Cyclosporin A and PSC 833 Prevent Up-Regulation of MDR1 Expression by Anthracyclines in a Human Multidrug-resistant Cell Line

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ABSTRACT
We have previously demonstrated that within 24 h of exposure of the CEM/A7R cell line to epirubicin (EPI), MDR1 gene expression is induced. The aim of the current study was to investigate the role of cyclosporin A (CyA) and PSC 833, two biochemical modulators of the classical multidrug-resistant phenotype, in this model.

CEM/A7R cells were exposed to EPI in the presence or absence of various concentrations of CyA or PSC 833. MDR1 expression was assessed using Northern blot analysis and quantitated using a phosphorimager. P-glycoprotein (P-gp) expression was analyzed by the determination of MRK16 binding using flow cytometry. P-gp function was measured in an assay of [3H]daunomycin accumulation.

The coinubcation of CyA or PSC 833 with EPI prevented the increase in MDR1 gene expression induced by EPI alone. This effect of the two modulators was dose dependent. Neither modulator alone had any significant effect on the expression of MDR1. In these experiments, changes in MDR1 expression correlated with changes in P-gp levels (based on MRK16 binding) and P-gp function. Thus, both PSC 833 and CyA appear to prevent the induction of MDR1 gene expression caused by the short-term exposure of CEM/A7R cells to EPI.

INTRODUCTION
Drug resistance (MDR) is a common problem in relapsed acute leukemia (1). One form of drug resistance commonly seen in this situation is the classical MDR phenotype due to the overexpression of P-gp (2) encoded by the MDR1 gene (3).

Expression of the MDR1 gene is often low or undetectable before treatment but increases after chemotherapy (4, 5). Low levels of drug resistance are usually accompanied by elevated expression of the MDR1 gene (2–7-fold) in the absence of gene amplification (6). However, it has been suggested that a 2–3-fold increase in resistance to chemotherapeutic agents may be sufficient to account for clinical resistance. A direct correlation between expression of the MDR1 gene and the outcome of treatment by cytotoxic drugs has recently been demonstrated (4, 7).

The acquisition of multidrug resistance during a course of chemotherapy, is usually thought to be due to the selection of drug-resistant cells. However, more recently, using a human leukemia cell line expressing very low levels of MDR1 in the absence of gene amplification, we were able to demonstrate a rapid increase in the expression of the MDR1 gene following treatment with anthracyclines (8). Increased P-gp levels were seen within 8 h of exposure of these cells to three anthracyclines (DOX, daunorubicin, and EPI). In this model, the rapid induction of MDR1 suggests that the development of clinical resistance may be due in part to the induction of drug resistance in addition to the selection of resistant cell populations.

The immunosuppressive agent CyA has been shown to be an effective chemosensitizing agent via the reversal of the classical MDR phenotype (9, 10). More recently, a nonimmunosuppressive analogue of CyA, PSC 833, has been used as a specific inhibitor of P-gp-mediated MDR. Compared to CyA, PSC 833 has been shown to be 10-fold more potent in the circumvention of resistance to Vinca alkaloids and anthracyclines in tumors expressing P-gp (11, 12).

Although the mechanism of action of many of these modulators is uncertain, it is generally thought that reversal of the MDR phenotype is related to their interference with the function of P-gp. Many of these modulators bind directly to P-gp and compete with the binding of unrelated drugs that also modulate P-gp function (13–16). However, drugs such as CyA are known to have effects at various levels in cells. In activated T cells, CyA prevents the induction of interleukin 2 mRNA in response to an antigen stimulus (17). In this study, we report the role of CyA and PSC 833 in preventing the induction of MDR1 expression in a human leukemia cell line (CEM/A7R) exposed to EPI.

MATERIALS AND METHODS
Materials. EPI was obtained commercially from Farmi- talia (Melbourne, Australia). PSC 833 and CyA were obtained from Sandoz Pharma Ltd. (Basel, Switzerland). These agents were initially dissolved in absolute alcohol before being diluted in RPMI 1640 to give a stock solution of 0.5 mg/ml (the final ethanol concentration was 35%). RPMI 1640 was purchased as a powder (GIBCO Labs) and supplemented with 10% FCS (Trace Biosciences Pty Ltd., Melbourne, Australia), gentamicin.
(80 µg/ml), minocycline (1 µg/ml), HEPES (20 mM), sodium bicarbonate (0.21%), and glutamine (0.8 mM). Monoclonal antibodies to P-gp were generously provided by Dr. Takashi Tsuruo (Division of Experimental Chemotherapy, Japanese Foundation for Cancer Research). Fluorescein-labeled Fab(1)2 fragment of sheep antimouse IgG was purchased from Silenus Laboratories (Melbourne, Australia). The cDNA probe pHDR5A was a gift from Dr. M. Gottesman and Dr. Ira Pastan (Laboratory of Molecular Biology, NIH, Bethesda, MD). The cDNA GAPDH was a gift from Dr. Mark Ross (Ludwig Institute for Cancer Research, Melbourne, Australia). Propidium iodide was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Lines and Culture Conditions. The low level DOX-resistant CEM/A7 cell line (18) was obtained by stepwise selection in increasing concentrations of DOX from the drug-sensitive CCRF-CEM parental cell line, originally derived from a patient with a T-cell lymphoblastic leukemia (19). The CEM/A7 line has been characterized as a classical MDR line with overexpression of P-gp and cross-resistance to a number of structurally unrelated cytotoxics including anthracyclines, Vinca alkaloids, and etoposide (18). The CEM/A7 line was maintained in RPMI 1640 supplemented with 10% FCS and 0.07 µg/ml DOX and cultured at 37°C in a humidified chamber containing 5% CO2 in air.

A variant of the CEM/A7 line was developed by culturing this cell line in the absence of DOX for more than 2 years. The expression of P-gp in the variant line was decreased to less than one quarter of that in the CEM/A7 line. The variant line was subcloned in 96-well plates by a limited dilution technique. A single clone was identified 2 weeks later, transferred to 24-well plates, and subsequently expanded. The cloned variant line, referred to as CEM/A7R, was used throughout this study. This line was not exposed to DOX or other Pgp substrates, except in the specific experiments detailed below. At the time of these experiments, all lines were Mycoplasma free based on the Mycoplasma T. C. Rapid kit (GEN-PROBE, Inc., San Diego, CA).

Drug Treatment and Sample Collection. The CEM/A7R cells in exponential growth phase were collected 2 days after subculture. The cells were washed, counted, and resuspended in 20 ml fresh medium to a total cell number of 5 × 10^6-1 × 10^7. The cells were preincubated with PSC 833 or CyA for 18 h and then washed three times before the addition of EPI in the presence or absence of CyA or PSC 833 for another 20 or 24 h. Cell viability was determined (after staining by trypan blue) using phase-contrast microscopy to detect cells of abnormal size or granularity. Nonviable cells were excluded from flow cytometric analysis using propidium iodide staining.

RNA Extraction and Northern Blot Analysis. RNA was isolated using the guanidinium thiocyanate method described by Chomczynski and Sacchi (20). Twenty µg of total cellular RNA were size fractionated on a 1.5% agarose gel under the high stringency conditions used in this study. The filters were prehybridized overnight at 42°C in hybridization buffer containing 50% formamide, 5 × SSPE (1 × SSPE containing 0.15 M NaCl, 0.001 M Na2HPO4, and 0.001 M EDTA), 5 × Denhardt’s solution, 0.5% SDS, and 1% skimmed milk powder. Hybridization was carried out in hybridization buffer. The pHDR5A and GAPDH cDNAs were randomly primed with [α-32P]dCTP. Labeled cdNA (10^6 cpm) was added to each ml of hybridization buffer. The filters were washed sequentially in 2 × SSPE with 0.1% SDS at 42°C for 15 min, 1 × SSPE with 0.1% SDS at 65°C for 30 min, and finally 0.1 × SSPE with 0.1% SDS at room temperature for 15 min. The filters were then exposed to X-ray film at −70°C using intensifying screens, or radioactive signals were quantitated by scanning on a phosphorimager using Image Quant software (Molecular Dynamics, Melbourne, Australia).

P-gp Expression. Flow cytometry was used to measure P-gp expression. Cells were collected and washed three times in media containing 10% FCS. MRK16, a monoclonal antibody to an external epitope of Pgp (final concentration 10 µg/ml), was added to cells at room temperature for 20 min. A nonspecific murine monoclonal antibody (IgG2a; Becton Dickinson, Sydney Australia) was used as the control. Following an additional three washes, cell pellets were resuspended in the same volume of PBS containing 10 µl of a 1:10 dilution of a fluorescein-conjugated Fab(1)2 fragment of sheep antimouse IgG antibody for 20 min at room temperature in the dark. Cells were washed once again (three times) and then analyzed in a FACScan flow cytometer (Becton Dickinson). Mean fluorescence intensity was recorded for each tested population (after correcting for nonspecific binding) to provide an estimate of relative MRK16 binding. The number of MRK16-binding sites was estimated using a sensitive flow cytometric bead assay (Qiifkit, Biocytex, France) in which beads with a known number of IgG2a molecules are indirectly labeled with fluorescein-conjugated sheep antimouse IgG in parallel with MRK16-labeled cells. These labeled beads were used to generate a standard curve which enabled mean fluorescence intensity values to be converted to the number of binding sites.

[3H]Daunomycin Accumulation. Changes in [3H]daunomycin accumulation were used as a functional assay of P-gp (22). Following treatment with EPI or EPI and PSC 833 (or CyA), CEM/A7R cells were washed three times with PBS and resuspended in fresh medium for 1 h at 37°C in a humidifier before performing drug accumulation studies. The cells were adjusted to a concentration of 5 × 10^6/ml, and viability was assessed with trypan blue. Cells were added to 96-well plates (Flow Labs) to give a final number of 5 × 10^5 cells/well and incubated at 37°C with tracer amounts of [3H]daunomycin (final concentration, 1.85 × 10^6 Bq/ml, 0.05 µg/ml). The cells were harvested onto glass fiber filters at designated times with an automated cell harvester (Cambridge; Technology, Inc.). The filter papers were dried and dissolved in 5 ml of a liquid scintillation cocktail (Ultima Cold; Packard) before radioactivity was measured. All assays were performed in triplicate.

Growth Assays. The sensitivity of each of the cell lines to a variety of chemotherapeutic drugs was determined by a standard growth inhibition assay (23). In brief, after determining cell viability, 2 × 10^5 of the tested cells were exposed to varying concentrations of EPI, PSC 833, or CyA in 12-well plates. The
cells were incubated at 37°C in a humidified chamber containing 5% CO2 in air for 3 days and counted using an automated Coulter counter (24). Results are expressed as the increase in the cell number of drug-exposed cells as a percentage of the increase in untreated control cells (23). The 50% inhibitory concentration for each drug was determined by calculating the drug concentration required to inhibit cell growth by 50%.

Statistics. Two-way ANOVA was used to compare the accumulation (over time) of [3H]daunomycin after exposure of cells to various experimental conditions. Analyses were performed on the software package GLIM. A P value <0.05 was considered significant.

RESULTS
CyA and PSC 833 Inhibit the Induction of MDR1 mRNA by EPI. Studies were carried out in the human MDR cell line CEM/A7R which expresses low levels of MDR1 mRNA and P-gp (Fig. 1). The comparison of MDRI expression in the various cell lines and the effect of CyA on the induction of the MDRI gene by EPI in the CEM/A7R line were assessed by Northern blot analyses (Fig. 1A) and quantitated by scanning Northern blots on a phosphorimager (Fig. 1B). The phosphorimager data were normalized with respect to the CCRF-CEM signal.

As expected, no expression of the MDRI gene was seen in the sensitive cell line CCRF-CEM (Fig. 1A). Progressively stronger signals were seen in the CEM/A7R and CEM/A7 lines (Fig. 1). Following the addition of 1 μg/ml EPI for 24 h, a 3.5-fold elevation of MDRI mRNA was observed in the CEM/A7R line compared to untreated controls (Fig. 1). This increase in MDRI levels (which has persisted for up to 3 weeks) correlated with the increase in resistance seen previously (8).

The addition of 2.5 μM CyA totally inhibited the induction of MDRI mRNA by EPI in the CEM/A7R cells (Fig. 1). In contrast, the presence of the same concentration of alcohol (in which the CyA was dissolved) had very little effect on MDRI mRNA levels following the exposure of these cells to EPI (Fig. 1).

The most dramatic effect of CyA was seen when cells were preincubated with CyA for at least 4 h prior to being exposed to both EPI and CyA (data not shown), although the preincubation without coincubation of cells with CyA had little effect on the increase in MDRI induced by EPI (Fig. 1). CyA alone had a minimal effect in MDRI expression in the CEM/A7R cells (data not shown).

Similar experiments were conducted using PSC 833. CEM/A7R cells in the exponential growth phase were incubated overnight with 1 μM PSC 833 before coincubation with EPI (1 μM/ml) and 1 μM PSC 833 (Fig. 2). Neither PSC 833 nor alcohol alone had any significant effect on MDRI expression in the CEM/A7R line. Exposure to 1 μg/ml EPI for 20 h in the presence or absence of alcohol (in which PSC 833 was dissolved) resulted in a 3-fold elevation in MDRI expression in the CEM/A7R line (Fig. 2). However, both the preincubation and coincubation of CEM/A7R cells with 1 μM PSC 833 each for 18 h completely inhibited the induction of MDRI mRNA by EPI in this cell line (Fig. 2). Of note, preincubation of CEM/A7R cells with PSC 833 without coincubation during the exposure to EPI had almost no impact on MDRI levels (data not shown). These experiments have been repeated and similar results obtained on at least three occasions.

![Fig. 1](http://www.cancerresearchuk.org/cancer-info/science/genes/secondary-active-efflux-pumps/images/mdr1-gene-expression.png)
Effect of CyA and PSC 833 on MDR1 Expression Is Dose Dependent. CEM/A7R cells were exposed to 1 μg/ml EPI for 20 h in the presence (or absence) of PSC 833 or CyA at concentrations ranging from 0.001 to 1 μM. Results were analyzed using Northern blot analysis (data not shown) and quantitated using a phosphorimager (Fig. 3). PSC 833 was 10-fold as effective as CyA in the prevention of MDR1 induction by EPI (Fig. 3). PSC 833 at 0.1 μM was almost as effective as 1 μM CyA in the inhibition of MDR1 mRNA expression induced by EPI. CyA alone, at 0.1 μM, had little effect on the expression of MDR1 induced by EPI (Fig. 3). Identical results were obtained in repeated experiments.

Ability of CyA and PSC 833 to Inhibit the Induction of MDR1 by EPI Correlates with Their Respective Activity as Modulators of the MDR Phenotype. Untreated CEM/A7R cells were exposed to increasing concentrations of EPI in a standard growth assay (see "Materials and Methods"). The effect of adding various concentrations of CyA and PSC 833 was then evaluated. PSC 833 was 10 times as effective as CyA in reversing the resistance of the CEM/A7R line to EPI. In the presence of 0.1 μM PSC 833, the 50% inhibitory concentration of EPI decreased to the same level as that achieved by 1 μM CyA (Fig. 4). Compared to untreated controls, 0.1 μM CyA only had a minimal effect on the growth of the CEM/A7R cells cultured in the presence of increasing concentrations of EPI (Fig. 4). This experiment has been repeated three times with similar findings.

Changes in MDR1 mRNA Levels Are Accompanied by Changes in P-gp Levels. To determine whether the changes in MDR1 mRNA levels were accompanied by corresponding
changes in P-gp levels, antigen density was measured with flow cytometry using a sensitive bead assay (Fig. 5). CEM/A7R cells were preincubated overnight with 1 μM PSC 833 and then exposed to 1 μg/ml EPI for 20 h in the presence of 1 μM PSC 833 or an alcohol control. These experiments have been repeated on several separate occasions with identical findings.

P-gp levels were compared to the parental line CCRF-CEM and the moderately resistant line CEM/A7. No expression of P-gp (compared to an isotype control) was seen in the CCRF-CEM line (Fig. 5). The number of MRK16-binding sites was approximately 5-fold less in the CEM/A7R line compared to the CEM/A7 cell line (9094/51492 binding sites). Compared to the untreated CEM/A7R line, a 3–4-fold increase in the number of MRK16-binding sites was observed following a 20-h exposure to 1 μg/ml EPI, corresponding to the relative change in MDR1 mRNA levels seen under similar conditions. Exposure of cells to alcohol, PSC 833, or CyA alone had almost no effect on the number of MRK16-binding sites in the CEM/A7R line (Fig. 5). However, the addition of 1 μM PSC 833 totally inhibited the increase in P-gp expression seen after exposure of the CEM/A7R cells to EPI (Fig. 5).

Changes in MDR1 Levels Correlate with the Functional Activity of P-gp. As a measure of the functional activity of P-gp, [3H]daunomycin accumulation was carried out in the CEM/A7R cells following overnight exposure to 1 μg/ml EPI with or without coincubation with 1 μM PSC 833. Cells were washed three times and incubated in standard tissue culture medium for 1 h before the functional assay of drug transport was undertaken. Compared to the untreated CEM/A7R cells, the intracellular levels of [3H]daunomycin were significantly lower.

Fig. 4 Effect of varying concentrations of PSC 833 and CyA on the cell number of CEM/A7R cells as a function of increasing concentrations of EPI. Growth curves in the absence (■) or presence of 1 μM PSC 833 (○), 1 μM CyA (▲), 0.1 μM PSC 833 (□), or 0.1 μM CyA (△) are shown.

Fig. 5 Flow cytometric analysis of P-gp expression using MRK16 binding (filled histograms) compared with an IgG2a control (unfilled histograms) in the CCRF-CEM, CEM/A7, and the CEM/A7R lines before and after exposure to EPI and PSC 833 or coexposure with EPI and PSC 833.
Fig. 6 Increase in the intracellular levels of [3H]daunomycin measured in the CEM/A7R line are plotted over time in the absence (△) or presence of 1 μM PSC 833 (□), 1 μg/ml EPI (●), or 1 μM PSC 833 plus 1 μg/ml EPI (○).

in cells treated with EPI (P = 0.0000; Fig. 6), consistent with the increase in P-gp levels seen previously (Fig. 5). In contrast, the intracellular levels of [3H]daunomycin in cells treated with 1 μg/ml EPI plus 1 μM PSC 833 (or 1 μM PSC 833 alone) appeared similar to that in untreated CEM/A7R cells (P = 0.2513 and P = 0.1974, respectively; Fig. 6).

DISCUSSION

The studies reported herein were carried out in a cloned variant MDR cell line CEM/A7R established by continuous culture of the CEM/A7 cell line in the absence of DOX for over 2 years. The CEM/A7 line was initially selected for resistance to DOX from the human T-cell leukemia line CCRF-CEM (18).

Because the subcloned CEM/A7R line expresses very low levels of P-gp (Figs. 1–3 and 5), does not have detectable amplification of the MDR1 gene (18), and expresses low levels of MDR1 mRNA in the absence of a continuous selective pressure, it appears to be a useful model for studying clinical drug resistance.

Using this model, we have recently demonstrated the rapid up-regulation of MDR1 mRNA expression following a brief exposure to several anthracyclines including DOX, daunorubicin, or EPI (8). These increases in MDR1 levels were associated with an increase in P-gp expression. These data suggest that the induction of drug resistance may be the first step in the development of clinical resistance, with selection occurring as a secondary event. The induction of MDR1 mRNA by anticancer agents may operate at either the transcriptional or posttranscriptional level (25). However, small increases in MDR1 mRNA levels after the exposure of CEM/A7R cells to EPI for as little as 4 h suggests that changes in the rate of transcription may be important in this model (8).

In the present study, we have extended these findings and examined the effect of modulators on this induction phenomenon. As previously demonstrated, an increase in MDR1 mRNA levels (Figs. 1–3) was observed after the exposure of CEM/A7R cells to EPI. The increase in MDR1 mRNA was accompanied by an increase in P-gp levels (Fig. 5) and decreased [3H]daunomycin accumulation (Fig. 6). Both CyA and PSC 833 inhibited the increase in MDR1 mRNA levels induced by EPI only when CyA or PSC 833 were coincubated with EPI (Figs. 1–3). The capacity of CyA and PSC 833 to prevent the induction of MDR1 mRNA by EPI was dose dependent and correlated with their ability to alter the resistance profile of the untreated CEM/A7R line in a standard growth assay (Figs. 3 and 4).

In view of the fact that the exposure of the CEM/A7R cells to EPI alone leads to an increase in MDR1 mRNA levels, we had assumed that an additional increase in MDR1 mRNA would be observed with the addition of either PSC 833 or CyA. We had reasoned that this effect would result from the increase in intracellular levels of EPI known to accompany the exposure of MDR cells to biochemical modulators (22). In fact, the opposite occurred, and the induction of MDR1 mRNA levels seen after exposure of these cells to EPI alone was prevented. This effect was observed within 24 h of such exposure, and as such is unlikely to be due to the selection of less resistant cells. This conclusion is reinforced by the flow cytometry data (Fig. 5), which suggested that under the various conditions used there was a shift in MRK16 expression in the entire population of CEM/A7R cells (rather than a subset of cells). Furthermore, in any given population, the least resistant cells are more likely to be killed by the combination of EPI and PSC 833 (or CyA), suggesting that these experiments would have resulted in an apparent increase in MDR1 mRNA levels if cell selection was the basis for the observations reported herein. The fact that these effects on P-gp expression were not only related to the dose of PSC 833 or CyA (Fig. 3), but required the modulators to be coincubated with EPI (Fig. 1), suggests that the process of modulation and P-gp expression may be linked in some way.

Although the role of biochemical modulators in the regulation of the MDR1 gene have not been studied extensively, previous studies have demonstrated that the human MDR1 gene can be induced by environmental stresses (26, 27), including agents (28) that activate PKC as well as cellular differentiation agents (29, 30). To suppress the emergence of resistant cells, PKC inhibitors have been used to prevent the induction of MDR1 by cytotoxics. For example, the PKC inhibitor staurosporine prevented the drug-induced increase in MDR1 mRNA in a K562 human leukemia cell line following exposure to cytotoxic agents (31). In addition, the PKC inhibitor quercetin has been reported to prevent the arsenite-induced increase in MDR1 expression in a hepatocarcinoma cell line (32).

The mechanism underlying this effect of CyA (and PSC 833) on P-gp expression is uncertain, but may be mediated by interaction with factors that control gene transcription. In activated T cells, CyA prevents the transcriptional induction of IL-2 by inhibiting the translocation of the NFAT transcription complex to the nucleus (33). The NFAT complex consists of the NFATp DNA-binding subunit and homodimers or heterodimers.
of the fos and jun family proteins (34, 35) and has been implicated in the transcriptional regulation of several cytokine genes (36, 37). CyA is also able to cause the down-regulation of interleukin 3 expression by destabilization of the mRNA 3’ untranslated region which contains the (A + U)-rich element (38). The role of various transcription factors and their potential interaction with CyA or PSC 833 in this model of MDR are under further investigation in our laboratory. The effect on MDR1 mRNA stability is also being studied.

These data suggest that in the clinical context, the administration of biochemical modulators such as CyA or PSC 833 should be considered early in a course of chemotherapy to prevent the induction of P-gp, a phenomenon which appears to precede the selection of drug-resistant cells.

REFERENCES


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