Cyclosporin A Enhances Paclitaxel Toxicity against Leukemia and Respiratory Epithelial Cancers

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ABSTRACT
Multidrug resistance is probably the single greatest obstacle to successful systemic therapy of human cancer. We have reported that cyclosporin A (CsA) can overcome multidrug resistance and improve the efficacy of etoposide in a murine model of drug-sensitive leukemia. The combination of CsA and paclitaxel (PCL) was also significantly superior to either drug alone against murine P388 (sensitive) and L1210 (resistant) leukemia. Lung cancer cells provide an ideal model system to study this phenomenon because both de novo and acquired drug resistance occur. Standard chemotherapy for advanced lung cancer is poorly effective, and although PCL is one of the most active new agents for this disease, responses occur in only 20% of patients. In vitro, CsA significantly enhanced the efficacy of PCL against lung (Lu-CSF-1 and 3LL) and oropharyngeal (CSCC-20) cancer cell lines. The combination also produced an increased expression of interleukin 1β, a cytokine known to inhibit the growth of Lu-CSF-1 cells. CsA alone had little or no anti-proliferative activity in vitro and did not alter PCL transport. These results indicate that the activity of chemotherapy modulators may extend beyond mitigation of drug resistance to enhancement of therapeutic efficacy against drug-sensitive tumor cells in vitro and in vivo.

INTRODUCTION
One of the most significant impediments to successful chemotherapy is drug resistance. Recent studies have demonstrated the ability of cyclosporine to reverse vincristine and daunorubicin resistance in several cell lines including a multidrug-resistant human lung cancer cell line (1) and to reverse cis-platinum resistance in xenotransplanted fresh human ovarian carcinoma (2). We have reported that the addition of CsA to etoposide enhances the survival of mice bearing drug-sensitive L1210 leukemias and can produce immunity to leukemia re-challenge (3). Only a few clinical trials have used CsA to modulate drug resistance. We conducted a Phase I/II trial of CsA/cis-platinum/etoposide in patients with advanced lung cancer (4). Although no overall increase in response rate was observed, patients treated with 1–2 mg/kg CsA had a significant improvement in two-year survival compared to historical controls.

Both Lu-CSF-1 and CSCC-20 cells express cytokines such as IL-1β and IL-6 that influence their growth and response to drug treatment. Experiments in our laboratory with Lu-CSF-1 and CSCC-20 cells have shown alterations in IL-1β expression that correlate with resistance to cis-platinum. A recent study has reported that endogenous IL-6 can also mediate resistance to cis-platinum and etoposide in prostate cancer cell lines (5). Cytokines have also been implicated in the mechanism of resistance to daunorubicin and PCL, and the expression of cytokine genes can be modulated by PCL in at least one cell type (6–7). Furthermore, CsA has been shown to modulate cytokine expression in keratinocytes, Burkitt lymphoma cell lines, and T lymphocytes (8–9).

We found that CsA significantly enhanced the effectiveness of PCL against experimental murine leukemia. Possible mechanisms of enhancement include pharmacological interaction in vivo, modulation of host immune response, direct antitumor effects (for example, by increasing intracellular PCL transport or optimizing tubulin binding), or indirect antitumor effects via alteration in autologous tumor growth factors. We studied these mechanisms using assays of cell proliferation, drug transport, and cytokine gene expression.

MATERIALS AND METHODS
Cell Lines. Lu-CSF-1 is a human lung adenocarcinoma and was a generous gift of H. P. Koeffler (Cedars-Sinai Medical Center, Los Angeles CA; Ref. 10), CSCC-20 is a human squamous oropharyngeal carcinoma and was a generous gift of J. Pitcock (University of California, Irvine, CA; Ref. 11), and 3LL is the murine Lewis lung carcinoma cell line (12). L0 and L100 are drug-sensitive and drug-resistant lymphatic leukemia cells (13). Lu-CSF-1, CSCC-20, and 3LL cells were grown under standard conditions in high-glucose DMEM (Sigma Chemical Co., St. Louis, MO) with 5% FCS and 5% newborn calf serum

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(Irvine Scientific, Irvine, CA). L0 and L100 were grown in RPMI 1640 with 20% FCS and 2 mM glutamine.

Cell Proliferation. Cells were plated in triplicate in T-25 flasks and were exposed for 4 h to 10–50 ng/ml PCL with or without 1 μM CsA on day 1. After removing the drugs from the media, cells were grown for the times indicated in the results and were counted using a Coulter counter (Coulter Electronics, Hialeah, FL).

MTT Assay. Cells were seeded in 96-well plates in sextuplicate and treated with PCL and/or CsA as described above. MTT assay was performed on days 5–7, according to standard protocol.

Leukemic Mice. Groups of 10 or more C57BL × DBA/2 F1 (hereafter called BD2F1) mice were inoculated i.p. with 1 × 10³ L1210 leukemia cells or 1 × 10⁶ P388 leukemia cells on day 0. Host mice were treated with PCL (9 mg/kg i.p.) with or without 10 mg/kg CsA i.p. on days 1, 5, and 9. Animal care was in accordance with institutional guidelines.

PCL Transport. Cells were seeded in triplicate in 24-well plates of 5 × 10³ cells/well and exposed to 30 nM [³H]PCL (1.0 mCi/ml, 14 Ci/mmol; Moravek Biochemicals, Brea, CA) with or without 1 μM CsA at 37°C. After the desired incubation time, cells were washed and then lysed in 10% SDS. Efflux measurements were obtained after a 4-h loading with 30 nM [³H]PCL and/or 1 μM CsA as described above. Cells were then washed twice with PBS, placed in fresh medium with or without CsA, and analyzed after the time intervals indicated. Incorporated [³H]PCL was determined by liquid scintillation counting.

Northern Blotting. Subconfluent cells in 75-cm² flasks were treated with PCL and/or CsA as described above. RNA was extracted by detergent lysis and phenol-chloroform extraction 48 h after treatment. Northern blots of 20 μg RNA/lane were probed as indicated in the figure legends. Blots were washed to 0.1 SSC at 65°C, and autoradiographs were exposed for 24–96 h at –80°C. Signals were quantitated using a scanning densitometer (Bio-Rad Laboratories) with Molecular Analyst software, and results were adjusted for loading by normalization to expression of the ribosomal gene rpL32.

cDNA Probes. GM-CSF cDNA probe (EcoRI, 0.8 kb) from pCSF1 and IL-6 cDNA probe (XhoI, 1.2 kb) from pXM309 were generous gifts of S. Clark (Genetics Institute, Cambridge, MA). IL-1β cDNA probe (PstI, 0.9 kb) was from pA-26 (14). β-actin probe was from pHFA3'UT (EcoRI/BamHI, 0.7 kb; Ref. 15). Transforming growth factor-B1 probe was from pBCL1 (EcoRI, 1.0 kb; Ref. 16). MDR1 cDNA probe was a generous gift of M. Gottesman (NIH). MDR3 cDNA probe was obtained from the American Type Culture Collection. plpL32 cDNA probe was a generous gift of A. Maity (University of Pennsylvania, Philadelphia, PA; Ref. 17). Probes were labeled with [³²P]dCTP (DuPont New England Nuclear) by random priming.

ELISA. Culture supernatants were collected, spun in a clinical centrifuge at 2000 rpm to remove cells and debris, and stored in aliquots at –20°C. Assays were performed according to the manufacturer’s instructions (R&D Systems, Inc.). Results are presented as picograms/10⁶ cells.

Statistical Analysis. Survival data for leukemic mice were analyzed using Kaplan-Meier methods as described previously (3). Data for MTT and cell proliferation assays were analyzed by single-factor ANOVA. Paired comparisons of means were evaluated by Student’s t test, using a Bonferroni adjustment for number of comparisons.

RESULTS

Based on our documentation of improved survival and development of tumor immunity in leukemic mice treated with the combination of etoposide and CsA, we hypothesized that CsA might also improve the efficacy of newer chemotherapy drugs. We have conducted a pilot study of CsA with etoposide and platinum in lung cancer patients (4) and elected to study PCL and CsA with the aim of devising another clinical trial if the combination seemed promising. Because demonstration of in vivo activity is essential to the development of a clinical trial, initial evaluation of PCL/CsA was performed using our established leukemia model in BD2F1, mice (3). Confirmation of in vitro activity and mechanistic studies were carried out in respiratory epithelial cancer cell lines.

CsA Improved in Vivo Efficacy of PCL in a Murine Leukemia Model System. BD2F1 mice bearing L1210 leukemia cells had a marked statistically significant survival advantage when treated with PCL + CsA compared to either drug alone (Fig. 1A). Median survival was increased from 8 days (untreated) to 9 days by PCL and to 13 days by PCL + CsA (P < 0.0003). Note that this regimen of PCL alone is ineffective against L1210 leukemia but is made highly effective by addition of CsA. Similar results were obtained for P388 leukemia (Fig. 1B). PCL enhanced the survival of leukemic mice from 9 to 18 days, and the combination of PCL and CsA further improved survival to 24 days (P < 0.0003). CsA alone did not affect the survival of mice bearing either L1210 or P388 leukemia.

PCL Toxicity against Respiratory Cancer Cell Lines Was Enhanced by CsA. Cells were treated in vitro with PCL ± CsA for 4 h and allowed to proliferate in the absence of drug for 5 days to mimic conditions of bolus administration in vivo. Drug doses were chosen to approximate achievable serum concentrations. Fig. 2 shows cell proliferation expressed as the percentage of control (untreated) cell number after PCL, CsA, or PCL + CsA treatment. All three cell lines were relatively insensitive to PCL alone, however, CsA increased the toxic effects of 50 ng/ml PCL by 2-fold in Lu-CSF-1 and 3LL (P < 0.00001 for PCL + CsA compared to PCL alone for both cell lines) and by 1.5-fold in CSCC-20 (P < 0.00001). CsA alone was nontoxic in vitro. Results were similar when CsA was replaced with the nonimmunosuppressive analogue PSC833.

Cremophor EL Was Nontoxic in Vitro. It has been reported that the diluent most commonly used for both PCL and CsA, cremophor EL, may contribute to the antiproliferative effects of PCL and CsA (18, 19). The maximum concentration of cremophor to which our cells were exposed was 0.002% (2.38 × 10⁻² μM). Although it is unlikely that such a low concentration would contribute to toxicity, it was necessary to rule out cremophor EL as an important contributor to the combined cytotoxicity of CsA + PCL. Lu-CSF-1, CSCC-20, and 3LL cells were exposed in triplicate to the maximum concentration of cremophor in the combination treatment arm in the absence of CsA/PCL and counted after 5 days. In all cases, the
Survival of leukemic mice treated with PCL ± CsA. Groups of 10 BD2F1 mice were inoculated i.p. with $1 \times 10^5$ L1210 leukemia cells (A) or $1 \times 10^6$ P388 leukemia cells (B) on day 0. Mice were treated with PCL (9 mg/kg i.p.) with or without CsA (10 mg/kg i.p.) on days 1, 5, and 9. Survival curves are shown.

Toxicity of cremophor EL was negligible (cell numbers were identical to control ± 10%).

**Long Exposures to CsA Were More Effective than Shorter Exposures.** We evaluated the effects of bolus versus continuous exposure to CsA after PCL bolus in vitro using an MTT assay (Fig. 3). CSCC-20 cells were used in this experiment because the previous data indicated the least effect of combined PCL + CsA in this cell line. Cells were treated with 50 ng/ml PCL with 0.5 μM CsA for 30 min before and during the 4-h 50 ng/ml PCL treatment (Lane 2), during PCL treatment and throughout the 5-day incubation (Lane 3), or during PCL treatment only (Lane 4). Control wells were untreated (Lane 1) or were exposed to CsA alone for 5 days (Lane 5). Antiproliferative effects were greatest in cells treated with PCL + CsA followed by continuous CsA exposure. Continuous CsA alone was also mildly antiproliferative, but much less so than the combination ($P < 0.01$ for CsA versus control; $P < 0.0003$ for CsA versus PCL).

**MDR Was Not Involved in Enhancement of PCL Toxicity by CsA in Vitro.** CsA is a known modulator of the MDR phenotype in certain cancer cells (20). To determine whether the mechanism of interaction between CsA and PCL in our cell lines involved modulation of the P-glycoprotein drug resistance membrane pump, Lu-CSF-1 and CSCC-20 cells were treated with 50 ng/ml PCL ± 1 μM CsA for 1, 4, and 24 h; RNA was extracted; and Northern blots were probed for MDR1 and MDR3 mRNA. No MDR expression was detected in either cell line. Fig. 4 shows lack of MDR1 expression in Lu-CSF-1 and CSCC-20, with the vincristine-resistant L100 cell line and its sensitive parent L0 shown as positive and negative controls.

To rule out expression of another efflux pump and evaluate the effect of CsA on intracellular PCL concentration, [3H]PCL uptake was measured. Fig. 5A shows intracellular PCL concentration for Lu-CSF-1 cells as determined by liquid scintillation counting. It is clear that CsA did not increase intracellular PCL concentration, which reached a plateau after about 4 h. Efflux studies showed that intracellular PCL concentration remained
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Fig. 3  Optimal duration of CsA exposure in CSCC-20 cells. Cells were plated in sextuplicate in 96-well plates. After 12 h, wells were untreated (Lane 1) or treated with 0.5 μM CsA for 30 min before and during the 4-h 50 ng/ml PCL treatment (Lane 2), during PCL treatment and throughout the 5-day incubation (Lane 3), or during PCL treatment only (Lane 4). Control wells were exposed to CsA alone for 5 days (Lane 5). Toxicity was determined by MTT assay. Data are shown as absorbance at 540 nm. SDs are shown.

stable for at least 24 h in the presence or absence of CsA (Fig. 5B). In contrast, [3H]PCL efflux from L100 cells, shown as a positive control, was essentially complete after only 1 h.

**PCL/CsA Modulated Autologous Growth Factor Expression \textit{in Vitro}**. Lu-CSF-1 and CSCC-20 produce autologous cytokines that influence growth rates and response to antiproliferative agents such as retinoids (Ref. 21 and data not shown). Fig. 6 shows cytokine mRNA levels 48 h after drug treatment. This time point was chosen because it likely represents a steady state. Transient nonspecific alterations in cytokine gene expression are common in these cells after acute toxic insults and usually subside within 24 h. Quantitation of cytokine gene expression was by densitometric analysis with correction for loading by normalization to the rRNA rpL32.

Neither CsA nor PSC833 (PSC) alone significantly affected cytokine mRNA levels with the exception of IL-6 in CSCC-20, which was reduced 33 and 23% by CsA and PSC, respectively. IL-1β mRNA was increased in a dose-dependent fashion by PCL in both cell lines but was much more affected in Lu-CSF-1 than in CSCC-20 (1.1- and 1.3-fold increase for 10 and 50 ng/ml for CSCC-20 compared to 2.5- and 4.7-fold increase for Lu-CSF-1). In Lu-CSF-1, CsA did not affect this increase, but in CSCC-20, CsA completely suppressed the increase in IL-1β by PCL and in fact resulted in a 20–30% suppression of IL-1β levels. In Lu-CSF-1, IL-6 behaved similarly to IL-1β. GM-CSF was also increased by 10 ng/ml PCL, but there was no further increase with 50 ng/ml. CSCC-20 does not express GM-CSF, but IL-6 was suppressed in a dose-dependent manner by PCL (42 and 50%), and suppression was even greater after addition of CsA (52 and 60%). Neither CsA nor PCL had any significant effect on expression of transforming growth factor β1.

ELISA was performed on culture supernatants from the cells from which RNA was extracted above. Results confirmed the Northern blot data showing a 4.2-fold increase in IL-1β after 50 ng/ml PCL for Lu-CSF-1 (25.6 versus 6.1 pg/10⁶ cells) and a 2.5-fold increase for CSCC-20 (11.5 versus 4.7 pg/10⁶ cells).

**DISCUSSION**

There is considerable interest in strategies for minimizing drug resistance in cancer treatment protocols. Non-small cell lung cancer is a prime candidate for such strategies because: (a) over 150,000 lung cancer deaths are expected in 1995; (b) the majority of patients are diagnosed only after tumors are too advanced to be cured with local treatment; (c) it is poorly responsive to systemic chemotherapy; and (d) it is often multidrug-resistant. Our data show that growth suppression by PCL, an effective new agent for lung cancer treatment, can be augmented \textit{in vivo} and \textit{in vitro} by cyclosporine. It is likely that several mechanisms contribute to this interaction, and it is possible that the crucial mechanisms for the \textit{in vivo} and \textit{in vitro} enhancement may differ.

The combination of PCL and cyclosporine was markedly more effective \textit{in vivo} against two experimental murine leuke-
mias than PCL alone. Surprisingly, addition of CsA overcame the primary resistance of L1210 leukemia to PCL. In vitro, CsA consistently enhanced PCL activity against lung and oropharyngeal cancer cells at a CsA dose that is achievable in patients (22) over a range of PCL concentrations likely to be clinically useful. In vitro increase of PCL toxicity by CsA indicates a probable contribution of a direct mechanism of action that does not involve pharmacokinetics or immune modulation. This conclusion is also supported by the similar activities of PSC and CsA in vitro. The implication is that neither pharmacokinetic nor immune effects alone completely explain the in vivo augmentation of PCL toxicity by CsA.

It has been reported that CsA can mitigate the effects of MDR expression in vitro (20). We show that a beneficial interaction occurs between CsA and PCL even in cells that do not express the MDR phenotype. The mechanism of action of the combination in non-MDR cells is not clear, but one possibility might involve alterations in tubulin binding and/or changes in cell cycle distribution.

Both PCL and CsA have been reported to influence production of cytokines under some circumstances. PCL has increased GM-CSF expression in murine B cells by stabilizing its mRNA (23). Conversely, CsA has slowed the growth of IL-3-dependent leukemic cells by destabilizing IL-3 mRNA (24). We have previously shown that IL-1β can be antiproliferative for Lu-CSF-1 cells and that the mechanism of growth inhibition by retinoids may involve augmentation of IL-1β expression in these cells (25). This may be one mechanism by which PCL inhibits Lu-CSF-1 proliferation as well. The data suggest, however, that modulation of cytokine gene expression is not likely
to contribute significantly to \textit{in vitro} enhancement of PCL toxicity by CsA in CSCC-20 cells. We have reported that induction of immunity to leukemia rechallenge in B2DF1 mice after CsA/etoposide treatment involves a population of CD8$^+$ lymphocytes. Modulation of cytokine expression by tumor cells and/or host immune cells could contribute to \textit{in vivo} efficacy of PCL + CsA by enhancing recruitment of these CD8$^+$ cells.

It is clear from the data described here that both \textit{in vitro} and \textit{in vivo} phenomena play a role in enhancement of PCL activity by CsA. It is still uncertain which, if any, of the interactions described can explain the beneficial antitumor effects of combined CsA + PCL. Alterations in tubulin binding or modulation of growth factor production by tumor cells themselves or by surrounding tissues may be profitable areas of investigation.

There has not, as yet, been agreement on the best method of administration of CsA as an adjunct to cytotoxic chemotherapy treatment in clinical trials. The most common routes have been i.v. bolus and continuous infusion. PCL is most often given as a 3-h infusion. Our data suggest a rationale for the use of repeated doses or continuous infusion CsA based on enhanced \textit{in vitro} antiproliferative activity.

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\section*{REFERENCES}


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