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Chemosensitisation of a drug-sensitive parental cell line by low-dose cyclosporin A

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Summary. We investigated the chemosensitisation of the parental EMT6 mouse mammary tumour cell line by low doses of cyclosporin A (CsA). This cell line has not previously been exposed to cytotoxic drugs but expresses low levels of P-glycoprotein. We produced >2-fold sensitisation to doxorubicin, colchicine and vincristine using 0.084 μM (0.1 μg/ml) CsA. Cellular accumulation of doxorubicin and daunorubicin was also increased by this dose. In the MDR subline EMT6/AR1.0, much higher doses of CsA were required to effect optimal restoration of doxorubicin or daunorubicin accumulation. The effects of CsA on the parent line could not be increased by extended preincubation of cells with the sensitiser. These effects of CsA in the EMT6 parent cell line occur at a dose that is 1 order of magnitude lower than those previously reported to produce significant chemosensitisation.

Introduction

It is now well established that most cell lines that display a multidrug-resistant (MDR) phenotype also hyperexpress P-glycoprotein in the cell membrane [5, 10]. Evidence that the mdr-1 gene that codes for P-glycoprotein is expressed at relatively high levels in a variety of human malignancies [7] has led to increased interest in the possibility of clinical chemosensitisation by agents that inhibit the action of P-glycoprotein. Several such drugs have undergone clinical trial, including verapamil, quinidine and cyclosporin A (CsA) [4, 9, 19]. The major mode of action of these sensitisers in MDR cells appears to be the partial reversal of the cytotoxic drug-accumulation deficit that accompanies P-glycoprotein hyperexpression [2, 17, 20]. In some cases it has been shown that the sensitisers have the ability to compete with cytotoxic drugs for binding sites on the P-glycoprotein molecule [3, 16]. In this regard, the affinity of CsA has been shown to be much higher than that of verapamil [13]. Although several reports have confirmed that CsA increases cytotoxic drug accumulation in MDR cells [2, 8, 16, 17], it has also been purported that its effect on some cell lines cannot be ascribed to this mechanism [18].

Selection of the best drugs for clinical use has been based on, amongst other factors, in vitro data concerning the drug concentrations necessary for optimal chemosensitisation together with estimates or clinical data regarding the achievable plasma levels. However, most of the in vitro data derive from cell lines exhibiting very high levels of resistance – possibly not a good model for the lower levels of resistance that may form the basis of the clinical problem.

High levels of P-glycoprotein are often seen in MDR cell lines derived in vitro by growth of the parent cells in increasing concentrations of an appropriate cytotoxic drug [usually doxorubicin (DOX), colchicine (COL) or vincristine (VCR)] [5, 10]. However, some parent cell lines that have never been exposed to a cytotoxic drug also express low, albeit detectable, levels of P-glycoprotein [11, 23]. Our recent studies using a panel of sublines of the EMT6 mouse tumour cell line have demonstrated chemosensitisation to be a complex, multifactorial process that depends on the cytotoxic drug under study, the sensitiser employed, the sensitiser dose and the amount of P-glycoprotein in the target cell line [23]. Major factors involved in this equation include the relative binding efficiencies of the cytotoxic drug and the sensitiser for P-glycoprotein.

We could recently show that a 2- to 3-fold sensitisation of the parent EMT6 cell line to COL could be brought about by concentrations of CsA as low as 0.25 μM [23]. However, no sensitisation of the highly resistant subline CR2.0 could be seen at concentrations of <0.84 μM CsA. It would therefore appear that low concentrations of sensitisers may produce significant effects in cells exhibiting low levels of P-glycoprotein. In the present study, we extended our studies using the parent cell line to examine the sensitisation to a range of cytotoxic drugs at CsA concentrations of between 0.0084 and 0.84 μM (0.01–1 μg/ml). In addition, we determined the effect of these low concentrations on cytotoxic drug accumulation in the

parent cell line together with the influence of the duration of sensitiser preincubation.

Materials and methods

Cells and medium. The EMT6/Ca/VJAC mouse mammary carcinosarcoma cell line [15, 21] grows as an attached monolayer on plastic and displays a doubling time of approximately 12 h during exponential growth. The parent cell line is hereafter referred to as EMT6/P. The MDR subline EMT6/AR1.0 was derived by in vitro growth of the parent line in increasing concentrations of DOX [23]. This subline is routinely maintained in 1 μg/ml DOX, exhibits a doubling time of 14–15 h and expresses high levels of P-glycoprotein [23]. Cells were grown in Eagle's minimal essential medium (MEM) with Earle's salts supplemented with penicillin and streptomycin and with 20% newborn calf serum (all from Gibco Biocult Ltd). Cells were maintained as stock cultures in 75-cm^2 flasks that were incubated at 37° C in an atmosphere consisting of 8% CO_2 and 92% air.

Cultures were routinely screened for mycoplasma contamination and tests were negative throughout the period during which these experiments were carried out. Subculture was carried out by rinsing the monolayer twice with a solution of 0.1% trypsin in phosphate-buffered saline (PBS) and then incubating it for 15 min at 37° C. Resuspension in medium and pipetting produced a single-cell suspension.

Drugs. The cytotoxic drugs studied included DOX (Farmitalia), COL (Sigma) and VCR (David Bull Labs). They were dissolved in sterile distilled water to a concentration of 500 μg/ml, stored as aliquots at −70° C and diluted with sterile water immediately before their use. CsA (Sandoz) was dissolved in absolute ethanol at 4.2 mM (5 mg/ml) and stored at 4° C; it was diluted with medium immediately before its use.

Drug-response assay. To determine the response of EMT6/P cells to the various cytotoxic drugs, we used the MTT colorimetric assay as previously described [23]. Briefly, cells were harvested from stock cultures and diluted to 3×10^3 cells/ml medium. Aliquots (200 μl) of this suspension were dispensed into wells on 96-well tissue-culture plates (Falcon) and incubated for 2 h. The appropriate doses of CsA (or solvent controls) were then added to the wells at a volume of 10 μl. After 1 additional h incubation, cytotoxic drugs (or solvent controls) were added at a further volume of 20 μl. In each experiment, seven 2-fold drug dilutions were employed to establish dose-response curves and four replicate wells were used at each point. The plates were then incubated for a period of 72 h. At the end of this period, 20 μl of a 5 mg/ml solution of MTT (Sigma) was added to each well and the plates were returned to the incubator for 5 h. The medium was then aspirated from each well and 200 μl dimethyl sulphoxide (DMSO) was added. The plate was agitated for 10 min on an automatic plate shaker and the optical density of each well was read on a Titertek Multiskan MCC plate reader at 540 nm (reference wavelength, 690 nm). From graphs of the data it was possible to determine the dose of cytotoxic drug required to reduce the final optical density and, hence, the cell number to 50% of control values (ID_{50}) [23].

Drug accumulation. To study the effect of CsA on drug uptake in EMT6/P and EMT6/AR1.0 cells, we generally used tritiated daunorubicin ([^{3}H]-DNR), an analogue of DOX. The use of labelled DNR enabled the experiments to be carried out at low molar drug concentrations similar to those used in cytotoxicity experiments. The labelled compound (1.4 Ci/mmol, New England Nuclear) was stored at −70° C in methanol. Cells (4×10^4/well in 2 ml medium) were inoculated into wells measuring 3 cm in diameter on 6-well multiplates (Sterlin Ltd) at 48 h prior to the experiments. To begin the investigations, the medium was aspirated from each well and replaced at 37° C with 2 ml medium containing the labelled compound (0.1 μCi/ml) supplemented with carrier DNR to a final concentration of 1 μM. After the appropriate incubation period, the medium was again aspirated from each well and the cell monolayer was washed three times with ice-cold PBS. Cells were lysed in 1 ml distilled water, and 0.5 ml was transferred to liquid scintillation vials containing 5 ml Quicksafe A (Zinsser Analytic). The vials were stored overnight and radioactivity was counted on the following day using a Beckman scintillation counter. Cell counts were carried out on three replicate wells such that the data could be expressed in terms of [^{3}H]-DNR uptake/cell.

In addition to experiments using [^{3}H]-DNR, we also studied the cellular accumulation of DOX using flow cytometry to measure the native fluorescence of this agent. Cells suspended in medium (2 ml/tube, 2×10^5 cells/ml) were exposed to 20 μM DOX in the presence of varying concentrations of CsA. After 60 and 120 min, samples of the suspension were analysed using the Cambridge flow cytometer as previously described [1]. Triplicate samples were analysed at each point.

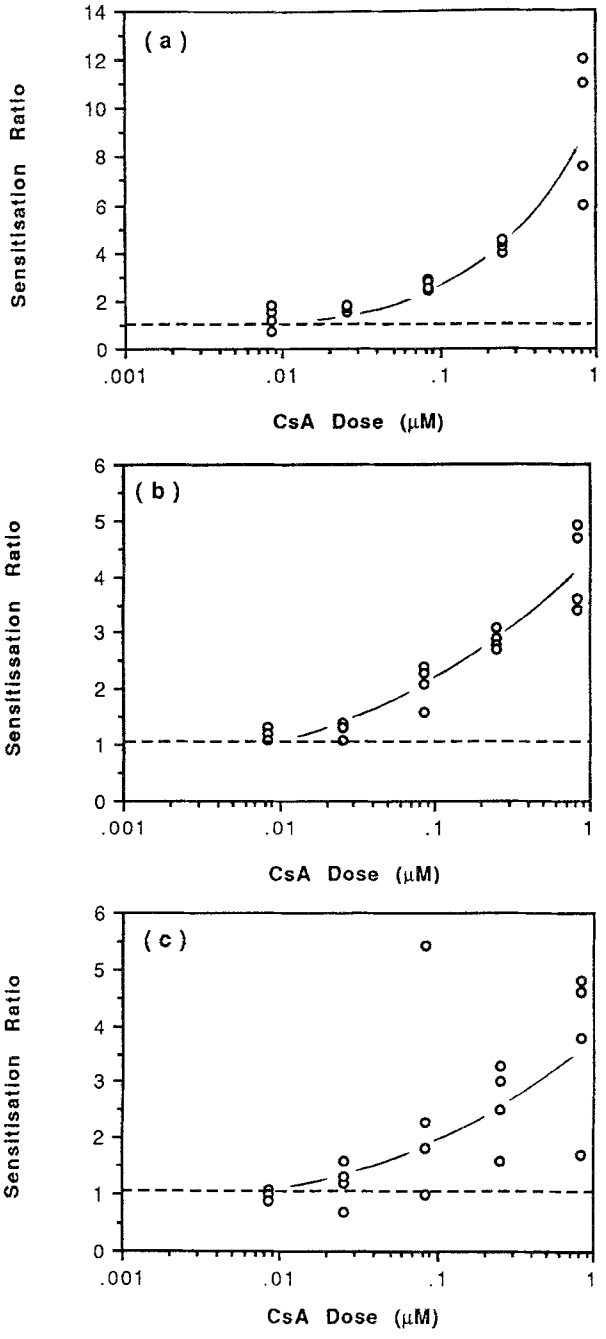

Fig. 1 a–c. Sensitisation ratio (SR = $\frac{ID_{50} \text{ in absence of CsA}}{ID_{50} \text{ in presence of CsA}}$) for EMT6/P cells exposed to **a** doxorubicin, **b** colchicine or **c** vincristine. Data points were taken from 4 independent experiments and the line was fitted by eye to the points

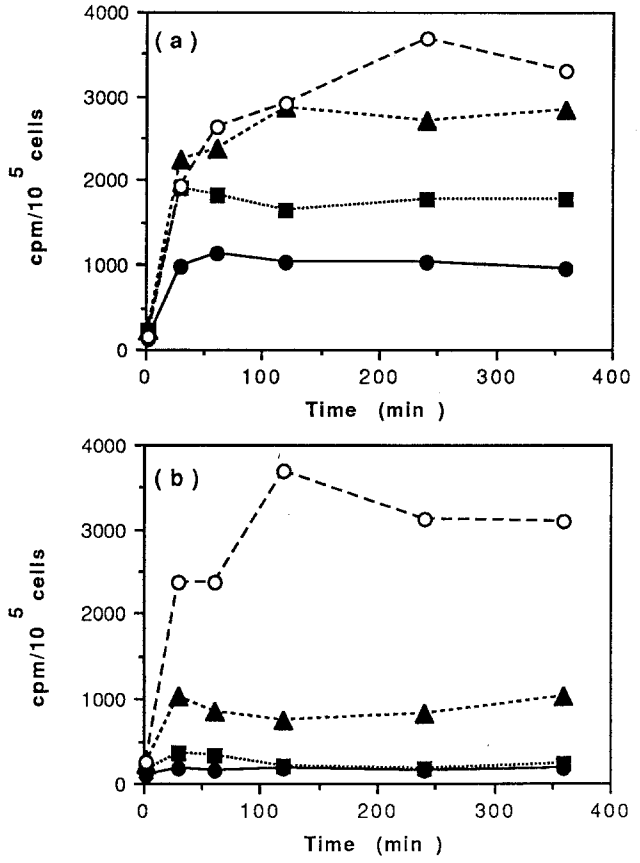

Fig. 2 a, b. Accumulation with time of [^{3}H]-daunorubicin by **a** EMT6/P or **b** EMT6/AR1.0 cells in the presence of different concentrations of CsA. ●, Control; ■, 0.084 μM; ▲, 0.84 μM; ○, 4.2 μM. Data represent mean values for 3 replicate wells

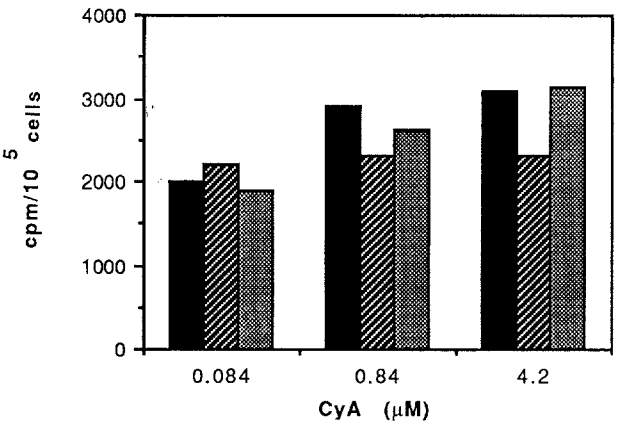

Fig. 3. Accumulation of [^{3}H]-daunorubicin by EMT6/P cells exposed to different doses of CsA for either 1 (■), 30 (▨) or 240 min (▩) prior to a 2-h exposure to the labelled compound in the continuing presence of CsA. Data represent mean values for 3 replicate wells. The accumulation in control wells in the absence of CsA was 1596 cpm

Results

The effects of low doses of CsA on the cytotoxic drug response of EMT6/P cells are shown in Fig. 1. The results are expressed as sensitisation ratios (SR), where

$$SR = \frac{ID_{50} \text{ in the absence of CsA}}{ID_{50} \text{ in the presence of CsA}} .$$

It can be seen that the SR values for all three drugs exceeded 2 at a dose of 0.084 μM CsA, increasing relatively slowly at higher CyA doses for COL and VCR but more rapidly for DOX. No effects of CsA alone were seen at doses of <1 μM.

The data in Fig. 2 show that the accumulation of [^{3}H]-DNR by EMT6/P cells was increased markedly by 0.084 μM CsA (approx. 1.7 times) and to a greater extent (approx. 2.7 times) by 0.84 μM CsA but exhibited little, if any, further increase at CsA concentrations of between 0.84 and 4.2 μM. In contrast, the drug-resistant cell line EMT6/AR1.0 displayed increased accumulation of [^{3}H]-DNR only at higher doses of CsA, with the greatest increase occurring at levels of between 0.84 and 4.2 μM. Similar results were obtained in repeat experiments using each of these cell lines. The data for DOX accumulation (Table 1) followed a trend similar to those observed for [^{3}H]-DNR, with the full effect on EMT6/P occurring at CsA doses lower than those at which it was seen in EMT6/AR1.0.

Results of an experiment in which the CsA preincubation period prior to the addition of [^{3}H]-DNR was varied are shown in Fig. 3. It is clear that equal effects on accumulation were obtained, irrespective of the duration of preincubation. Similar results were obtained in a repeat experiment.

Discussion

The data presented herein demonstrate that clear chemosensitisation (>2-fold reduction in ID_{50}) of the EMT6 parent cell line occurred at doses of CsA that were considerably lower than those previously reported to be effec-

Table 1. Effect of CsA on doxorubicin accumulation

Cell line	Time (min)	Doxorubicin content (arbitrary fluorescence units)			
		Control	+0.084 μM CsA	+0.84 μM CsA	+4.2 μM CsA
EMT6/P	60	711±27	944±38	1105±24	1009± 61
	120	1303±18	1975±45	2482±48	2267±117
EMT6/AR1.0	60	266± 8	302±14	844±15	1033± 63
	120	304± 4	422± 8	1207±26	1995±115

Data represent mean values (± SE) for 3 replicate samples as determined using flow cytometry

tive. We also found that in this cell line, the accumulation of radiolabelled DNR was increased by 0.084 μM CsA and that this is therefore likely to be the basis of the observed chemosensitisation.

In our investigation of the level of P-glycoprotein expression in the EMT6 parent cell line and a number of in vitro-derived MDR sublines, we could detect a low level of expression in the parent line by using a long exposure period for the autoradiographs of Western blots [23]. This contrasts with our parent human small-cell lung cancer line H69/P, in which no P-glycoprotein expression could be detected [14]. In the latter line, the use of 4.2 μM CsA resulted in a 1.5- and 2.2-fold sensitisation of the cells to DOX and VCR, respectively [22].

Most of the reported studies using CsA as a modifier of MDR have applied doses that were considerably in excess of 1 μM. In their initial publication, Slater et al. [18] found no sensitisation of parental human acute lymphatic leukaemic cells by 2.8 or 5.5 μM CsA, although a small effect was seen at 11 μM. These authors claimed that the high degree of sensitisation to DNR seen in a resistant line was not attributable to changes in drug accumulation. However, in a study using Ehrlich ascites cells, the same group reported some enhancement of DNR effects by 1 μM CsA in both parent and resistant cells [12]; no effect was seen in either line at 0.1 μM CsA. Gavériaux et al. [6] found considerable (6- to 16-fold) sensitisation to COL, VCR and DNR in a parental Chinese hamster cell line at 0.84 μM CsA; the MDR subline exhibited 9- to 38-fold sensitisation to these drugs. The parent cell line used in these studies (AUX B1) had previously been shown to express low levels of P-glycoprotein [11]. The same parent cell line was subsequently studied by Silbermann et al. [17] to determine the enhancement of DNR accumulation by CsA. A 20% increase in accumulation was observed at 0.5 μM CsA, but this was reversed at higher CsA doses. However, a line exhibiting low-level MDR showed considerable enhancement of accumulation at both 0.1 and 0.5 μM, with no further increase occurring at higher doses. In contrast, the highly resistant line CHR C5 reached maximal accumulation of DNR only at CsA doses of >3 μM. A recent study by Hu et al. [8] has examined DNR accumulation in the human T-cell lymphoblastic leukaemia CEM/CCRF and in two sublines displaying moderate (CEM/VLB100) or high (CEM/VLB 1000) levels of MDR. No change in accumulation in the parent line was seen in the presence of CsA doses of up to 10 μM. Whereas some increase in DNR accumulation was seen at 1.2 μM CsA in CEM/VLB 100, higher doses were required to produce an increase in CEM/VLB 1000.

From these previously reported findings it can be seen that in the cases in which CsA effects have occurred in parent cell lines (EMT6/P and AUX B1), the presence of low-level P-glycoprotein expression has been reported [11, 23]. Unfortunately, however, among the cases in which CsA effects have been absent in parent lines, the P-glycoprotein status has been published only for H69/P.

There is a general trend amongst these various data sets for CsA effects to reach a plateau at lower doses in parent or less resistant cells than in more highly resistant cells. Such an observation is compatible with our previous argument that low levels of P-glycoprotein can more easily be inhibited than high levels by a given concentration of CsA. Only the data of Silbermann et al. [17] indicate effects of low doses of CsA that are comparable with those used in the present study; these investigators observed a clear effect at 0.1 μM CsA in parent and less resistant lines but not in the highly resistant line. This finding is in agreement with our data on the accumulation of DNR in EMT6/P and AR1.0 in the presence of different CsA concentrations.

Our data regarding preincubation of cells with CyA prior to treatment with drugs involved in the MDR phenotype are the first of which we are aware. It appears that the concentration of CyA at the time at which the exposure to DNR occurs is the important parameter and that the development of a lesion during an extended period of preincubation does not occur. Therefore, the present study shows that chemosensitisation of the EMT6 parent cell line occurs at doses of CyA that are 1 order of magnitude lower than those previously reported to bring about this effect. An effect on drug accumulation is likely to be the basis of this "low-dose" effect. The chemosensitisation of cells expressing low levels of P-glycoprotein at extremely low doses of sensitisers should be borne in mind during the comparison of sensitisers or the design of clinical trials.

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