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ORIGINAL ARTICLE

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Modulation of Adriamycin cytotoxicity and transport in drug-sensitive and multidrug-resistant Chinese hamster ovary cells by hyperthermia and cyclosporin A

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Abstract *Purpose*: Chemosensitizers such as cyclosporin A can increase intracellular accumulation of chemotherapeutic agents such as Adriamycin in certain multidrug-resistant (MDR) cell lines with overexpression of P-glycoprotein. It is likely that, when combined with cyclosporin A, hyperthermia could increase membrane permeability to Adriamycin and enhance its cytotoxic effects. The ability of both hyperthermia and cyclosporin A to modulate the cytotoxicity, transport and subcellular distribution pattern of Adriamycin was studied in a pleiotropic MDR Chinese hamster ovary cell line (CH^RC5) and in the drug-sensitive parent line (AuxB1). *Methods*: Adriamycin cytotoxicity was evaluated by clonogenic cell survival, drug transport using [^{14}C]-labeled Adriamycin and intracellular drug distribution by fluorescence microscopy. *Results*: Adriamycin cytotoxicity was increased in both drug-sensitive and MDR cells by cyclosporin A (5 μM) alone, and by hyperthermia alone (41–43 °C) only in sensitive cells. However, when cyclosporin A and 42 °C hyperthermia were used in combination, a large increase in drug cytotoxicity occurred in both cell lines. This effect increased with time and was temperature-dependent. The increase in Adriamycin cytotoxicity caused by cyclosporin A and hyperthermia was accompanied by alterations in membrane permeability to the drug. Cyclosporin A increased [^{14}C]Adriamycin uptake, while drug efflux decreased, for both AuxB1 and CH^RC5 cells and nuclei. For AuxB1 cells only, drug distribution studies showed that cyclosporin A promoted an increase in both nuclear and cytoplasmic drug accumulation. Hyperthermia, combined with cyclosporin A, increased [^{14}C]Adriamycin uptake. This effect was seen as an increase in intensity of nuclear and cytosolic drug fluorescence in both cell lines. Cyclosporin A alone diminished drug efflux and caused Adriamycin to remain firmly bound in the nucleus of AuxB1 cells, while it remained primarily in the cytoplasm of CH^RC5 cells. *Conclusions*: Hyperthermia alone had little effect on Adriamycin cytotoxicity and transport in MDR cells, in contrast to drug-sensitive cells. This suggests that P-glycoprotein is fully functional in these MDR cells. Our findings suggest that cyclosporin A and hyperthermia could be beneficial by increasing intracellular drug accumulation, thus improving the effectiveness of Adriamycin against both drug-sensitive and MDR cells within a localized target region.

Keywords Adriamycin · Cyclosporin A · Hyperthermia · Drug efflux · Multidrug resistance

Abbreviations *BSA* bovine serum albumin · *CHO* Chinese hamster ovary · *FBS* fetal bovine serum · *MDR* multidrug resistance · *MEM* minimum essential medium · *PBS* phosphate-buffered saline · *SEM* standard error of mean

Introduction

The multidrug resistance (MDR) phenotype confers cross-resistance to a wide variety of structurally and functionally unrelated cytotoxic agents in many cancer cell lines [17, 31]. P-glycoprotein plays an important role in MDR. This 170-kDa membrane protein acts as an energy-dependent efflux pump and is responsible for the decreased steady-state intracellular drug levels in MDR cells [18, 32]. Current evidence indicates that P-glycoprotein acts as a multidrug transporter by binding to many different anticancer drugs, thus exporting them to the exterior of the cell [26]. Hydrolysis of ATP by the ATPase activity of P-glycoprotein provides the energy that drives this reaction [17, 31].

In vitro, several compounds have been shown to reverse the MDR phenotype associated with overex-

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pression of P-glycoprotein. Among the most effective MDR-reversing agents are the calcium channel blocker verapamil [28, 40, 49], the immunosuppressive agent cyclosporin A [7, 19, 43, 52] and the indole alkaloid quinine [5]. These compounds appear to reverse the MDR phenotype by directly binding to P-glycoprotein, thus inhibiting its ability to bind and expel anticancer drugs from cells [45]. However, in vivo, the efficacy of these chemosensitizers remains to be clarified [46, 51, 55, 58].

Adriamycin, a potent antineoplastic agent, is effective against leukemias and solid tumors [12]. It binds strongly to DNA causing DNA damage [15], and also causes alterations to cellular membranes [48]. Thus, both interactions with DNA and cellular membranes have been proposed as a basis for cytotoxicity [6, 15, 44, 48]. Adriamycin undergoes redox cycling leading to formation of reactive oxygen species such as superoxide, hydrogen peroxide and hydroxyl radicals [15]. These reactive species could account for damage to DNA and/or cellular membranes and have been implicated in the mechanism of Adriamycin cytotoxicity [14, 36, 41, 42]. However, the development of drug resistance and cardiotoxic side effects are two major obstacles to the clinical efficacy of Adriamycin. Resistance to Adriamycin has been associated with the MDR phenotype involving overexpression of P-glycoprotein in several different cellular systems [2, 9, 13].

The combination of regional hyperthermia with radiotherapy or systemic chemotherapy has potential value in cancer treatment since localized heating may enhance cytotoxic activity within a well-defined target region [11, 53]. Hyperthermia increases the cytotoxicity of a wide variety of chemotherapeutic agents, including Adriamycin, melphalan, BCNU, bleomycin and cisplatin, both in vitro and in vivo [2–4, 10, 11, 20, 23, 25, 30, 34, 37]. Therefore, the combined use of hyperthermia with cytotoxic drugs has potential for improving the therapeutic ratio by increasing the antitumor effect, relative to normal tissue damage. Furthermore, hyperthermia itself is able to eliminate pleiotropic MDR cells [2]. Hyperthermia increases the cytotoxic effects of several anticancer agents, including cisplatin, methotrexate and mitomycin C, against cells selected for primary resistance to these specific drugs [22, 23, 37, 47, 56, 57].

In this study the possibility was explored that cyclosporin A can increase intracellular accumulation of Adriamycin in pleiotropic MDR cells with overexpression of P-glycoprotein, thus allowing marked sensitization by hyperthermia (39–43 °C) to drug cytotoxicity. The combination of cyclosporin A and hyperthermia could be beneficial by increasing the effectiveness of Adriamycin against drug-resistant cells in a localized target region.

Material and methods

Tissue culture

The drug-resistant cell line CH^RC5 was selected for resistance to colchicine from the drug-sensitive parental CHO cell line, AuxB1 [32]. This cell line also displays resistance to a wide variety of chemotherapeutic agents such as melphalan, Adriamycin and vinca alkaloids. AuxB1 and CH^RC5 cells were grown in monolayer in minimum essential medium Alpha (MEM Alpha; Gibco Canada, Burlington, Ontario) containing 10% fetal bovine serum (FBS; Gibco Canada) and 1% penicillin (50 U/ml)/streptomycin (50 μg/ml) (Gibco Canada), in plastic tissue culture flasks (Starstedt, St Laurent, Québec) in a humidified atmosphere containing 5% CO_2 in a water-jacketed incubator at 37 °C [2]. The culture medium for CH^RC5 cells contained colchicine (5 μg/ml). Cells were cultured without colchicine for one passage prior to experiments. Experiments were carried out using cells grown to confluence and incubated for 24 h at 37 °C in fresh culture medium. Cells were harvested with phosphate-buffered saline (PBS) containing 0.015 *M* sodium citrate, washed by centrifugation (1000 *g*, 3 min) and resuspended in MEM Alpha containing 10% FBS.

Cytotoxicity experiments

Freshly harvested cells (10^6/ml) were incubated with Adriamycin (Sigma Chemical Co., St Louis, Mo.) in a final volume of 1 ml MEM-Alpha containing 10% FBS, either with or without cyclosporin A (5 μ*M*) (Sigma Chemical Co.). Cells were incubated for the appropriate time in tubes in a temperature-controlled circulating waterbath at temperatures from 37 °C to 43 °C. To stop the incubation, the cells were washed twice by centrifugation (1000 *g*, 2 min, 2 °C), resuspended, diluted to the appropriate concentration, plated in culture dishes and incubated at 37 °C for 8 days (AuxB1 cells) or 10 days (CH^RC5 cells). The dishes were washed with PBS, fixed with 95% ethanol and stained with methylene blue before counting macroscopic colonies (> 50 cells). Plating efficiencies in controls were greater than 70% and 50% for AuxB1 and CH^RC5 cells, respectively. Percentage survival was expressed as the mean number of colonies obtained relative to the mean number of colonies obtained in the control. In the control, 200 cells per plate were seeded, but where there was a loss of cell survival, cells were plated at different densities to ensure that countable colonies would be obtained, and the results were corrected accordingly. In this system, there is linearity between the number of cells plated and colonies formed over the range 10 to 10^4 [2].

Adriamycin uptake

Adriamycin labeled with ^{14}C (specific activity 44.9 μCi/mg) was a generous gift from Mr. Maurice Leaffer of SRI International, Menlo Park, Calif. Freshly harvested CHO cells (5×10^6/ml) were incubated with [^{14}C]Adriamycin (1 μg/ml), with or without cyclosporin A (5 μ*M*), in a final volume of 0.2 ml in MEM Alpha containing 10% FBS, in glass tubes in a waterbath. Drug uptake was stopped by the addition of 4 ml ice-cold PBS/1% BSA, followed by three centrifugation (1000 *g*, 1 min, 2 °C) steps with PBS/1% BSA to remove extracellular Adriamycin. Drug content was determined either in dry cell pellets, or in nuclei following their extraction (see below). The radioactivity was determined in the cell pellet using a liquid scintillation analyzer (Packard 2200 CA, Canberra-Packard Canada, Montreal, Québec).

Adriamycin efflux

Freshly harvested cells (10^7/ml) were preloaded with [^{14}C]Adriamycin (5 μg/ml) for 30 min at 37 °C in MEM Alpha containing 10% FBS. The cells were immediately washed three times by centrifugation (1000 *g*, 2 min, 2 °C) to remove extracellular Adriamycin and were then resuspended in Adriamycin-free incubation medium. Drug efflux from cells (2×10^6/ml) was allowed to proceed immediately in a final volume of 0.5 ml MEM Alpha/10% FBS, with or without cyclosporin A (5 μ*M*), at 37 °C or 42 °C. Efflux was stopped by the addition of 4 ml ice-cold PBS/1% BSA, followed by centrifugation (1000 *g*, 1 min). Drug content was

determined either in dry cell pellets (see above) or in nuclei following their extraction (see below).

Isolation of nuclei from cells with accumulation of [^{14}C]Adriamycin

Following [^{14}C]Adriamycin uptake or efflux experiments (see above), the dry cell pellet was resuspended in cold 10 m*M* Tris-HCl (pH 7.5)/1.5 m*M* $MgCl_2$/10 m*M* NaCl buffer containing 1% Igepal Ca-630 (Sigma Chemical Co.). The radioactivity was determined in the cell pellet as described above.

Intracellular Adriamycin distribution

Freshly harvested CHO cell suspensions were plated onto sterile coverslips in petri dishes at a concentration of 10^5 cells per petri dish and incubated overnight at 37 °C in an atmosphere containing 5% CO_2. For drug uptake experiments, cells were incubated with Adriamycin (20 μg/ml) for 1 h at 37 °C or 42 °C, with or without 5 μ*M* cyclosporin A in the incubation medium. Prior to drug efflux experiments, cells were preloaded with Adriamycin (50 μg/ml) in MEM Alpha containing 10% FBS during 30 min. For determination of efflux, cells were washed twice with medium and incubated for 1 h at 37 °C or 42 °C, with or without 5 μ*M* cyclosporin A in drug-free medium. Following uptake and efflux experiments, the coverslips were washed twice with ice-cold PBS, inverted onto slides and viewed for fluorescence under UV illumination using a Zeiss microscope equipped with a mercury lamp. The UV illumination induced an orange fluorescence at sites of Adriamycin accumulation.

Data analysis of interactions between Adriamycin and heat

In combination experiments, the type of interaction between Adriamycin and heat was assessed according to the method of Drewinko et al. [16]. For this evaluation, the two cytotoxic agents, A (heat) and B (Adriamycin) 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 Adriamycin, 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.

Results

The ability of the chemosensitizer cyclosporin A to reverse resistance to Adriamycin was determined in MDR CH^RC5 cells (Fig. 1B), relative to their drug-sensitive

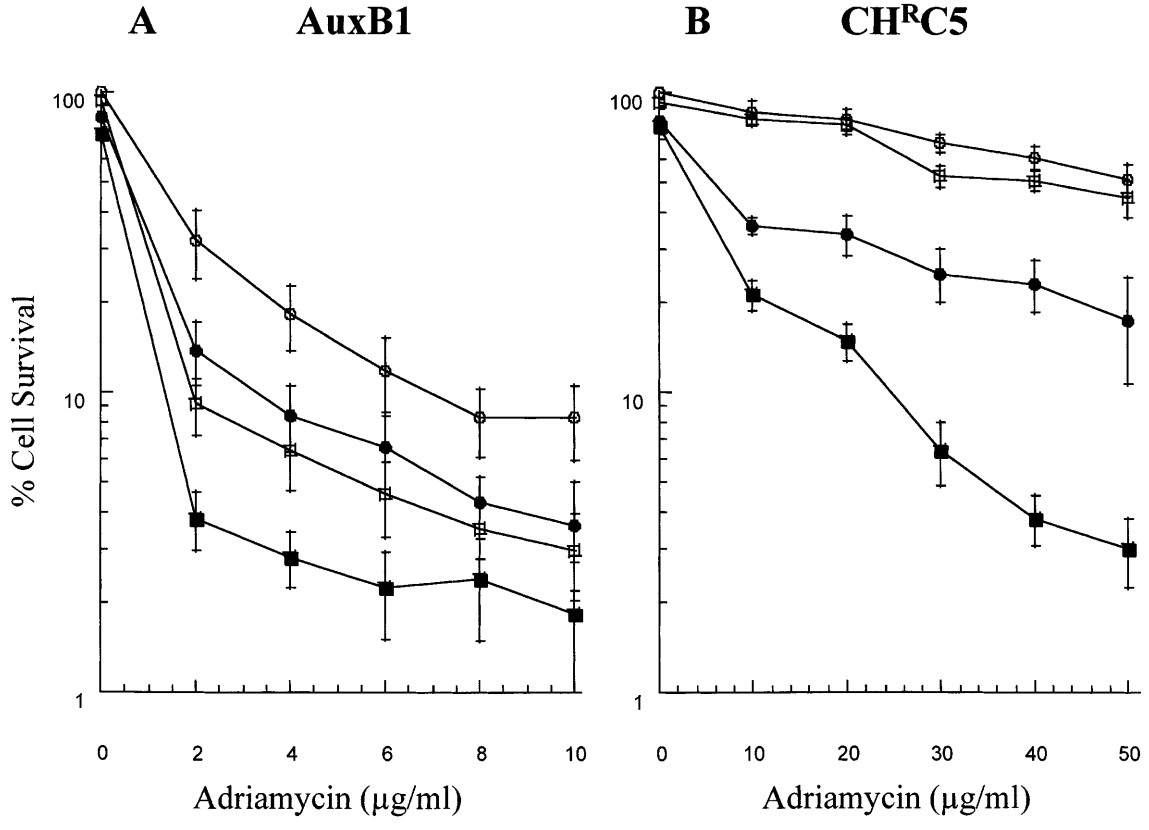

Fig. 1A,B Sensitization to Adriamycin cytotoxicity by cyclosporin A and hyperthermia in AuxB1 cells (**A**) and CH^RC5 cells (**B**). Cells (10^5/ml) were incubated with Adriamycin during 20 min in MEM Alpha containing 10% FBS, at 37 °C with (●) or without (○) 5 μ*M* cyclosporin A, or at 42 °C with (■) or without (□) 5 μ*M* cyclosporin A. Controls were incubated without Adriamycin. Please note different drug concentration scales for **A** and **B**. Means and SEM are shown from three independent experiments with multiple estimations per point

counterparts, AuxB1 cells (Fig. 1A). Dose response curves showed that for AuxB1 cells, the percentage cell survival declined for Adriamycin concentrations from 0 to 10 μg/ml during 20 min at 37 °C (Fig. 1A). In contrast, CH^RC5 cells showed a small response to the drug even when the concentration was as high as 50 μg/ml (Fig. 1B). The resistance factor to Adriamycin in CH^RC5 cells was about 30-fold relative to AuxB1 cells. When cyclosporin A was present, Adriamycin cytotoxicity increased in CH^RC5 cells (Fig. 1B) and also, although to a lesser extent, in AuxB1 cells (Fig. 1A). Cyclosporin A alone did not cause cytotoxicity in either of the cell lines during the 20-min incubation (data not shown).

We investigated whether cyclosporin A and 42 °C hyperthermia, in combination, could increase cytotoxic responses of AuxB1 (Fig. 1A) and CH^RC5 cells (Fig. 1B) to Adriamycin. When the drug was not present, hyperthermia alone (42 °C) caused a very small loss of viability (7%) in both cell lines. Hyperthermia alone increased Adriamycin cytotoxicity relative to 37 °C, in AuxB1 cells (Fig. 1A). Cyclosporin A caused an increase in Adriamycin cytotoxicity in drug-sensitive cells at 42 °C. In CH^RC5 cells, Adriamycin cytotoxicity was slightly higher at 42 °C compared to 37 °C (Fig. 1B). When hyperthermia was combined with cyclosporin A, there was a large increase in Adriamycin cytotoxicity in MDR cells, which largely exceeded the levels of enhancement obtained when either heat or chemosensitizer was used separately. However, cyclosporin A, either alone or combined with 42 °C hyperthermia, only afforded partial reversal of resistance to Adriamycin (compare drug concentration of 10 μg/ml between Fig. 1A and 1B).

Sensitization of AuxB1 cells to Adriamycin cytotoxicity by heat or by cyclosporin A, used separately or in combination, increased steadily as a function of time from 0 to 60 min (data not shown). For MDR cells, the cytotoxicity of Adriamycin increased markedly with time, but only when cyclosporin A was present, at both 37 °C and 42 °C (data not shown).

Cyclosporin A-induced sensitization to Adriamycin cytotoxicity was dependent on temperature, from 37 °C to 43 °C in AuxB1 (Fig. 2A) and CH^RC5 cells (Fig. 2B). In AuxB1 cells, Adriamycin cytotoxicity was enhanced at temperatures from 41 °C to 43 °C (Fig. 2A). This effect increased further when cyclosporin A was present, at all temperatures shown. In MDR cells, there was a tendency for a very small increase in Adriamycin cytotoxicity at temperatures above 41 °C (Fig. 2B). However, when the chemosensitizer was present, cytotoxicity increased markedly at temperatures from 39 °C to

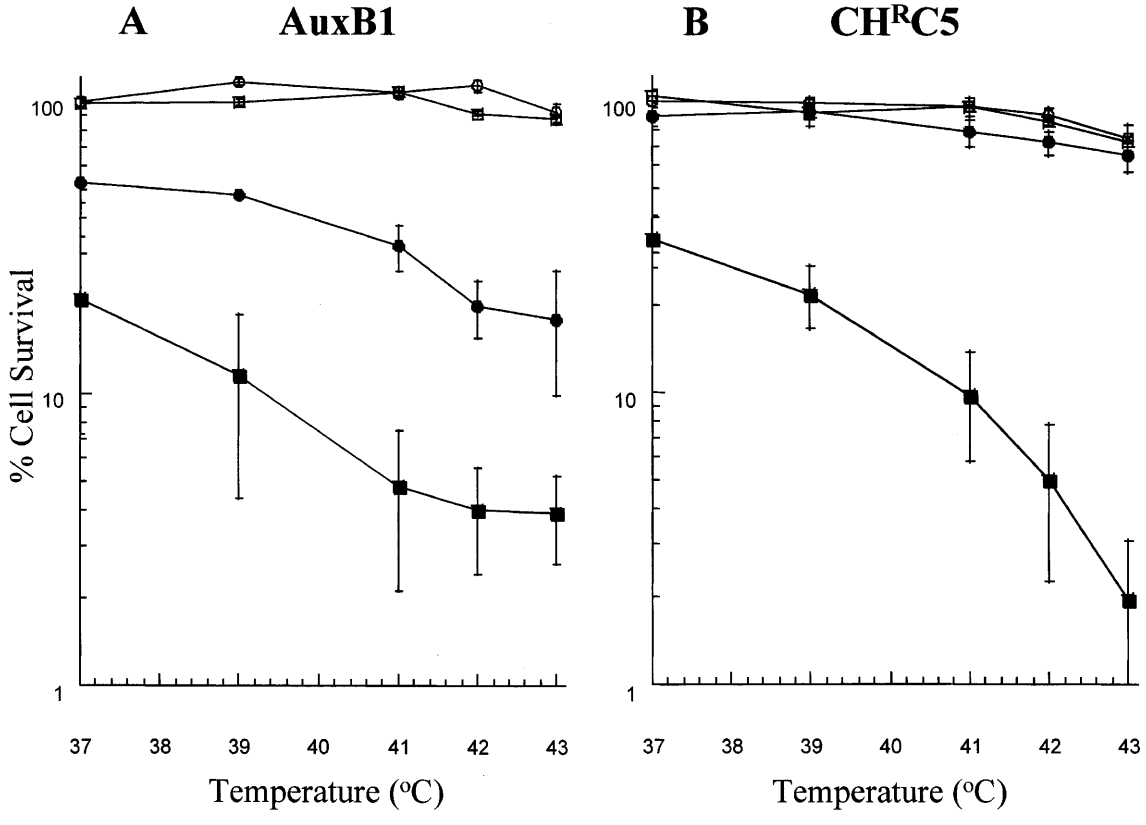

Fig. 2A,B Sensitization to Adriamycin cytotoxicity by cyclosporin A increases with temperature in AuxB1 (**A**) and CH^RC5 cells (**B**). Cells (10^5/ml) were incubated at different temperatures with Adriamycin (1 μg/ml for AuxB1 cells, 30 μg/ml for CH^RC5 cells), either with (■) or without (●) 5 μ*M* cyclosporin A, during 20 min in MEM Alpha containing 10% FBS. Controls without Adriamycin are shown, and they represent the effect of short exposures (20 min) to heat alone (○), or the effect of cyclosporin A alone (□) at different temperatures. Means and SEM are shown from three independent experiments with multiple estimations per point

43 °C. Heat alone or cyclosporin A alone essentially caused no loss of cell survival at the different temperatures, as shown in the controls (no Adriamycin present) in AuxB1 (Fig. 2A) and CH^RC5 cells (Fig. 2B).

We determined whether the interactions between Adriamycin and hyperthermia were of a potentiative or additive nature. This was evaluated in AuxB1 cells (Table 1) and CH^RC5 cells (Table 2), either with or without a noncytotoxic concentration of cyclosporin A. In drug-sensitive cells, a potentiation effect occurred between heat and Adriamycin at temperatures from 39 °C to 43 °C, both with and without cyclosporin A (Table 1). In MDR cells, Adriamycin and hyperthermia exhibited an additive effect at all temperatures (Table 2). However, when cyclosporin A was present, there was a potentiation effect from 39 °C to 43 °C.

We predicted that sensitization to Adriamycin by heat and cyclosporin A could be explained by increased intracellular drug accumulation. Such an increase could arise from alterations in drug uptake and/or efflux. Therefore, we investigated the effect of heat and cyclosporin A, used separately or in combination, on [^{14}C]Adriamycin transport processes (Figs. 3, 4 and 5) and intracellular drug distribution (Figs. 6 and 7).

First, [^{14}C]Adriamycin uptake was studied as a function of time in AuxB1 (Fig. 3A) and CH^RC5 cells (Fig. 3B). In drug-sensitive cells, [^{14}C]Adriamycin uptake increased gradually during 60 min at 37 °C (Fig. 3A). Drug uptake was enhanced by 42 °C hyperthermia alone and by cyclosporin A alone (Fig. 3A). When used in combination, cyclosporin A and hyperthermia caused a twofold increase in [^{14}C]Adriamycin uptake relative to 37 °C in AuxB1 cells. In CH^RC5 cells, [^{14}C]Adriamycin uptake increased slowly and had reached a maximum intracellular level after about 10–20 min (Fig. 3B). The intracellular level of Adriamycin in CH^RC5 cells (~20 pmol/10^6 cells) after 60 min was threefold lower than that in AuxB1 cells (~60 pmol/10^6 cells). Hyperthermia alone did not appear to modulate [^{14}C]Adriamycin uptake in CH^RC5 cells (Fig. 3B). In MDR cells, cyclosporin A caused a threefold increase in drug uptake after 60 min (~70 pmol/10^6 cells), restoring

Table 1 Interactions between heat and Adriamycin in drug-sensitive CHO cells. The experimental values represent the effects in AuxB1 cells of two cytotoxic agents, A (heat) and B (Adriamycin, 1 μg/ml), either separately or together. Cyclosporin A (5 μ*M*) was not cytotoxic at any of the temperatures tested (Fig. 4A). The values are means ± SEM from two independent experiments

Temperature (°C)	Percentage cell survival				
	Heat only	Adriamycin		Adriamycin with cyclosporin A	
	Experimental (E_A)[a]	Theoretical ($E_A \times E_{B1}$)[d]	Experimental	Theoretical ($E_A \times E_{B2}$)[d]	Experimental
37	100.0 ± 0	52.47	52.47 ± 7.47[b]	20.95	20.95 ± 13.65[c]
39	117.45 ± 2.95	61.62	48.23 ± 1.02	24.60	11.50 ± 7.10
41	107.45 ± 1.75	56.38	32.11 ± 5.80	22.51	4.80 ± 2.70
43	91.7 ± 7.00	48.11	18.03 ± 8.19	19.21	3.90 ± 1.30

[a] Experimental values of E_A representing the effects of a 20-min heat exposure alone
[b] Experimental value of E_{B1} representing the effect of a 20-min drug exposure alone at 37 °C
[c] Experimental value of E_{B2} representing the effect of a 20-min drug exposure at 37 °C with 5 μ*M* cyclosporin A
[d] Expected levels of percentage survival calculated from the independent effects of a 20-min heat exposure (E_A) and a 20-min drug exposure at 37 °C (E_{B1} or E_{B2}) without or with cyclosporin A, respectively

Table 2 Interactions between heat and Adriamycin in multidrug-resistant CHO cells. The experimental values represent the effects in CH^RC5 cells of two cytotoxic agents, A (heat) and B (Adriamycin, 30 μg/ml), either separately or together. Cyclosporin A (5 μ*M*) was not cytotoxic at any of the temperatures tested (Fig. 4B). The values are means ± SEM from three independent experiments

Temperature (°C)	Percentage cell survival				
	Heat only	Adriamycin		Adriamycin with cyclosporin A	
	Experimental (E_A)[a]	Theoretical ($E_A \times E_{B1}$)[d]	Experimental	Theoretical ($E_A \times E_{B2}$)[d]	Experimental
37	100.0 ± 0	88.27	88.27 ± 6.71[b]	33.43	33.43 ± 6.73[c]
39	98.97 ± 3.43	87.36	91.68 ± 9.47	33.08	21.76 ± 5.29
41	95.69 ± 1.43	84.46	78.00 ± 8.53	31.99	9.76 ± 3.96
43	74.86 ± 8.04	66.08	65.05 ± 8.25	25.02	1.95 ± 1.16

[a] Experimental values of E_A representing the effects of a 20-min heat exposure alone
[b] Experimental value of E_{B1} representing the effect of a 20-min drug exposure alone at 37 °C
[c] Experimental value of E_{B2} representing the effect of a 20-min drug exposure at 37 °C with 5 μ*M* cyclosporin A
[d] Expected levels of percentage survival calculated from the independent effects of a 20-min heat exposure (E_A) and a 20-min drug exposure at 37 °C (E_{B1} or E_{B2}) without or with cyclosporin A, respectively

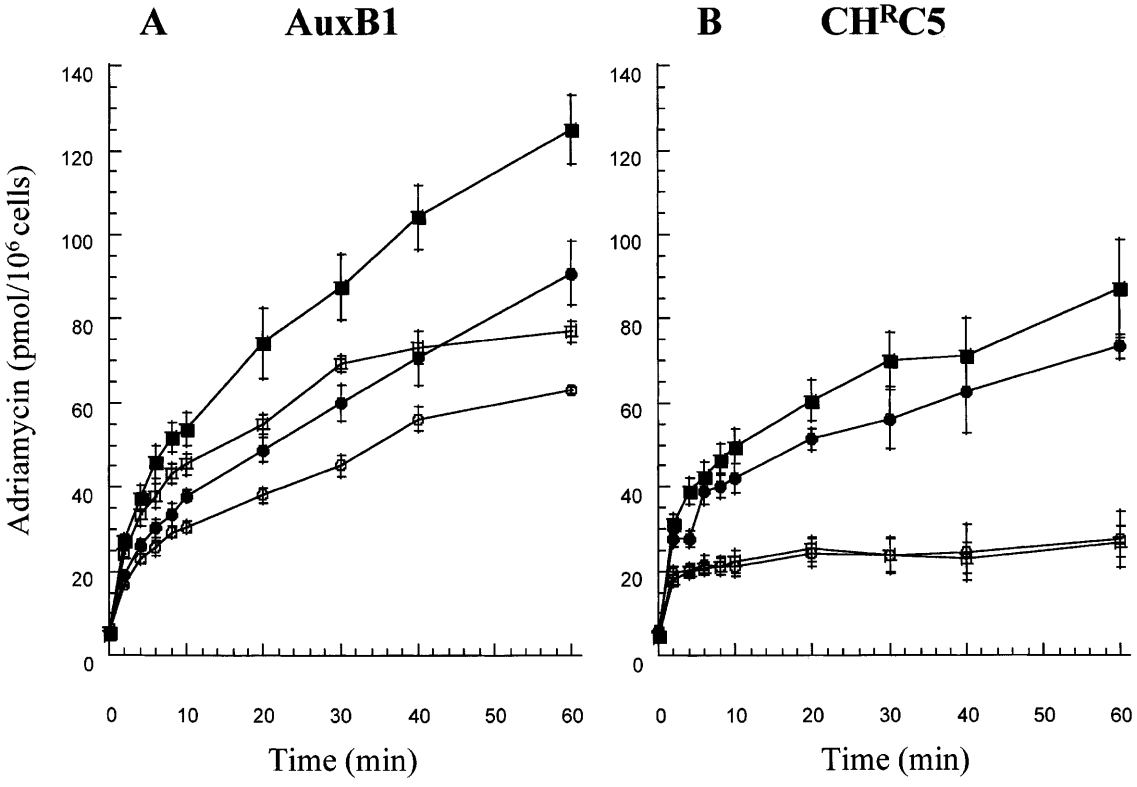

Fig. 3A,B Effect of cyclosporin A and hyperthermia on Adriamycin uptake by AuxB1 cells (**A**) and CH^RC5 cells (**B**). Cells (10^6/ml) were incubated with [^{14}C]Adriamycin (1 μg/ml) in MEM Alpha containing 10% FBS, at 37 °C with (●) or without (○) 5 μ*M* cyclosporin A, or at 42 °C with (■) or without (□) cyclosporin A. Means and SEM are shown from four independent experiments with multiple estimations per point

uptake to the level obtained in drug-sensitive cells. When cyclosporin A was used in combination with 42 °C hyperthermia, [^{14}C]Adriamycin accumulation increased even further in MDR cells (~80 pmol/10^6 cells; Fig. 3B). Heat caused a relatively small increase in drug uptake when cyclosporin A was present, relative to the larger increase caused by cyclosporin A alone at 37 °C.

Second, the ability of cyclosporin A and hyperthermia to modulate efflux of [^{14}C]Adriamycin from AuxB1 cells (Fig. 4A) and MDR cells (Fig. 4B) cells was determined. The intracellular Adriamycin concentration decreased gradually with time in AuxB1 cells, tending towards a plateau level of drug remaining inside the cells (Fig. 4A). However, for CH^RC5 cells, [^{14}C]Adriamycin efflux occurred at a much higher rate (see first 10 min) than for AuxB1 cells, to reach a plateau drug level after only 10–20 min (Fig. 4B). Hyperthermia (42 °C) caused an increase in the rate of [^{14}C]Adriamycin efflux from both cell lines, relative to 37 °C. In contrast, cyclosporin A caused a very pronounced increase in the plateau drug level in AuxB1 cells, at both 37 °C and 42 °C (Fig. 4A). Following efflux for 60 min at 37 °C and 42 °C, respectively, cyclosporin A increased the plateau drug levels from about 35–45% of the initial value, to about 75–80% (Fig. 4A). For MDR cells, cyclosporin A caused a clear decrease in the rate of [^{14}C]Adriamycin efflux (first 10 min; Fig. 4B). With the chemosensitizer, the rate of efflux was slower at 37 °C, relative to 42 °C. Cyclosporin A also affected the steady-state drug level after efflux. In MDR cells, about 40% and 35% of the [^{14}C]-labeled drug remained inside the cells, at 37 °C and 42 °C, respectively (Fig. 4B). When cyclosporin A was present, these steady-state levels increased to 50% and 45%, respectively.

The effect of cyclosporin A on [^{14}C]Adriamycin accumulation in the nuclei of AuxB1 and CH^RC5 cells was determined as a function of time at 37 °C (Fig. 5A). Nuclei were isolated from cells immediately following incubation with [^{14}C]Adriamycin (1 μg/ml). [^{14}C]Adriamycin accumulation increased gradually with time to reach a maximum concentration of 40 pmol/10^6 nuclei isolated from AuxB1 cells after 40 min (Fig. 5A). Cyclosporin A increased [^{14}C]Adriamycin accumulation in AuxB1 nuclei and this continued to increase even after 90 min. In nuclei from CH^RC5 cells, [^{14}C]Adriamycin accumulation was lower than in AuxB1 nuclei, and had reached a maximum concentration after about 10 min (Fig. 5A). However, addition of cyclosporin A increased [^{14}C]-labeled drug accumulation in the nuclei of CH^RC5 cells by a factor of 3.5 (90 min).

We then investigated the effect of cyclosporin A on [^{14}C]Adriamycin efflux from nuclei of AuxB1 and CH^RC5 cells (Fig. 5B). For AuxB1 cells, cyclosporin A caused 85% of [^{14}C]Adriamycin to remain firmly bound inside the nuclei after 90 min, compared to only 35% without the chemosensitizer (Fig. 5B). For CH^RC5 cells, efflux occurred much more rapidly, and after 10 min,

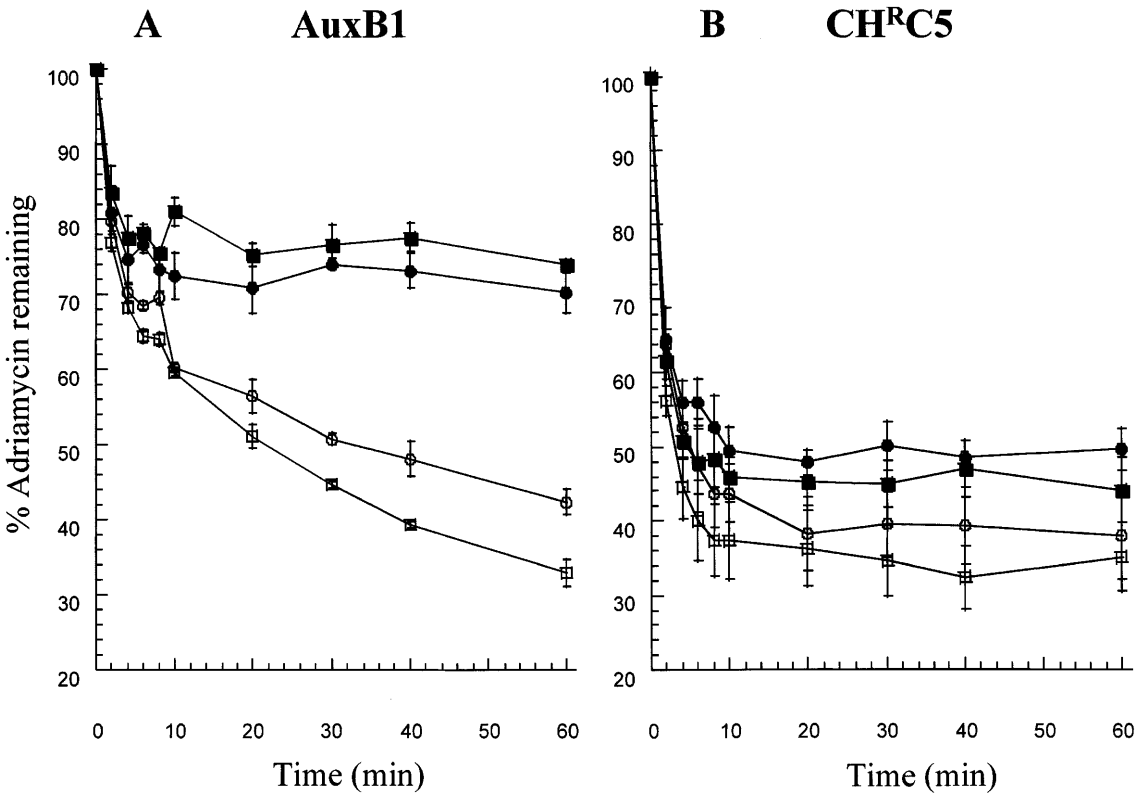

Fig. 4A,B Cyclosporin A decreases Adriamycin efflux from AuxB1 (**A**) and CH^RC5 cells (**B**). Cells (10^7/ml) were preloaded with [^{14}C]Adriamycin (5 μg/ml) during 30 min in MEM Alpha containing 10% FBS and then centrifuged (1000 *g*, 2 min) to remove extracellular drug. Efflux measurements were carried out immediately in a final volume of 0.5 ml (2×10^6 cells/ml), during 60 min under different conditions. Relative to a common starting point for each of the two cell lines, the four different conditions compared were: (1) 37 °C, either with 5 μ*M* cyclosporin A (●) or (2) without cyclosporin A (○), or (3) 42 °C, either with 5 μ*M* cyclosporin A (■) or (4) without cyclosporin A (□). Efflux experiments were performed without extracellular Adriamycin in the incubation medium. The data point at time zero represents the Adriamycin content in the cells prior to efflux (100%). Each time-point represents the residual intracellular Adriamycin concentration at various times during the efflux process and is expressed as a percentage of the zero time-point. Note that the Adriamycin concentration used for preloading cells prior to efflux was five times higher than that used for the uptake studies the results of which are shown in Fig. 3. Means and SEM are shown from three to six independent experiments with multiple estimations per point

a plateau was reached, where about 30% of the [^{14}C]-labeled drug remained bound to the nucleus (Fig. 5B). When cyclosporin A was present, this level increased to 40–45% of [^{14}C]Adriamycin remaining bound to the nucleus.

Finally, the ability of hyperthermia and cyclosporin A to alter intracellular drug distribution was evaluated. The subcellular Adriamycin distribution pattern was studied in the parental and MDR cells following a 60-min exposure to Adriamycin (20 μg/ml) (Fig. 6). Intense nuclear fluorescence was clearly distinguishable in the parental cells (Fig. 6A). In contrast, resistant cells displayed only faint nuclear fluorescence (Fig. 6E). Hyperthermia caused an increase in the intensity of both nuclear and cytosolic fluorescence in AuxB1 cells (Fig. 6C). Hyperthermia did not affect fluorescence intensity in CH^RC5 cells, but there was an increase in cytoplasmic relative to nuclear fluorescence (Fig. 6G). Cyclosporin A caused a marked increase in nuclear fluorescence in CH^RC5 cells, at both 37 °C and 42 °C (Fig. 6F and H, respectively). In addition, there was increased drug in the cytosol at 42 °C (Fig. 6H). In AuxB1 cells, cyclosporin A caused an increase in nuclear and cytosolic fluorescence, at both 37 °C and 42 °C (Fig. 6B and D, respectively).

The effect of cyclosporin A and hyperthermia on the subcellular Adriamycin distribution pattern following drug efflux was then investigated. Following 60 min of efflux at 37 °C, only faint nuclear fluorescence remained in AuxB1 cells (Fig. 7A), while the cytosolic fluorescence remaining in CH^RC5 cells was hardly detectable (Fig. 7E). After 60 min of efflux at 42 °C, the subcellular Adriamycin distribution pattern remained the same as at 37 °C, the only difference being that the fluorescence intensity was much fainter (Fig. 7C and G). With cyclosporin A, however, strong nuclear fluorescence remained after 60 min of efflux from AuxB1 cells, at both 37 °C and 42 °C (Fig. 7B and D, respectively). In CH^RC5 cells, more drug fluorescence remained in the cytosol when efflux was carried out in the presence of cyclosporin A at 37 °C (Fig. 7F). At 42 °C, drug fluorescence was hardly detectable in MDR cells (Fig. 7G), although intensity was slightly higher with the chemosensitizer (Fig. 7H).

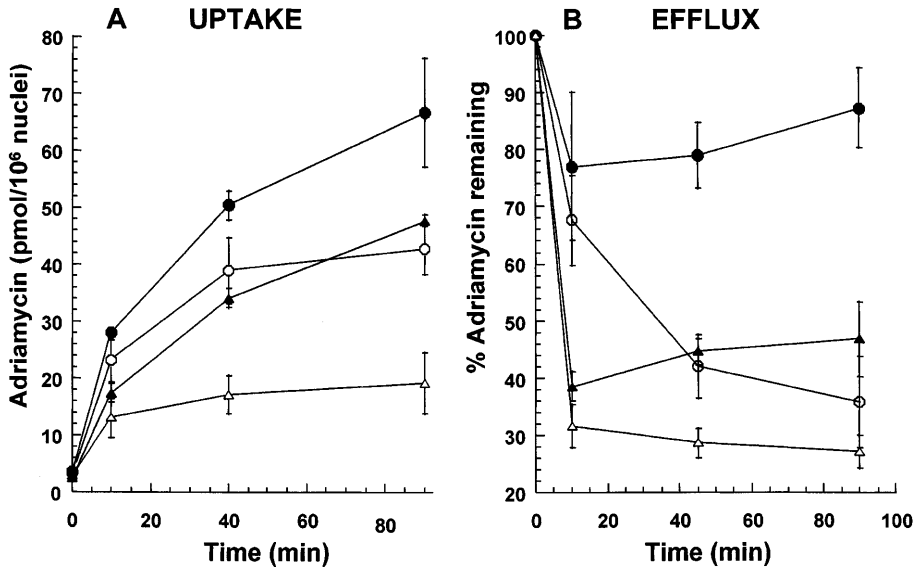

Fig. 5A,B Cyclosporin A enhances Adriamycin accumulation (**A**) and decreases Adriamycin efflux (**B**), for nuclei isolated from AuxB1 and CH^RC5 cells. **A** [^{14}C]Adriamycin content was determined in nuclei isolated from cells (10^6/ml) immediately following exposure to [^{14}C]Adriamycin (1 μg/ml) for different times at 37 °C in MEM Alpha containing 10% FBS. Data are for AuxB1 cells, either with (●) or without (○) 5 μ*M* cyclosporin A, and for CH^RC5 cells, either with (▲) or without (△) 5 μ*M* cyclosporin A. Means and SEM are shown from two independent experiments with multiple estimations per point. **B** Cells (10^7/ml) were preloaded with [^{14}C]Adriamycin (5 μg/ml) during 30 min in MEM Alpha containing 10% FBS at 37 °C. Adriamycin efflux measurements from cells were carried out for different times at 37 °C in drug-free MEM Alpha/10% FBS. Nuclei were isolated immediately from cells and [^{14}C]Adriamycin content was determined. Data are for AuxB1 cells, either with (●) or without (○) 5 μ*M* cyclosporin A, and for CH^RC5 cells, either with (▲) or without (△) 5 μ*M* cyclosporin A. The data point at time zero represents the Adriamycin content in nuclei prior to efflux (100%). Each time-point represents the residual Adriamycin concentration in nuclei at various times during the efflux process and is expressed as a percentage of the zero time-point. Means and SEM are shown from three independent experiments with multiple estimations per point

Discussion

Resistance to Adriamycin has been associated with several different mechanisms of MDR, including those involving overexpression of P-glycoprotein, alterations in glutathione metabolism, amplification of glutathione S-transferases and changes in nuclear enzyme DNA topoisomerase II [13, 15, 31]. Cells which exhibit the P-glycoprotein-based form of MDR often show reduced accumulation of anthracyclines such as Adriamycin and daunorubicin [17, 31]. In addition to reduced drug accumulation, many MDR cell lines also exhibit altered intracellular distribution and compartmentalization of the drug into vesicles away from the critical target site [24, 27, 59]. The well-characterized colchicine-resistant MDR cell line, CH^RC5, exhibits cross-resistance to Adriamycin and decreased drug accumulation [32]. P-glycoprotein is thought to be responsible, at least in part, for the resistance to Adriamycin through increased drug efflux out of the cells [2].

We showed in the study reported here that the chemosensitizer cyclosporin A increased intracellular Adriamycin accumulation and sensitized CH^RC5 cells to Adriamycin. We report a near tenfold factor (Fig. 1B, 50% cell survival) for modification of Adriamycin resistance by cyclosporin A. It has been clearly demonstrated that cyclosporin A directly binds and interacts with P-glycoprotein [19, 45]. Our findings suggest that cyclosporin A decreases Adriamycin resistance by blocking the activity of the energy-dependent P-glycoprotein efflux pump in MDR CH^RC5 cells. There was a decrease in the rate of [^{14}C]Adriamycin efflux as well as an increase in the steady-state level of drug, thus leading to an overall increase in intracellular drug accumulation. We showed further that the chemosensitizer promotes a marked increase in Adriamycin accumulation in the nucleus, where the drug is considered to exert its cytotoxic effect. However, cyclosporin A did not completely restore the cytotoxic response of MDR cells (Fig. 1B) to Adriamycin, to that obtained in drug-sensitive cells without the modulator (Fig. 1A), despite the correction of the deficit in drug accumulation, both at the cellular and nuclear levels. Clearly, other factors are also involved. Adriamycin induces a multitude of effects in cells, including inhibition of DNA topoisomerase II catalytic activity, stimulation of apoptosis, activation of protein kinase C-mediated signal transduction pathways and generation of reactive oxygen species [15]. However, the mechanism(s) of action of Adriamycin are not entirely clear [15].

We also demonstrated that cyclosporin A enhanced Adriamycin uptake and cytotoxicity in drug-sensitive AuxB1 cells. We report an approximately threefold factor (Fig. 1A, 50% cell survival) of sensitization to Adriamycin cytotoxicity by cyclosporin A. The en-

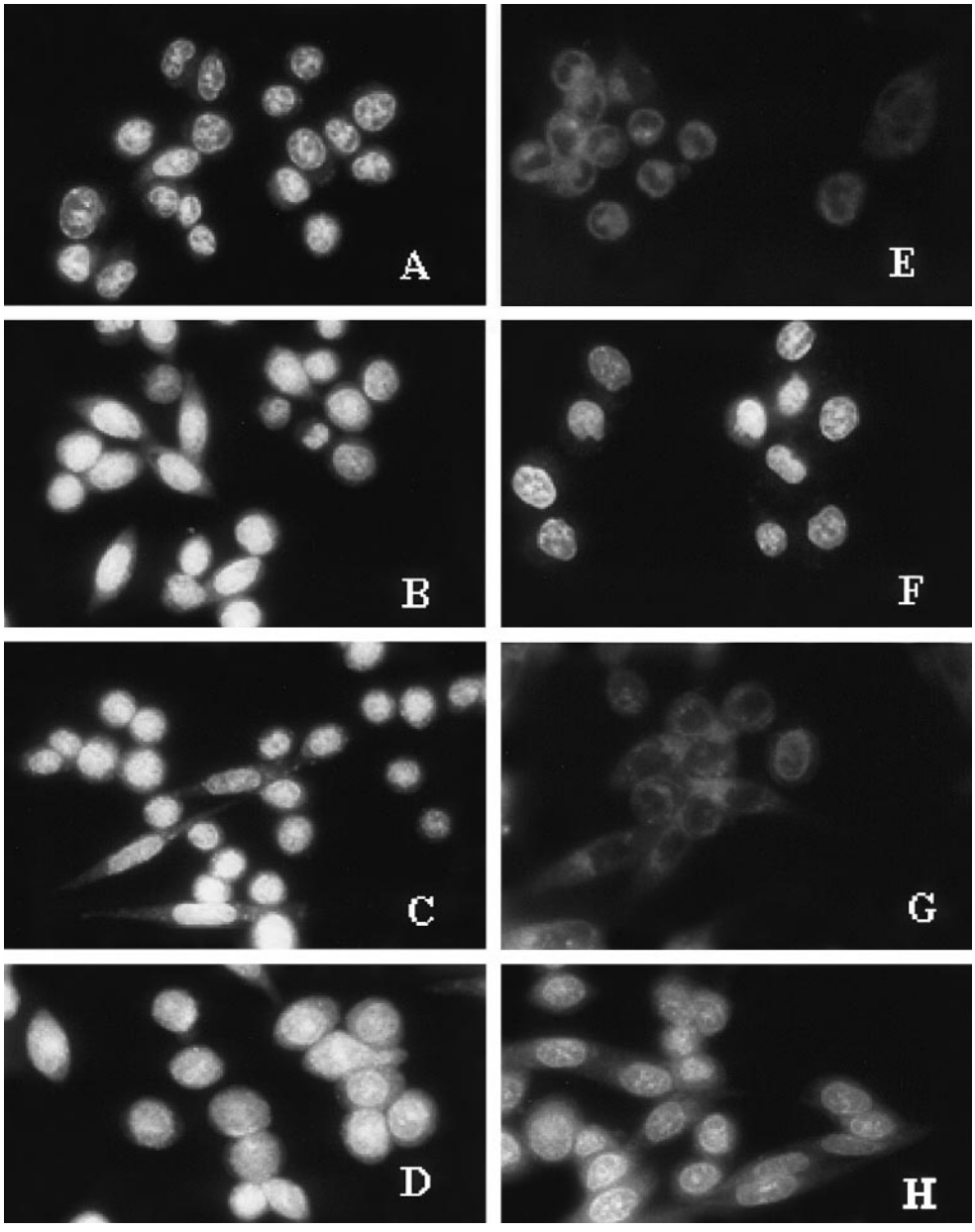

Fig. 6A–H The subcellular Adriamycin distribution pattern in AuxB1 (**A**–**D**) and $CH^{R}C5$ cells (**E**–**H**) following drug uptake showing the effects of cyclosporin A and hyperthermia. Cells were incubated with Adriamycin (20 μg/ml) during 60 min in MEM Alpha containing 10% FBS, at 37 °C either with (**B** and **F**) or without (**A** and **E**) 5 μ*M* cyclosporin A, or at 42 °C either with (**D** and **H**) or without (**C** and **G**) 5 μ*M* cyclosporin A. Magnification ×400. In order to visualize Adriamycin fluorescence using the microscope, note that higher drug concentrations were used relative to transport studies using radiolabeled drug the results of which are shown in Fig. 3. Experiments were performed on three occasions

hancement of Adriamycin cytotoxicity by cyclosporin A in drug-sensitive cells was accompanied by increased intracellular [^{14}C]-labeled drug accumulation. This arose from a very pronounced increase in the content of drug firmly bound inside the cells following efflux. We showed further, in drug-sensitive cells, that cyclosporin A promoted increased [^{14}C]Adriamycin transport into the nuclear compartment, as well as a marked increase in nuclear drug retention following efflux from nuclei. Drug distribution data showed that cyclosporin A promoted an increase in Adriamycin in both the nuclear and cytoplasmic compartments. These changes could be responsible, at least in part, for the sensitization to Adriamycin cytotoxicity by cyclosporin A. However, the nature of the modifying effect of cyclosporin A on cellular response to Adriamycin in drug-sensitive cells is unclear. In addition to their interactions with DNA, anthracyclines also bind strongly to cell membranes [15, 48] and part of their cytotoxicity may be at the membrane level. Cyclosporin A is also a highly lipophilic, membrane-interactive drug which can modify membrane potentials and may thus alter intracellular ion gradients [54]. This could alter the intracellular distribution and membrane actions of Adriamycin, thus accounting for the modification of cytotoxicity by cyclosporin A. It is also possible that alternative energy-dependent transport mechanisms, apart from P-glycoprotein, may be involved [8, 15].

Cyclosporin A exhibits chemosensitizing properties towards anticancer drugs such as anthracyclines and vinca alkaloids, in several different cellular systems, in vitro [5, 19, 21, 43, 50]. The ability of cyclosporin A to

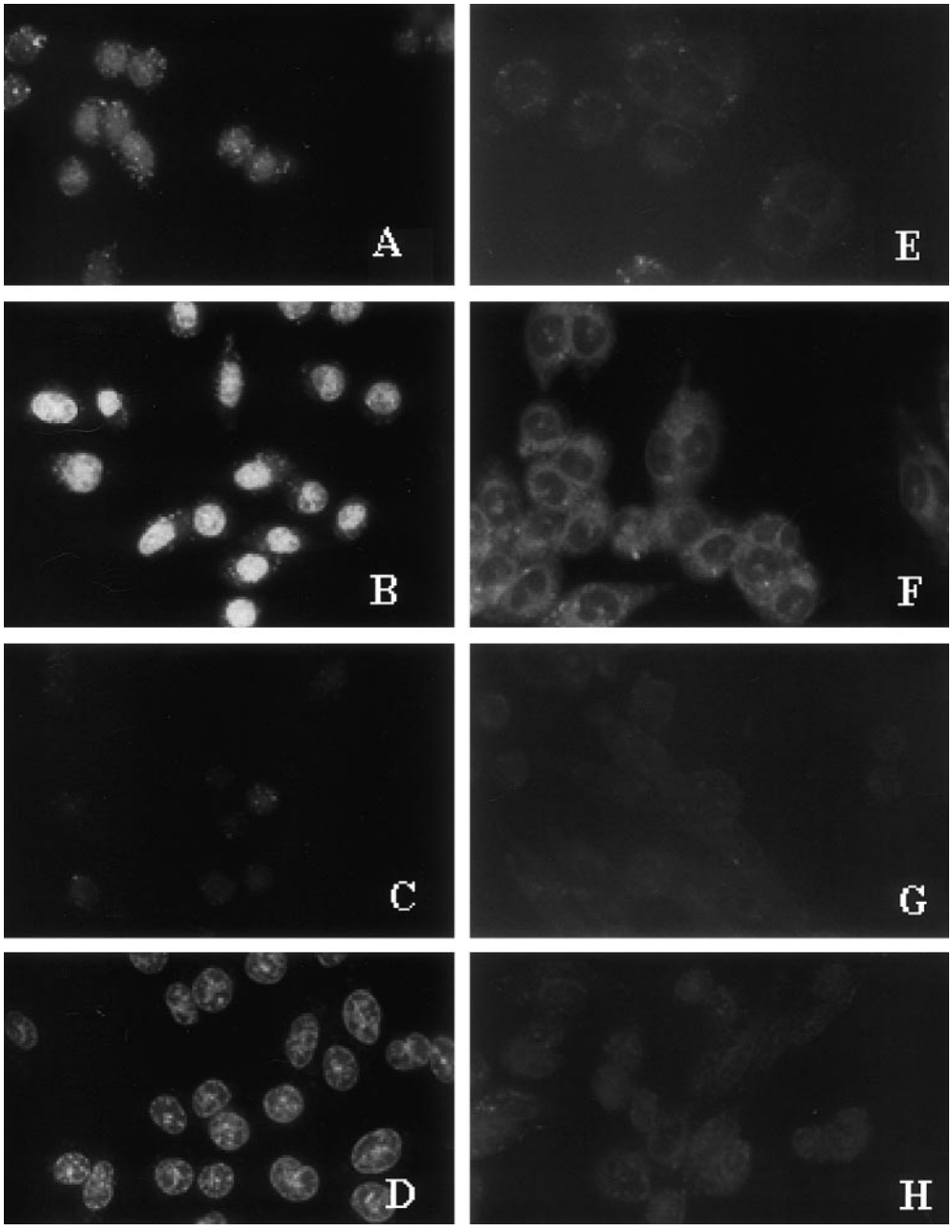

Fig. 7A–H The subcellular Adriamycin distribution pattern in AuxB1 (**A**–**D**) and CH^RC5 cells (**E**–**H**) following drug efflux showing the effects of cyclosporin A and hyperthermia. Cells were preloaded for 30 min with 50 μg/ml of Adriamycin in MEM Alpha containing 10% FBS. Efflux was carried out during 60 min without Adriamycin in the incubation medium under different conditions. Relative to a common starting point for each of the two cell lines, the four different conditions compared were: (1) 37 °C, either with 5 μ*M* cyclosporin A (**B** and **F**) or (2) without cyclosporin A (**A** and **E**), or (3) at 42 °C, either with 5 μ*M* cyclosporin A (**D** and **H**) or (4) without cyclosporin A (**C** and **G**). Magnification ×400. In order to visualize Adriamycin fluorescence using the microscope, note that higher drug concentrations were used, relative to transport studies using radiolabeled drug the results of which are shown in Fig. 4. Experiments were performed on three occasions

act as a sensitizer has been described in only a few drug-sensitive systems such as L1210 murine leukemia [35], CHO cells [7], Ehrlich ascites and murine hepatoma [33] and H2 T hamster pancreatic carcinoma [39]. However, chemosensitization by cyclosporin A does not usually occur in the large majority of drug-sensitive cell lines [43, 50, 52].

The mechanism of cell death due to hyperthermia is not well understood. Heat causes changes to many cellular constituents including proteins, cytoskeletal elements and chromosomal structures [29]. Heat also causes changes in membrane permeability and alters many transport functions of the cellular membrane. It can also increase the generation of reactive oxygen species due to its effects on electron chain transport in mitochondria, or because of inhibitory effects of heat on free radical-scavenging systems [29].

Hyperthermia can increase cell killing by several different anticancer drugs in both drug-sensitive and drug-resistant cells [2, 3, 11, 22, 23, 37, 47, 56, 57]. Heat potentiation of Adriamycin cytotoxicity and uptake has been reported in several cell lines [2, 38]. CH^RC5 cells are as sensitive to heat-induced cell killing at 43 °C and 45 °C as their drug-sensitive parent cells [2]. Furthermore, hyperthermia enhances melphalan cytotoxicity in these MDR cells [1, 3]. In all of these studies, however, hyperthermia alone could not totally overcome drug resistance.

Hyperthermia and cyclosporin A, when combined, caused a beneficial effect by increasing Adriamycin cy-

totoxicity in drug-sensitive cells, relative to their individual modulating effects. This was accompanied by a marked decrease in Adriamycin efflux from the nucleus (Fig. 4A and Fig. 7D) and an overall increase in [^{14}C]-labeled drug accumulation (Fig. 3A), in both the nuclear and cytoplasmic compartments (Fig. 6D).

Against MDR cells, 42 °C hyperthermia alone caused only a very small increase in Adriamycin cytotoxicity, whereas a beneficial effect was obtained when hyperthermia was combined with cyclosporin A. This combination resulted in an approximately 15-fold (Fig 1B, 50% cell survival) increase in Adriamycin cytotoxicity in MDR cells. Potentiation of Adriamycin cytotoxicity by heat and cyclosporin A in MDR cells can be explained by several factors. Our findings suggest that the enhancement of Adriamycin cytotoxicity by hyperthermia and cyclosporin A could be caused, at least in part, by changes in membrane permeability to the drug. This appears to be an important reason since cyclosporin A essentially completely corrected the deficit in drug accumulation in MDR cells to the level obtained in drug-sensitive cells. However, there must be other explanations for the marked ability of hyperthermia to increase the cytotoxicity of Adriamycin in the presence of cyclosporin A, since hyperthermia caused a relatively small increase in drug accumulation when the chemosensitizer was present. This small increase was in the cytoplasm. Further explanations for the increased Adriamycin cytotoxicity in MDR cells could be due to increased rates of reactions of the drug with cellular constituents (e.g. proteins, DNA, membranes) at elevated temperatures, leading to increased damage to critical targets. Heat could also inhibit repair of Adriamycin-induced damage to cellular targets such as DNA, or could increase inhibition of the catalytic activity of topoisomerase II. It may be that heat enhances the ability of Adriamycin to exert its oxidative toxicity to cells.

The findings reported here show that the combination of cyclosporin A and clinically achievable elevated temperatures (39–43 °C) allowed more Adriamycin accumulation in AuxB1 and CH^{R}C5 cells. This was accompanied by an increase in the cytotoxic effect of the drug in both drug-sensitive and MDR cells. Furthermore, hyperthermia alone had little effect on Adriamycin cytotoxicity and transport in MDR cells, in complete contrast to its effect in the drug-sensitive cells. These results suggest that P-glycoprotein is fully functional in these MDR cells at elevated temperatures. These important findings should be verified in other MDR cell lines with overexpression of P-glycoprotein. In conclusion, our results indicate that hyperthermia, when combined with cyclosporin A which increases intracellular Adriamycin accumulation, could prove very useful in eliminating both drug-sensitive and MDR cells. Furthermore, hyperthermia has considerable potential to increase the cytotoxic effects of this chemotherapeutic drug in a localized target region, thus leading to an improved therapeutic index.

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