Modulation of Multidrug Resistance in Cancer by Immunosuppressive Agents

Preclinical Studies

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This is a brief summary of the status of known immunosuppressive drugs describing their potential and mode of action to reverse the function of the MDR1 gene product, the P-glycoprotein. Different aspects of these immunosuppressors have been reviewed in the recent literature. This summary will focus only on those studies which relate to the effect of these drugs on the P-glycoprotein. In addition, studies which may explain the mode of action, but do not deal directly with P-glycoprotein, are also summarized. (Pathology Oncology Research Vol 1, No1, 64-70, 1995)

**Key words:** multidrug, resistance, cancer, immunosuppression

**Introduction**

Cancer patients treated with cytotoxic chemotherapy often show resistance to several chemically unrelated agents as well. One major reason for this resistance was shown to be the overexpression of a transmembrane glycoprotein of 170 kDa (P-170) the product of the multi-drug resistance 1 (MDR1) gene. There is evidence to support that this P-glycoprotein acts as a drug efflux pump, as shown in Fig.1. Cells expressing P-glycoprotein accumulate less drug than similar cells that lack this P-glycoprotein. Anti-cancer drugs which are pumped out include daunorubicin, doxorubicin, etoposide, actinomycin D, vincristine and taxol. Other compounds which are not anti-cancer drugs, such as valinomycin, rhodamine 123, colchicine, verapamil and quinidine are also substrates of the P-glycoprotein.

Overexpression of the MDR1 gene in tumor cells may have two major reasons. One, the tumor cells originate from tissues which naturally express this gene. The natural physiological function of these tissues require the pumping activity of P-glycoprotein. For example, renal cell carcinoma, tumors of the proximal tubules of the kidney and colon cancers frequently show high basal level of P-glycoprotein expression. Second, there are indications that P-glycoprotein expression is the result of malignant transformation. It seems, therefore, that MDR1 gene expression in tumors not treated with chemotherapeutic agents may indicate differentiation and various levels of malignant transformation. Usually, MDR1 gene expression is low in untreated prostate, gastric, esophageal, breast and ovary cancer cells.

Attention should be called to the fact that cancer cells expressing the MDR1 gene are sometimes resistant to anti-cancer drugs which are not substrates of the P-glycoprotein. Such resistance indicates that there are mechanisms other than P-glycoprotein related resistance (i.e. altered membrane permeability or DNA repair, etc.).

Studies on P-glycoprotein expression in tumors of patients who were found to be resistant to drug therapy lead to the conclusion that P-glycoprotein levels in cells may be important for choosing a treatment for cancer patients. This observation initiated research to discover agents that block P-glycoprotein and thereby increase the concentration of cancer chemotherapeutic agents in resistant cancer cells. Verapamil was the first such agent used clinically. However, it was soon found in a number of clinical trials that it was difficult to achieve sufficiently high plasma levels of verapamil that block P-glycoprotein function without causing cardiotoxic side effects, therefore other agents able to block P-glycoprotein had to be considered for clinical use.

**Relevant Studies on the Mechanism of Action of P-glycoprotein Blockers**

Searching for potent P-glycoprotein blockers initiated numerous studies with a number of compounds. Several Ca²⁺ channel blockers, including nimodipine, nitrendipine,
nifedipine and diltiazem were found to block P-glycoprotein function to different extents. The verapamil analog, niquidipine also was found to be active. Perhexiline maleate was found to increase adriamycin concentrations in MCF-7 cells. Besides these compounds, some diterpenes, such as forskolin, detergents, such as Tween 80 and Cremophor, some phenothiazines, the peptides valinomycin and gramicidin and certain cephalosporin antibiotics were found to be blockers of P-glycoprotein.

A recent study attempted to correlate different cellular-function-affecting agents with the ability of those agents to block P-glycoprotein function. Correlation was analyzed between chemical charge, influence on specific ion channels, altering membrane potential and the blocking of P-glycoprotein function in L5178Y mouse T lymphoma cells, infected with pHa MDRI/A retrovirus. Among the different agents active on ion channels compounds that alter K⁺, Na⁺, Ca²⁺, H⁺ and Cl⁻ movements were investigated. Agents which affected K⁺ and Na⁺ effluxes were the most active. These findings are summarized in Table I. In these studies the blocking of efflux of the substrates rhodamine 123 and daunorubicin from cells was measured by a flow cytometric method. No correlation among the above described parameters could be found. Depolarizing buffer, (50mM K⁺), did not affect P-glycoprotein function. Among other types of agents, protected di- and tri-peptides, cytochalasin and the protein kinase inhibitor H-7 were inactive, but tamoxifen and estradiol were active.

In the above study all tested immunosuppressors were active. This class of drugs includes compounds with different chemical structures. For example several cyclosporin (Cs) analogs, such as CsA, an eleven amino acid cyclic peptid and FK506, a macrolide antibiotic and rapamycin, a triene type polyene, with some similarities to FK506, as shown in Fig.2. All these compounds are lipophilic and each depolarized L5178Y/MDR cells. This finding is interesting for several reasons. First, all these three compounds hyperpolarize human and mouse lymphocytes, although rapamycin only at low concentrations. The mechanism by which this depolarization occurs in L5178Y cells is unclear. Second, blocking the P-glycoprotein by CsA was previously observed to be associated with hyperpolarization. Third, CsH, which has no immunosuppressive and no membrane potential shifting ability in human lymphocytes, depolarized the L5178Y/MDR cells as CsA did and also blocked P-glycoprotein function.

The effects of the compounds tested in this study on membrane potential and on P-glycoprotein function showed no clear correlation. In addition, the depolarizing 50mM K⁺ buffer depolarized both the parental L5178Y cells and L5178Y/MDR cells to about the same degree. These
Table 1. Effects of K⁺ and Ca²⁺ active compounds on MDR pump activity in L5178Y vMDR cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration</th>
<th>Charge</th>
<th>R123</th>
<th>Daunorubicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verapamil</td>
<td>6</td>
<td>3′amine</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Quinine</td>
<td>95</td>
<td>3′amine</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>50</td>
<td>2′amine</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>4-Aminopyridine</td>
<td>300</td>
<td>1′amine</td>
<td>I</td>
<td>A</td>
</tr>
<tr>
<td>Valinomycin</td>
<td>0.4</td>
<td>neutral</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Nigericin</td>
<td>1.4</td>
<td>neutral</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Triethylammonium-Cl</td>
<td>20 mM</td>
<td>4′amine</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>R-Verapamil</td>
<td>10</td>
<td>3′amine</td>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td>S-Verapamil</td>
<td>10</td>
<td>3′amine</td>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td>Nor-Verapamil</td>
<td>10</td>
<td>3′amine</td>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td>Niguldipine</td>
<td>5</td>
<td>2′amine</td>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td>50 Mm K⁺ buffer</td>
<td>-</td>
<td>-</td>
<td>I</td>
<td>-</td>
</tr>
<tr>
<td>Ca²⁺ affecting compounds</td>
<td>-</td>
<td>-</td>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td>Stauroporine</td>
<td>2</td>
<td>2′amine</td>
<td>AA¹</td>
<td>A</td>
</tr>
<tr>
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<td>50</td>
<td>2′amine</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Flunarizine</td>
<td>50</td>
<td>2′amine</td>
<td>AA</td>
<td>A</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>0.6</td>
<td>COO⁻</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>Propanol</td>
<td>500</td>
<td>neutral</td>
<td>I</td>
<td>-</td>
</tr>
</tbody>
</table>

Concentrations of drugs are given in μM; ¹Type of charge group: 1′, primary; 2′, secondary; 3′, tertiary; 4′, quaternary amines respectively; AA, very active; A, active in reversing MDR phenotype; I, inactive; -, not determined; R123: rhodamine 123 (substrate indicator).

results clearly indicate that effects on membrane potential and the functional status of the P-glycoprotein are unrelated.

Other studies attempted to correlate the cellular effects of these drugs with those on the P-glycoprotein. For example, it was found that CsA enhanced the translocating nucleolar phosphoprotein B23 into the nucleus, but no correlation between this effect and P-glycoprotein activity could be demonstrated. The effect of CsA on intracellular pH was also studied. In some cells intracellular pH change paralleled P-glycoprotein blockade, but in other cells it did not. The finding that CsA may hinder protein kinase activity, perhaps by complexing with calmodulin, is an interesting one. This line of investigation is based on the assumption that some protein kinase isoenzymes are involved in P-glycoprotein expression or activation.

It was previously reported, based on patch - clamp studies, that P-glycoprotein functions as a Cl⁻ channel under hypoxosmotic conditions. This possibility was tested in our laboratory on intact cells, and we found that under such conditions the P-glycoprotein functions in its normal mode and can be blocked by P-glycoprotein blockers, even in the presence of Cl⁻ channel blockers.

Photoaffinity labeling was used to investigate the interaction of P-glycoprotein with its substrates. Specifically, a photoaffinity analog of vinblastine could label P-glycoprotein in MDR Chinese hamster ovary cells. Binding was specific, since it could be inhibited by various substrates of P-glycoprotein and the label could be immunoprecipitated with P-glycoprotein. Similar studies were performed with azidopine, verapamil, and forskolin among others. These labeling studies seem to explain part of the mechanism of P-glycoprotein function for some, but not for all substrates, as we will see in section 3, below.

**Mechanism of Action of cyclosporin A and its analogues on MDR cells**

One earlier study assessed the effect of CsA on [¹H] daunorubicin uptake and cell proliferation in sensitive and resistant acute lymphatic leukemia cell lines. Uptake and proliferation was not affected by CsA in parental cells but proliferation was greatly reduced in the resistant cells. Inte-

Figure 2. Chemical structure of CsA analogs.
restingly, daunorubicin uptake and efflux was not altered in resistant cells by CsA. However, CsA treatment increased the sensitivity of these cells to daunorubicin four fold, in the otherwise five fold resistant cells. Similarly, studies with P388 cells revealed that CsA sensitizes the MDR line, but has no differential effect on daunorubicin accumulation between the parental and the MDR line. In another cell line, H69/LX4, which is 100 fold resistant to cytotoxicity shows a 2.5 fold lower doxorubicin accumulation as compared to the parental line. CsA restored drug accumulation completely in the resistant line with a 20 fold sensitization. The parental line was not affected by CsA in its sensitivity or drug accumulation. These and similar studies indicate that the effect of CsA on P-glycoprotein expressing cells cannot always be correlated with increased drug uptake.

Other effects of CsA, such as changes in cellular drug distribution or membrane alterations could also be considered. Investigating this latter effect by electron spin resonance spectrometry, we found that CsA alters the "fluidity" of plasma membranes of mouse and human lymphocytes. These alterations indicate membrane dynamic changes, possibly transmitted to transmembrane proteins because of the specific lipid-protein interactions in biological membranes. Changes in the pumping activity of P-glycoprotein may be explained, at least in part, by such membrane effects of CsA. It should be added here that for anthracycline type anti-tumor agents, cytotoxicity is partially based on membrane effects, and so is for CsA, as we discussed above.

Cyclosporin analogues with different or no immunosuppressive effects were also studied in respect to blocking P-glycoprotein pumping. CsH, which has no immunosuppressive activity, blocks P-glycoprotein pumping as well as CsA does in L-51787 T lymphoma cells.

Another study showed that the Cs analogues A, C, D and H are all active as P-glycoprotein blockers. The order of activity was D > A > C > H, in MDR ovarian cancer cells. Analogues CsD and CsH have no immunosuppressive activity. Similar results were obtained by other groups, who studied Cs analogues A, C, G, H and B3-243. The order of activity in the MDR-H69 human small cell lung cancer line was A = G = B3-243 > C > H. The analog B3-243 has no immunosuppressive activity. These findings indicate, that binding to different intracellular proteins (immunofins) by the immunosuppressor compounds, CsA and CsC, is not associated with changes on P-glycoprotein function. This is further supported by the fact that a ten-fold higher concentration of CsA is required to block P-glycoprotein function than to affect lymphocyte proliferation. It is interesting to point out that CsH was as active as CsA as a P-glycoprotein blocker at doses of 0.8 μM in L51787 v MDR cells while showing relatively less activity than CsA in MDR H69 cells.

Studies aiming to clarify the mode of action of CsA on P-glycoprotein function applied binding studies or drug accumulation assessment in parental versus MDR cells. One such study concluded that [H] CsA binds more to MDR Chinese hamster ovary cells than to its parental cells, and in the MDR cells CsA binds preferentially to P-glycoprotein. The differential labeling could be inhibited by non-radioactive CsA and by verapamil. In agreement with this study, others have shown that MDR Chinese hamster ovary cells accumulate only about half of [H] CsA than the corresponding parental cells. This finding would indicate either that CsA is a substrate of P-glycoprotein or that MDR cell membranes bind less CsA than the membranes of the parental cells. Contrary to the above results, an equal amount of [H] CsA accumulation was observed in MDR and parental P388 leukemia cell lines. This result also indicates that besides direct binding of CsA to P-glycoprotein or being its substrate, other non-specific membrane effects influence accumulation of CsA in cells. This notion is supported by the studies of Loe and Sharam, who investigated the effect of positively charged and neutral amphiphilic molecules on the function of P-glycoprotein. They concluded that the membranes of P-glycoprotein containing cells are different than that of the parental cells, and therefore drug accumulation can be different in these two types of cells.

One of the leading cyclosporin analog for possible clinical use is PSCB33. This 3'-keto-But[1][val] cyclosporin showed 10 times more effectiveness in reversing resistance against doxorubicin, daunorubicin, vincristine and etoposide in several cell lines. These results could be confirmed by other laboratories. In the 200 fold resistant P388 resistant leukemia cell line 0.08 and 0.25 M PSCB33 restored sensitivity 60 and 140 fold, respectively. PSC833 had no significant effect on parental cells, indicating perhaps a selective action of this agent on P-glycoprotein.

**Studies of FK506 and rapamycin with MDR cells**

FK506 and rapamycin are structurally distinct from Cs (Fig.2). Nevertheless, these compounds were found to reverse MDR in the T cell lymphoblastic leukemia cell line CEMVBL2SO. The amount of FK506 and rapamycin needed to restore daunorubicin accumulation in these cells was about 1000 fold greater (M range) than the amount needed to suppress T lymphocyte activation. The mode of action of FK506 and rapamycin involves competition binding with the photoaffinity analog of 1-iodoaryl azidoprazosin to the P-glycoprotein. Another study indicated that as little as 0.6 μM FK506 and 0.5 μM rapamycin can restore daunorubicin and rhodamine 123 accumulation in L51787 v MDR T-lymphoma cells to the level of the parental L51787 cells. A concentration of about 1 μM FK506 was shown to increase doxorubicin accumulation and drug sensitivity in TAOV/A0.2 ovarian cancer cells and in K562/ADM myelocytic leukemia cells. In Chinese hamster ovary cells, FK506 and rapamycin competitively inhibited the photoaffinity labeling of plasma
membranes by iodomycin, indicating that these immunosuppressive agents bind to P-glycoprotein.\textsuperscript{53}

Mitosis is blocked by 0.5% to 2.0 nm concentrations of these compounds. As mentioned above, FK506 and rapamycin require 100-1000 fold higher concentrations to block P-glycoprotein function compared to inhibition of T cell activation by mitogens. Such high concentrations of FK506 were shown to inhibit P-glycoprotein function in isolated hepatocytes, where its pumping activity is responsible for bile acid transport. Therefore, it is suggested that the concentration of FK506 needed to sensitize resistant cancer cells would block normal physiological functions and it would be too toxic.\textsuperscript{46} The difficulty in using FK506 for blocking P-glycoprotein function in MDR cells was further demonstrated by showing that this compound inhibits certain amino acid transporters, at least in yeast cells.\textsuperscript{53} One would predict then, that FK506 and rapamycin would not be useful clinical agents to reverse MDR in cancers. However, nonimmunosuppressive analogues and analogues with less effects on physiologically important P-glycoprotein functions might be developed.

\textit{In vivo studies}

In vivo experiments were initiated after successful in vitro selection of potential P-glycoprotein blockers. One early in vivo experiment used BALB/C mice bearing parental (drug sensitive) or MDR Ehrlich ascites carcinoma cells.\textsuperscript{41} The mean survival time of untreated mice bearing the parental cells was 18.4 ± 0.6 days, compared to mice bearing the MDR cells which was 19.0 ± 1.0 days. These mice treated with 0.3 mg/kg daunorubicin had mean survival times of > 60 days and 21.1 ± 1.4 days, respectively. Treatment of MDR tumor-bearing mice with 80 mg/kg CsA, in five divided daily doses, resulted in mean survival time of 24.0 ± 2.6 days. If these latter mice were also treated with 0.3 mg/kg daunorubicin, the mean survival time increased to > 60 days, about equal to that of mice bearing the drug - sensitive cells. This is an encouraging result since the dose of CsA was clinically tolerable.

Sandoz laboratories investigated the effectiveness of PSC 833, the immunologically inactive Cs analog, in doxorubicin resistant P388 cell bearing mice.\textsuperscript{40} For this purpose, MDR-P388 cells, which are in vitro 150 fold more resistant to doxorubicin than the corresponding sensitive parental line, were grafted to DBA/2 or B6DF1 mice, 10\textsuperscript{4} cells i.p., at day 0 of treatment. These mice were treated with vincristine or vinblastine in the presence or absence of CsA or PSC 833. In mice, inoculated with the sensitive P388 tumor-cell line, T/C values of 133 to 140 could be achieved with vinblastine (100 mg/kg) and with or without CsA (100 mg/kg), or PSC 833 (100 mg/kg), p.o. However, when mice were inoculated with MDR-P388 cells, T/C values around 150 or higher could be achieved only in the presence of PSCB33, but not with CsA. The protocol used in these experiments involved injection of cells 4h before time 0 and CsA or PSCB33 was added with vincristine or vinblastine on days 0, 2 and 4. More significant results could be achieved with doxorubicin (2 mg/kg, i.p.) with PSCB33 (25 or 50 mg/kg, p.o.) in MDR-P388 bearing BCD2F1 mice, in the same laboratory. In these experiments 10\textsuperscript{4} cells were grafted 4h before time 0 and PSCB33 and doxorubicin treatment occurred at days 0, 4 and 8. Doxorubicin was given 4h after PSCB33 treatment in each day. This experiment yielded T/C values close to 400 with the combined drug treatment, while with doxorubicin alone T/C values were around 100.

Additional in vivo studies also showed the efficacy of CsA and PSCB33 as P-glycoprotein blockers. In searching for a pharmacological explanation for the in vivo mode of action of these, Cs-s, CsA and PSCB33 were found to affect the pharmacokinetics of antitumor drugs.\textsuperscript{46} This was demonstrated in BALB/C nude mice, bearing drug sensitive, s.c. administered human colon carcinoma xenographs. Treatment involved different schedules of etoposide and PSCB33. When 50 mg/kg dose of PSCB33 was combined with 31 mg/kg etoposide, significant tumor growth suppression was observed. Similarly, in the same type of mice, treated with 31 mg/kg etoposide and different concentrations,12.5, 25 and 50 mg/kg of PSCB33, dose dependent suppression of tumor growth was seen. Long term mice survival studies indicated that the dose of etoposide had to be significantly reduced when PSCB33 or CsA was administered concurrently. For example, BALB/C nude mice survived a dose of 39 mg/kg etoposide alone, but in combination with CsA (50 mg/kg) the dose had to be reduced to 25 mg/kg. CsA alone had no effect on long term survival. These findings were attributed to the fact that the blood level of etoposide in PSCB33 or CsA treated animals increased about 10 fold compared to levels observed with etoposide administration alone. This study concluded that there are elevated blood levels of etoposide and doxorubicin\textsuperscript{26} in the presence of P-glycoprotein blockers because of the competition for a common metabolic pathways in the liver and kidney between these two different types of drugs. This competition was frequently demonstrated in P-glycoprotein containing cells. This consideration applies not only to kidney and liver cells, but also to the blood-brain barrier as well. P-glycoprotein is expressed in kidney (tubules) and liver hepatocytes. Both the Cs-S and anti-cancer agents are competing for that protein. This competition can alter blood levels of these agents due to different elimination kinetics from these cells. Also, different metabolic pathways in liver (cytochrome p450, glutathione reductase, etc) contribute to the metabolism of anti-cancer drugs as well as that of the P-glycoprotein blockers. Therefore, the metabolism of anti-cancer drugs is reduced, resulting in higher blood levels.

In summary, studies presented in this paper suggest that one can achieve improved efficacy of anti-cancer drugs in...
MDR cancers, with the concurrent use of P-glycoprotein blockers, in vivo. The necessity to conduct pharmacological studies with the simultaneous application of P-glycoprotein blocker and the anti-tumor drug before clinical trials was also demonstrated.

References


