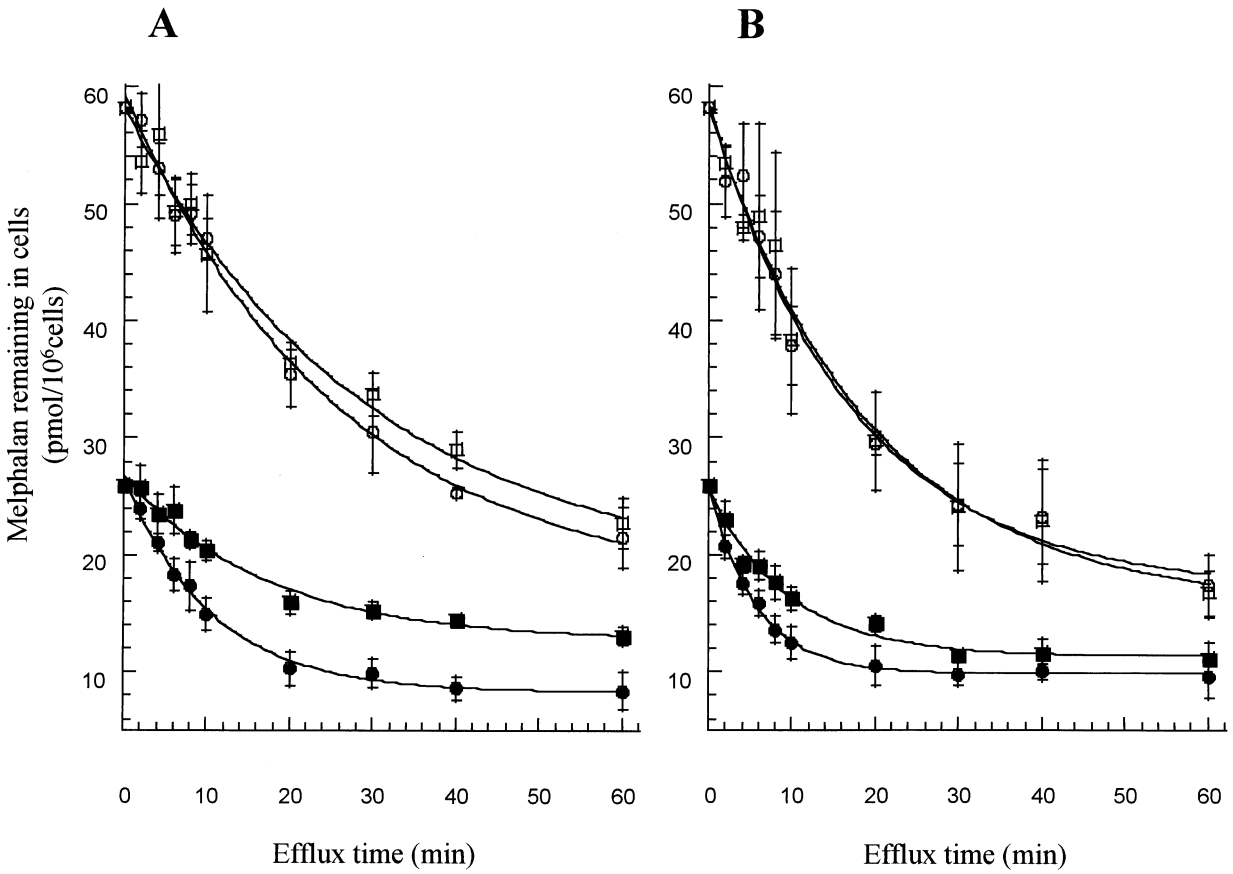

FIG. 8. Time course for melphalan efflux in AuxB1 and CH^{R}C5 cells at 37° (panel A) and 42° (panel B). Cells (10^7/mL) were preloaded with [^{14}C]melphalan (3.25 μM) during 20 min in PBS–1% BSA–10 mM glucose. Efflux measurements were carried out using aliquots of 0.1 mL (10^6 cells) of AuxB1 cells with (□) or without (○) 5 μM cyclosporin A, or in CH^{R}C5 cells with (■) or without (●) cyclosporin A. Efflux experiments were performed in the absence of extracellular melphalan in the incubation medium. Means ± SEM are shown from four independent experiments.

We investigated whether cyclosporin A, which is known to bind to P-glycoprotein, and hyperthermia could reverse melphalan resistance in pleiotropic multidrug-resistant CH^{R}C5 cells. Cyclosporin A has been shown to sensitize many MDR cell lines [10–12], including CH^{R}C5 cells [8, 13], to the effects of a wide variety of antineoplastic agents, including anthracyclines and *Vinca* alkaloids. Our results show clearly that cyclosporin A can potentiate melphalan cytotoxicity in CH^{R}C5 cells, but not in AuxB1 cells. The increase in intracellular melphalan accumulation in the MDR cell line caused by cyclosporin A most likely accounts for the increase in cytotoxicity. Melphalan uptake occurs via an active carrier-mediated process involving two distinct amino acid transport systems [46, 63]. It was reported that efflux occurs by a different mechanism than that for influx, that is, by passive diffusion through the membrane [37, 64]. However, for the CH^{R}C5 cell line, melphalan efflux occurs more rapidly than for AuxB1 cells, due to a

TABLE 3. Rate constants (κ) for efflux of melphalan from drug-sensitive and drug-resistant CHO cells in the presence of cyclosporin A

Temperature (°)	Cyclosporin A (μM)	AuxB1 cells		CH^{R}C5 cells		
		κ (min^{-1})*	κ (relative)†	κ (min^{-1})*	κ (relative)†	κ (relative)‡
37	0	0.0384 ± 0.0046	1.00	0.0954 ± 0.0070	2.48	1.00
37	5	0.0342 ± 0.0077	0.89	0.0595 ± 0.0099	1.55	0.62
42	0	0.0572 ± 0.0082	1.49	0.1768 ± 0.0072	4.61	1.86
42	5	0.0522 ± 0.0086	1.36	0.1057 ± 0.0130	2.76	1.11

*Values for κ and corresponding error bars (SD) were determined as described in Materials and Methods from fitted curves (Eq. 1) for the data in Fig. 8, from four independent experiments.

†Values of κ are relative to that obtained at 37° in AuxB1 cells (without cyclosporin A), designated as 1.

‡Values of κ are relative to that obtained at 37° in CH^{R}C5 cells (without cyclosporin A), designated as 1.

TABLE 4. Steady-state values (parameter A) for efflux of melphalan from drug-sensitive and drug-resistant CHO cells in the presence of cyclosporin A

Temperature (°)	Cyclosporin A (μM)	AuxB1 cells		CH^RC5 cells	
		A* (pmol melphalan)	A (%)†	A* (pmol melphalan)	A (%)‡
37	0	16.851 ± 2.181	28.9	8.288 ± 0.384	31.9
37	5	18.135 ± 4.120	31.1	12.761 ± 0.788	49.1
42	0	17.060 ± 2.036	29.3	9.851 ± 0.164	37.9
42	5	15.705 ± 2.533	27.0	11.335 ± 0.484	43.6

*Values for parameter A and corresponding error bars (SD) were determined as described in Materials and Methods from fitted curves (Eq. 1) for the data in Fig. 8 from four independent experiments.

†Values of parameter A are relative to the initial concentration of melphalan at 37° in AuxB1 cells (58.2 pmol/10^6 cells) prior to efflux.

‡Values of parameter A are relative to the initial concentration of melphalan at 37° in CH^RC5 cells (26.0 pmol/10^6 cells) prior to efflux.

plasma membrane alteration [39]. It would be expected that melphalan efflux from CH^RC5 cells should occur by two mechanisms, passive diffusion and active transport mediated by P-glycoprotein. Cyclosporin A increased intracellular melphalan accumulation by slowing drug efflux, but only from CH^RC5 cells, which have overexpression of P-glycoprotein. Our findings suggest that cyclosporin A binds to P-glycoprotein, thus preventing binding of melphalan to the efflux pump. Since two agents, verapamil and cyclosporin A, which are able to reverse the MDR phenotype involving P-glycoprotein, increase melphalan cytotoxicity and reduce efflux, this strengthens further the implication of P-glycoprotein in the mechanism of resistance to melphalan in these cells.

Heat causes changes to many cellular constituents including cytoskeletal elements, chromosomal structures, and the plasma membrane [65]. For example, heat can inhibit protein synthesis and energy metabolism [26], induce the synthesis of HSPs [61, 62, 66, 67], cause damage to DNA [68], and cause extensive denaturation of cellular proteins [69]. The critical target involved in cell death, however, remains unidentified.

Several studies in human and rodent cells showed that hyperthermia can partially overcome drug resistance [see review in Ref. 70]. For example, heat sensitises cells to killing by mitomycin C [71], *cis*-diamminedichloroplatinum(II) [72–74], and methotrexate [75], in cells selected for primary resistance to each drug. We reported previously that MDR CH^RC5 cells with overexpression of P-glycoprotein are equally sensitive to heat-induced killing as their drug-sensitive parent cells [41]. Moreover, heat sensitises these MDR cells to melphalan [24]. We have observed here a potentiation effect between hyperthermia and melphalan in MDR cells when cyclosporin A was present. Melphalan cytotoxicity was enhanced by either heat or the chemosensitiser, when used separately, but to a much lesser extent than the combined modalities. Potentiation of melphalan cytotoxicity was achieved at mild temperatures such as 41°, which can be attained easily in the body when considering clinical applications. Enhancement of melphalan cytotoxicity by heat and the chemosensitiser can be explained by several factors. A major reason is likely to be the increased intracellular drug accumulation arising from inhibition of melphalan efflux by cyclosporin A. In addition, melphalan uptake was increased by 42° hyperthermia, probably due to altered membrane permeability. The rates of reactions generally increase with temperature. The increased quantity of drug inside cells would therefore become more reactive at elevated temperatures. For a given dose of melphalan, this would lead to increased damage to critical cellular targets, such as DNA, ultimately leading to cell death.

In conclusion, photoaffinity labelling has demonstrated the role of P-glycoprotein in the mechanism of resistance to melphalan in the MDR cell line CH^RC5. Moreover, cyclosporin A reverses melphalan resistance in these cells by slowing drug efflux and thus increasing intracellular drug accumulation. Hyperthermia alone could prove to be very useful in eliminating both drug-sensitive and MDR cells. However, the effectiveness of hyperthermia and chemotherapeutic drugs such as melphalan against MDR cells has even better potential when combined with chemosensitisers such as cyclosporin A or verapamil. The cytotoxic action of anticancer drugs could be enhanced significantly in a localised target region, with the major advantage of diminishing drug dose and, consequently, undesirable side-effects to normal tissues.

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