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ORIGINAL ARTICLE

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A reexamination of PSC 833 (Valspodar) as a cytotoxic agent and in combination with anticancer agents

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Abstract *Background*: The cyclosporins have been thought as being mainly immunosuppressive agents which interfere with the function of the MDR pump and thus play a role in resistance to drug anticancer effects. We reexamined their cytotoxicity in defined cell lines both as single agents and in combination with agents which may be of value in human malignant disease. *Methods*: Cells were grown to confluence following inoculation at 5000–8000 cells/well in 96-well dishes, and growth patterns and death were determined by an MTT assay. Median effect analysis was used to look for synergy, additive effects, or antagonism between the cyclosporins and drugs with antitumor effects in humans. *Results*: Cyclosporin A and PSC 833 were found to have cytotoxic activity at clinically achievable concentrations in breast, leukemia, and prostate cell lines. Synergistic or additive effects were demonstrated in all three prostate cell lines when PSC 833 was combined with estramustine, etoposide, ketoconazole, suramin, or vinorelbine in the prostate cancer cell lines. Cell line-selective additive effects or synergism were also identified with bicalutamide, carboplatin, cisplatinum, *cis*-retinoic acid, dexamethasone, 5-fluorouracil, liarozole, and *trans*-retinoic acid. *Conclusions*: PSC 833 or cyclosporin alone or in combination with other agents may have an anticancer effect independently of their modulatory action on MDR. Several of the synergistic combinations which are not mediated by the MDR pump need to be tested in vivo for efficacy.

Key words Cyclosporin · Drug resistance · Cytotoxicity

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Introduction

Intrinsic drug resistance and the poor therapeutic index of the currently available agents have limited the treatment of solid tumors with pharmacological agents. This problem is especially true in prostate cancer where significant antitumor responses to chemotherapeutic agents have been unusual [1] until the advent of estramustine phosphate-docetaxel combinations [2]. However, despite evidence of an antitumor effect of this combination in humans, there is an urgent need for new, more effective agents and combinations of agents in this disease [3]. In preclinical models and some human hematopoietic malignancies [4], energy-mediated glycoprotein pumps have been associated with resistance to commonly used cytotoxic substances. However, the use of agents to block this pump has demonstrated a disappointing lack of success in the treatment of human malignancy [5]. These results have been attributed to the discovery of additional mechanisms of resistance involving additional proteins associated with multiple drug resistance (MDR) [6], kinetic resistance, tumor vascularity, and inhibition of the apoptotic process [7].

However, the currently available agents known to block the MDR pump may have additional effects on the growth of tumor cells [8–11]. Therefore, if combined with the appropriate agent, they still may be of use in human malignant disease by directly affecting cells expressing MDR and by indirectly inhibiting other pathways. As the non-immunosuppressive cyclosporin derivative, PSC 833, is currently in clinical trials, well tolerated, and may have additional antitumor attributes [8], we used our previously described cell systems [12] to evaluate the potential of PSC 833 in the treatment of prostate cancer. In addition, as cell effects may be either tissue-specific or cell line-specific, we also examined the effect of PSC 833 in the breast cancer cell lines MCF_7 and MCF_7ADR (an anthracycline-resistant line), and the leukemia cell lines CCRF-SB, MOLT-4, and AML-193.

Materials and methods

The reagents and methodology have been previously described in detail [12, 13]. In brief, three human prostate cancer cell lines (DU 145, PC 3, LnCaP), the leukemia lines (CCRF-SB, MOLT-4, and AML-193) and the human breast cancer cell line (MCF_7) were obtained from the ATTC (Rockville, Md.). Of the three prostate cancer-derived cell lines tested for androgen receptor activity with radioactive R 1881 (methyltrienolone), only LnCaP cells show substantial binding to this radioactive ligand [14]. The anthracycline-resistant breast cancer cell line MCF_7ADR was a gift from Dr. Kenneth Cowan (National Cancer Institute, Bethesda, Md.). Cells were grown to confluence in RPMI-1640 (Gibco, Grand Island, N.Y.) with 10% fetal bovine serum in T 150 tissue culture flasks (Corning Glass Works, Corning, N.Y.). For the LnCaP cells, the medium was McCoy's 5A (Gibco). All cell lines were cultured in an atmosphere containing 5% CO_2 with the medium containing penicillin 100 μg/ml, streptomycin 0.25 μg/ml, and 2 m*M* glutamine (Gibco). All cell lines were tested for mycoplasma contamination. Cell viability by Trypan blue exclusion was maintained greater than 95%. Cells were inoculated at 5000–8000 cells/well in 96-well Falcon dishes (Baxter Scientific, McGraw Park, Ill.) in a total volume of 200 μl/well. The cells were washed and recultured in fresh medium daily [13].

After 72 h of incubation, centrifugation and removal of 100 μl medium, 50 μl of a 1 mg/ml solution of MTT (3-[4,5-dimethylthiazole 2-yl]-2,5-diphenyl tetrazolium bromide; Sigma, St. Louis, Mo.) was added and the plates read in a colorimetric assay at 595 mm on a Biorad Microplate reader (Biorad, Hercules, Calif.) [12]. All IC_{50} (drug dose inhibiting 50% of cell growth) determinations were done with cells in log-phase growth. The growth curves were evaluated over 72 h using the EZ ED50 program (Perrella Scientific, Conyers, Calif.) with all points determined at least in triplicate. Vehicle alone with the initial solvent used to dissolve the test agent diluted to the final incubation concentration was used as a control. In addition, as an internal control, the EZ ED50 program automatically subtracts the control incubation (solvent with cells) from the test sample values. The following drugs were obtained from Sigma: cyclosporin, doxorubicin, etoposide, 5-fluorouracil, dexamethasone, all-*trans*-retinoic acid, hydroxyflutamide, and paclitaxel. Additional agents were PSC 833 (Novartis), docetaxel (Rhone-Poulanc Rorer), estramustine (Kabi Pharmacia), vinorelbine (Glaxo Wellcome), liarozole (Janssen Research), suramin (NCI), bicalutamide (AstraZeneca), and ketoconazole (Janssen).

The presence of synergism, additive effects, or antagonism between two or three agents was tested in these cell lines using median effect analysis [15] and the previously described computer program of Chou and Chou [16]. As described previously [13], fixed drug combinations above and below the IC_{50} were examined with a minimum of eight points to determine the median effect curve. All points were done in triplicate. This model is based on the median effect equation of Hill:

$$f_a/f_u = [D/D_m]^m$$

where f_a is the fraction of cells affected, f_u the fraction not affected ($1 - f_a$), D the dose of the drug, D_m the dose needed to cause a median effect, and m the slope of the median effect curve [15]. The combination index (CI) is further defined as:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} + \frac{\alpha(D)_1(D)_2}{(D_x)_1(D_x)_2},$$

where $\alpha = 0$ for drugs with mutually exclusive modes of action (no drug or biologic interaction between agents), and $\alpha = 1$ for drugs with nonexclusive actions (drug interaction).

Plots of log dose versus $\log(f_a/1 - f_a)$ gave intersecting slopes suggesting interaction [15] between the PSC 833 or cyclosporin A and the other agents. The mutually nonexclusive assumption was therefore chosen with the CI determined where $CI < 1$ is synergistic, $CI = 1$ is additive, and $CI > 1$ is antagonistic. In tabular form, the CI is at the point at which 50% of the cells are affected (fa_{50}) since the median effect formula may be unreliable at the extremes as it represents a linear approximation of a nonlinear function [17].

Results

The IC_{50} values for 20 agents for 24, 48, and 72 h incubation times in the three prostate cell lines were determined. Vehicle alone with the initial solvent used to dissolve the test agent and then diluted to the final incubation concentration did not give cytotoxic effects in this assay system. Table 1 shows the values for a 72-h exposure to a given drug. All the cell lines were particularly sensitive to doxorubicin and docetaxel with intermediate sensitivity in the micromolar range to PSC 833, cyclosporin A, paclitaxel, vinorelbine, and

Table 1 IC_{50} values (in μ*M*) for 72-h incubation with daily change of medium. Values are means ± SD from three to seven experiments

Drug	DU 145	PC 3	LnCaP
Bicalutamide	170.92 ± 43.66	106.97 ± 14.74	25.625 ± 7.541
Carboplatin	92.955 ± 6.566	79.805 ± 9.269	40.912 ± 3.755
Cisplatin	4.646 ± 1.461	9.949 ± 3.346	5.943 ± 1.676
Cyclosporin A	6.805 ± 0.847	7.363 ± 1.690	4.793 ± 1.107
Dexamethasone	168.24. ± 52.28	382.19 ± 125.38	213.41 ± 69.54
Docetaxel	0.4713 ± 0.0592	0.2504 ± 0.0458	0.2844 ± 0.0306
Doxorubicin	0.0069 ± 0.0021	0.0258 ± 0.0168	0.0202 ± 0.0010
Estramustine	20.521 ± 4.140	15.185 ± 0.917	10.970 ± 3.348
Etoposide	0.2782 ± 0.0061	0.1218 ± 0.0220	2.4119 ± 0.3289
5-Fluorouracil	5.730 ± 2.319	2.367 ± 1.606	9.208 ± 1.227
Hydroxyflutamide	164.93 ± 36.40	242.68 ± 58.31	92.11 ± 5.87
Ketoconazole	53.31 ± 11.55	15.762 ± 3.335	33.25 ± 4.67
Liarozole	116.64 ± 3.50	72.47 ± 5.10	74.83 ± 9.43
Paclitaxel	12.352 ± 0.665	15.733 ± 4.653	8.449 ± 0.794
PSC 833	5.185 ± 0.561	3.330 ± 0.344	2.891 ± 0.772
Cis-retinoic acid	57.68 ± 9.94	51.32 ± 5.48	39.03 ± 8.38
Trans-retinoic acid	40.86 ± 5.06	37.99 ± 1.37	23.046 ± 9.416
Suramin	88.575 ± 22.405	64.470 ± 18.271	34.156 ± 7.531
Vinblastine	0.0025 ± 0.0009	0.0044 ± 0.0016	0.0072 ± 0.0011
Vinorelbine	4.560 ± 0.472	5.322 ± 1.319	3.765 ± 1.562

5-fluorouracil (Table 1). Hormonal agents and retinoids demonstrated less inhibitory activity with LnCaP being the most sensitive cell line. Cyclosporin A was significantly less active than PSC 833 in these cell lines (Table 1). These effects were independent of the origin of the malignant cells as PSC 833 and cyclosporin A also demonstrated cytotoxic activity in the cell lines MCF_7, MCF_7ADR, CCRF-SB, MOLT-4, and AML-193 (Table 2).

Because of the interest in the non-immunosuppressive activity of PSC 833, and the finding of significant cytotoxic effects against all three prostate cancer cell lines, MCF_7, and the leukemia cell lines, both PSC 833 and cyclosporin were used as the initial agent and various compounds were then added simultaneously in the cell culture to look for synergy. The results for PSC 833 using the median effect analysis at fa_{50} are shown in Table 3. Some of the effects were cell line-specific so that synergism may have occurred in one cell line and antagonism in another under identical conditions. However, PSC 833 demonstrated synergism or additive effects with estramustine, etoposide, ketoconazole, suramin, and vinorelbine in all three cell lines. MDR-mediated drugs such as doxorubicin or paclitaxel did not exhibit a synergistic effect when combined with PSC 833 in these cell lines (Table 3). This is in contrast to the findings with vinorelbine which also has one form of resistance which is MDR-mediated. Cyclosporin A, in combination with liarozole, was synergistic in two of the three prostate cancer cell lines. Cyclosporin also was synergistic with estramustine in all three cell lines (Table 4).

As the median effect equation allows three different agents to be evaluated for synergy in combination, PSC 833 was also combined with several doublets that have been in clinical trials or suggested for such trials. No advantage was seen for triplet therapy (Table 5) in that the synergistic effect was not increased for most of the combinations tested. However, in the case of sequential exposure of the prostate cancer cell lines to estramustine with PSC 833 followed by exposure to *trans*-retinoic acid, marked cytotoxic synergism was identified. Simultaneous exposure of these three agents was synergistic in one prostate cell line and antagonistic in LnCaP and DU 145 (Table 5), suggesting schedule dependency.

Table 2 IC_{50} values (in μM) for 72-h incubation with daily change of medium for eight cell lines. Values are means ± SD from two to seven experiments

Cell line	PSC 833	Cyclosporin A
DU 145	5.185 ± 0.561	6.805 ± 0.847
PC 3	3.330 ± 0.344	7.363 ± 1.690
LnCaP	2.891 ± 0.772	4.793 ± 1.107
MCF_7WT	6.840 ± 1.210	1.0798 ± 0.0763
MCF_7ADR	3.743 ± 0.352	4.7125 ± 0.4511
CCRF-SB	2.3651 ± 0.9455	5.549 ± 0.608
MOLT-4	1.4897 ± 0.1758	2.848 ± 0.345
AML-193	1.5186 ± 0.2875	2.536 ± 0.313

Discussion

The antitumor activity of cyclosporin A was first identified in our laboratory in mice bearing S 180, P815, Ehrlich, L1210 Leukemia, and Taper liver tumors [18]. Antiproliferative activity of cyclosporin in lymphoid neoplasia has also been noted by other investigators [19–21]. Part of the cytotoxic effect of cyclosporin has been attributed to depletion of polyamines [22]. However, cyclosporin A has not assumed a role as a cytotoxic agent in the treatment of human malignancies.

The cyclosporin analogue PSC 833 is of interest as it, unlike cyclosporin A, is non-immunosuppressive. Although this agent was developed to inhibit the MDR protein (MDR-1), our studies have suggested that PSC 833 has activity similar to more classical cytotoxic drugs in our three prostate cell lines, in two human breast

Table 3 CI values at fa_{50} for 72 h simultaneous two-drug combinations with PSC 833 with a mutually nonexclusive assumption. Values are means ± SD from two to four experiments

Drug combination	DU 145	PC 3	LnCaP
PSC 833 + bicalutamide	1.04 ± 0.06	1.03 ± 0.04	2.97 ± 0.20
PSC 833 + carboplatin	0.58 ± 0.02	0.63 ± 0.03	1.58 ± 0.18
PSC 833 + cisplatin	1.14 ± 0.15	1.36 ± 0.11	1.02 ± 0.10
PSC 833 + cyclosporin A	1.76 ± 0.25	1.14 ± 0.05	3.65 ± 0.37
PSC 833 + dexamethasone	2.62 ± 0.08	2.01 ± 0.08	0.56 ± 0.01
PSC 833 + docetaxel	3.20 ± 0.11	1.77 ± 0.13	1.91 ± 0.07
PSC 833 + doxorubicin	2.53 ± 0.27	1.90 ± 0.09	2.51 ± 0.39
PSC 833 + estramustine	0.56 ± 0.02	0.81 ± 0.01	0.50 ± 0.02
PSC 833 + etoposide	0.95 ± 0.11	0.67 ± 0.07	0.81 ± 0.04
PSC 833 + 5-fluorouracil	0.47 ± 0.14	2.16 ± 0.59	0.84 ± 0.01
PSC 833 + hydroxyflutamide	1.54 ± 0.10	1.94 ± 0.13	1.89 ± 0.06
PSC 833 + ketoconazole	1.05 ± 0.05	0.42 ± 0.03	0.73 ± 0.03
PSC 833 + liarozole	0.81 ± 0.02	0.90 ± 0.01	1.68 ± 0.05
PSC 833 + paclitaxel	3.46 ± 0.20	5.82 ± 0.48	8.90 ± 0.51
PSC 833 + *cis*-retinoic acid	0.66 ± 0.07	1.74 ± 0.09	2.72 ± 0.21
PSC 833 + *trans*-retinoic acid	2.07 ± 0.10	0.98 ± 0.05	0.76 ± 0.01
PSC 833 + suramin	1.02 ± 0.03	0.99 ± 0.02	0.78 ± 0.01
PSC 833 + vinblastine	1.01 ± 0.13	1.39 ± 0.11	1.07 ± 0.06
PSC 833 + vinorelbine	0.69 ± 0.03	0.88 ± 0.08	1.01 ± 0.08

Table 4 CI values at fa_{50} for 72 h two-drug combinations with cyclosporin A with mutually nonexclusive assumptions. Values are means ± SD from two to four experiments

Drug combination	DU 145	PC 3	LnCaP
Cyclosporin A + dexamethasone	0.76 ± 0.02	2.13 ± 0.06	0.72 ± 0.01
Cyclosporin A + etoposide	1.63 ± 0.27	1.38 ± 0.09	1.35 ± 0.18
Cyclosporin A + liarozole	0.93 ± 0.02	1.24 ± 0.05	0.80 ± 0.01
Cyclosporin A + PSC 833	1.76 ± 0.26	1.14 ± 0.05	3.65 ± 0.37
Cyclosporin A + estramustine	0.63 ± 0.02	0.46 ± 0.01	0.85 ± 0.02
Cyclosporin A + vinblastine	1.40 ± 0.20	1.42 ± 0.12	1.45 ± 0.14

Table 5 CI values at fa_{50} for 72 h simultaneous and sequential three-drug combinations with PSC 833 with a mutually nonexclusive assumption. Values are means ± SD from two to four experiments (*DOC* docetaxel, *EST* estramustine, *PAC* paclitaxel, *SIM* simultaneously)

Drug combination	DU 145	PC 3	LnCaP
PSC + EST + liarozole (SIM)	0.52 ± 0.04	0.94 ± 0.02	1.17 ± 0.03
PSC + EST + *trans*-retinoic acid (SIM)	1.35 ± 0.04	0.60 ± 0.02	1.13 ± 0.02
EST/PSC to *trans*-retinoic acid 24 to 48 h	0.16 ± 0.09	0.74 ± 0.02	0.33 ± 0.01
Trans-retinoic acid to EST/PSC 24 to 48 h	1.05 ± 0.03	1.35 ± 0.04	1.34 ± 0.07
PSC + DOC + EST (SIM)	1.63 ± 0.04	1.44 ± 0.03	2.64 ± 0.10
PSC + PAC + EST (SIM)	1.58 ± 0.20	0.85 ± 0.14	0.97 ± 0.13
EST/DOC to PSC 24 to 48 h	0.73 ± 0.02	0.94 ± 0.01	1.20 ± 0.03
PSC to EST/DOC 24 to 48 h	0.78 ± 0.03	1.20 ± 0.04	0.72 ± 0.02
EST/PSC to DOC 48 to 24 h	0.25 ± 0.07	1.34 ± 0.09	0.62 ± 0.03
DOC to EST/PSC 24 to 48 h	1.28 ± 0.08	2.39 ± 0.07	1.16 ± 0.04
PSC + DOC + dexamethasone (SIM)	1.37 ± 0.05	2.19 ± 0.06	0.85 ± 0.01
PSC + PAC + dexamethasone (SIM)	1.17 ± 0.12	0.61 ± 0.07	1.52 ± 0.12

cancer cell lines (one with high resistance to anthracyclines), and in three leukemia cell lines. Prior studies of our lines have identified the presence of MDR in DU 145 and PC-3 but not in LnCaP cells [23] and we therefore utilized all three cell lines in our drug combination studies. PSC 833 has been reported to alter glycolipid metabolism of tumor cells [8], and activate ceramide synthesis [24]. We have shown that the polyamines putrescine, spermine, and spermidine reverse PSC 833 cytotoxicity in the prostate cancer cell lines. Furthermore, PSC 833 does not seem to complex with these polyamines [25]. Hence, the effect of this agent may be pleiotropic in malignant cells and not confined to resistance reversal. Further evidence for this conclusion that PSC 833 has actions beyond MDR reversal comes from the finding of nonexclusive drug interaction between PSC 833 and other agents in non-MDR-positive cell lines by graphing log-dose versus $\log(f_a/1 - f_a)$ and finding convergent lines in this current study. Therefore, it is rational to further test this agent in model systems of metastatic prostate cancer with the ultimate intent of human trials as the pharmacokinetics and toxicities in humans are known [26].

The concentration of PSC 833 needed in vitro to achieve biologic effects in our system is in the same range as can be achieved clinically. Given either by the oral route (5 mg/kg every 6 h) or as an intravenous infusion (10 mg/kg over 2 h), this agent achieves trough plasma levels in humans of 1000 ng/ml (0.82 μM) [27–29].

We extended our studies to evaluate PSC 833 with anticancer agents which have been previously tested in human prostate cancer. Many of these agents by themselves have only marginal activity in prostate cancer but are used in hormone-refractory disease due to the dearth of effective single agents with high activity [1]. In these studies, we used median effect analysis to identify additive or synergistic effects. Using this assay, PSC 833 showed additive or synergistic activity with estramustine, ketoconazole, suramin, and vinorelbine. The combination of PSC 833 and estramustine was particularly synergistic. To date, these combinations have not been clinically evaluated or have they been studied in xenograft models.

Median effect analysis allows three different agents to be combined to look for synergy. In the present studies, estramustine and PSC 833 preceding *trans*-retinoic acid resulted in a synergistic cytotoxic effect in three prostate cell lines. These effects were sequence-dependent. As estramustine combined with docetaxel shows significant clinical activity in prostate cancer patients [2], we tested the addition of PSC 833 to this doublet combination. The triplet in this in vitro system demonstrated additive effects or synergism which were sequence-dependent. This triplet also has not been explored in preclinical models or human trials.

Overall, these studies provided potential new avenues for therapeutic investigations in the treatment of prostate cancer.

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