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Direct Demonstration of High Affinity Interactions of Immunosuppressant Drugs with the Drug Binding Site of the Human P-Glycoprotein

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SUMMARY

The interactions between the human P-glycoprotein (Pgp) and two different types of immunosuppressant drugs known to modulate multidrug resistance in tumor cells have been directly investigated using our newly developed drug-stimulated ATPase assay for Pgp function. The macrolides FK506 and FK520 stimulate the Pgp-ATPase activity with affinities in the 100 nM range, nearly 10 times higher than that of verapamil, a well known Pgp substrate. On the other hand, the cyclic peptides cyclosporin A and dihydrocyclosporin C do not stimulate the Pgp-ATPase activity at all. They do, however, act as potent competitive inhibitors of verapamil-stimulated Pgp-ATPase activity, with affinity constants in the 20–25 nM range. Thus, although these two classes of immunosuppressant drugs affect the Pgp in different ways, they both probably interact with high affinity at the transported drug binding site(s) of the Pgp, which would explain their ability to resensitize multidrug-resistant cells to the killing action of certain antitumor drugs. Possible implications of these findings for Pgp function, cancer chemotherapy, and immunosuppression are discussed.

The development of multidrug resistance in cancer cells is a major obstacle to the successful chemotherapy of cancer. In many cases, multidrug resistance is thought to arise as a result of overexpression of the Pgp, a membrane transporter that acts to pump a variety of chemically different antitumor agents out of resistant tumor cells, thus sparing them from the killing action of these drugs (1–4). Certain drugs, termed chemosensitizers, have been shown to be able to resensitize multidrug-resistant cells to cytotoxic agents (5, 6), and it is generally agreed that such drugs exert their effects by interfering with cytotoxic drug extrusion catalyzed by the Pgp.

The discovery of new efficient chemosensitizers with minimal side effects is an important objective for the future of cancer chemotherapy. *A priori*, it can be expected that the most effective chemosensitizers will be drugs that interact with the Pgp with high affinity and selectivity, thereby precluding its ability to extrude cytotoxic drugs. Unfortunately, the traditional assays for identifying Pgp-reactive agents are somewhat limited. Cell growth experiments designed to identify drugs that resensitize drug-resistant cells to cytotoxic agents are biased toward drugs capable of penetrating the cell plasma membrane. Also, drug binding assays do not always accurately reflect the ability of compounds to interfere with the transport function of the Pgp.

We have recently reported a new cell-free drug-stimulated Pgp-ATPase assay that more directly assesses drug interactions with the Pgp (7). Expression of the human *mdr1* cDNA in cultured insect cells via a recombinant baculovirus generates an ATP-hydrolytic activity in isolated membranes that requires drugs known to be transported by the Pgp. In the present study, this new assay system is used to characterize the nature of the interactions between the Pgp and several of the most effective chemosensitizers currently known, including the calcium channel blocker verapamil and the immunosuppressant agents cyclosporin A and FK506 (8–10). The results obtained confirm that these agents do indeed interact effectively with the Pgp, and they partially define the nature of these interactions. They also provide useful guidelines for future studies designed to identify more effective chemosensitizers. And, finally, they raise several other interesting questions relevant to Pgp function, immunosuppressant action, and the search for better immunosuppressant agents.

Experimental Procedures

Preparation of Pgp-Containing Membranes

Sf9 insect cells were grown and infected with a recombinant baculovirus carrying the *mdr1* cDNA as described previously (7). The membrane fraction from the infected Sf9 insect cells was prepared and

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ABBREVIATIONS: Pgp, P-glycoprotein; DMSO, dimethylsulfoxide.

resuspended in a buffer containing 50 mM TRis, pH 7.0, with HCl, 50 mM mannitol, 2 mM EGTA, 2 mM β-mercaptoethanol and protease inhibitors (7) with the addition of 30% (w/v) glycerol.

Pgp-ATPase Activity Determination

Drug-stimulated Pgp-ATPase activity. The drug-stimulated Pgp-ATPase activities of the membranes were determined in the presence of various drugs, as described previously (7).

Inhibition of drug-stimulated ATPase activity. Solutions with varying amounts of drugs that stimulate the Pgp-ATPase activity were prepared in DMSO and 1-μl aliquots were added to 5 μl of the membrane suspension (average protein concentration, 2–4 mg/ml), on ice. One-microliter aliquots of solutions with varying amounts of the two cyclosporins prepared in DMSO were then added, and the reactions were started by the addition of 95 μl of the ATPase assay reaction mixture, as described previously (7). Addition of DMSO alone to the membranes as described above did not have any effect on the drug-stimulated ATPase activity.

Each of the experiments shown in the figures was carried out three or four times using membranes prepared from different batches of cells, with essentially the same results. The data points indicate the average of duplicate determinations in the individual experiments.

Protein Estimation

Protein was determined by the method of Lowry *et al.* (11) as modified by Bensadoun and Weinstein (12), using bovine serum albumin as the standard.

Materials

Cyclosporin A and dihydrocyclosporin C were from Sandoz Research Institute (Hanover, NJ). FK506 was from Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). FK520 was from Merck Research Laboratories (Rahway, NJ). Vinblastine and vincristine were from Sigma. Hoechst 33342 and 7-aminoactinomycin D were from Molecular Probes.

Results

As we have recently reported (7), infection of cultured Sf9 insect cells with a recombinant baculovirus containing the human *mdr1* cDNA leads to the expression of functional Pgp molecules at levels amounting to several percent of the total membrane protein. The Pgp expressed in this way exhibits a high-capacity ATPase activity that is dependent on the presence of numerous drugs known to be transported by the Pgp. Fig. 1 shows the effects of two macrolide immunosuppressants, FK506 and FK520, on the Pgp-ATPase activity in such isolated membranes. The effects of our standard Pgp-ATPase substrate, the calcium channel blocker verapamil, are also shown; for the purposes of comparison, the results are presented as a percentage of the Pgp-ATPase activity seen in the presence of 10 μM verapamil. The basal ATPase activity in this experiment was 20 nmol of P_i released/mg of membrane protein/min, which was stimulated by verapamil to a level of 90 nmol/mg of membrane protein/min. All of the experiments reported in this paper had similar basal and verapamil-stimulated Pgp-ATPase activities. Although these macrolides were originally developed as immunosuppressant agents to prevent allograft rejection, they have since been shown to act also as chemosensitizers for multidrug-resistant cells (13, 14). The results indicated, as expected, that both FK506 and FK520 are potent substrates of the Pgp-ATPase. FK506 stimulates the Pgp-ATPase to levels roughly two thirds that of verapamil, one of the best stimulators yet found. FK520 is an even more effective Pgp-ATPase substrate, equaling the effectiveness of verapamil. Importantly, both FK506 and FK520 interact with the Pgp-ATPase with an

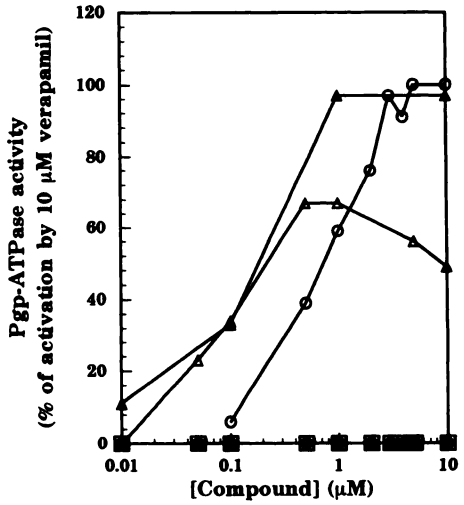

Fig. 1. Effects of immunosuppressants and verapamil on the ATPase activity of the Pgp. ○, Verapamil; ●, cyclosporin A; □, dihydrocyclosporin C; △, FK506; ▲, FK520. The Pgp-ATPase activity measured in the presence of 10 μM verapamil was taken as 100%. Activities are expressed as the percentage of the activity measured in the presence of 10 μM verapamil.

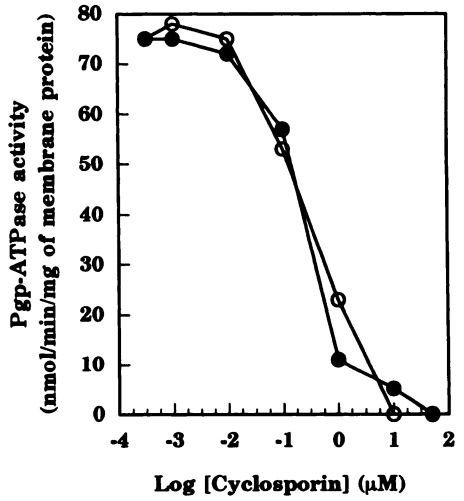

Fig. 2. Effects of cyclosporins on the verapamil-stimulated Pgp-ATPase activity. Increasing concentrations of cyclosporins were included in the Pgp-ATPase assay mixture containing 10 μM verapamil, and the ATPase activities were measured as described in Experimental Procedures. ○, Cyclosporin A; ●, dihydrocyclosporin C.

affinity about 10 times higher than that of verapamil. Control experiments indicated that the stimulation of the Pgp-ATPase activity by FK506 and FK520 is inhibited by vanadate and is absent in control membranes prepared from cells infected with a baculovirus containing *Escherichia coli* β-galactosidase cDNA. Fig. 1 also shows the effects of two other potent immunosuppressant agents, the cyclic undecapeptides cyclosporin A and dihydrocyclosporin C (15), which likewise have been shown to act as chemosensitizers (16, 17). Interestingly, neither of these drugs elicits any Pgp-ATPase activity.

Because cyclosporin A has been shown to interact with the Pgp at its *Vinca* alkaloid binding site (9), it was somewhat surprising to find that cyclosporin A does not stimulate the Pgp-ATPase activity, because the *Vinca* alkaloids clearly do (7). It was therefore considered possible that cyclosporins A and C might be inhibitory substrates of the Pgp. The effects of

cyclosporins A and C on the verapamil-stimulated ATPase activity of the Pgp were thus investigated. Fig. 2 shows the results of these experiments. The results clearly show that both cyclosporin A and dihydrocyclosporin C are potent inhibitors of the Pgp-ATPase activity. For both of these cyclic peptides, half-maximal inhibition of the activity seen in the presence of 10 μM verapamil occurs at an approximately 50-fold lower concentration.

To further characterize the nature of the cyclosporin inhibitions, kinetic analyses of the effects of the two cyclosporins on the verapamil-stimulated Pgp-ATPase activity were carried out. The results of these analyses are shown in Fig. 3 as double-reciprocal plots. Both cyclosporin A and dihydrocyclosporin C act predominantly as competitive inhibitors of the verapamil-stimulated Pgp-ATPase activity. The K_i for cyclosporin A inhibition of the Pgp-ATPase activity is about 20 nM. The corresponding value for dihydrocyclosporin C is about 25 nM. Although not shown, cyclosporin A also inhibits vinblastine- and vincristine-stimulated Pgp-ATPase activity in a similar fashion, with K_i values of approximately 30 nM.

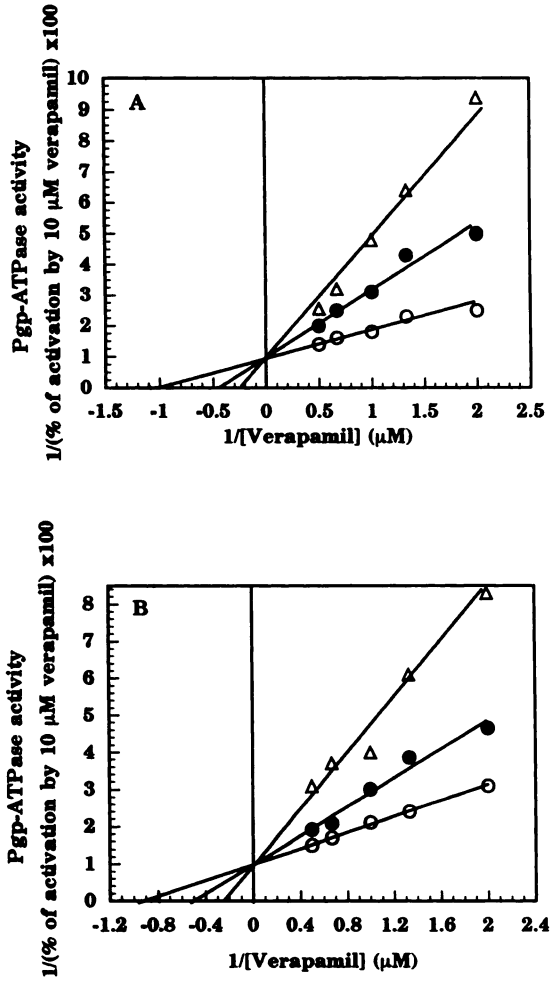

Fig. 3. Kinetics of inhibition of the verapamil-stimulated Pgp-ATPase activity by cyclosporins. The verapamil concentration was varied, with constant cyclosporin concentrations of 0 nM (○), 25 nM (●), and 50 nM (Δ). Velocities of the reactions are expressed as the percentage of the drug-stimulated Pgp-ATPase activity measured in the presence of 10 μM verapamil. A, Cyclosporin A; B, dihydrocyclosporin C.

Discussion

The results presented herein indicate that the popular immunosuppressants FK506 and cyclosporin A and their respective analogues FK520 and dihydrocyclosporin C probably all interact at the drug substrate binding site(s) of the human multidrug resistance transporter. The macrolides FK506 and FK520 both activate the Pgp-ATPase activity in much the same way as its well known substrate verapamil, with a substantially higher affinity. On the other hand, neither of the cyclic undecapeptide cyclosporins activates the Pgp-ATPase activity but both are high affinity competitive inhibitors at the verapamil and *Vinca* alkaloid binding site(s). These results provide an important lesson regarding any search for chemosensitizing agents using our newly developed cell-free baculovirus/insect cell Pgp-ATPase assay system for measuring Pgp function. That is, they show that compounds exist that can interact with high affinity at the drug binding site(s) of the Pgp without eliciting a full catalytic cycle leading to ATP hydrolysis. These effects are not confined to the cyclosporins, because we have found other compounds with similar properties, including 7-aminoactinomycin D and Hoechst 33342 (data not shown). The effects are also not simply due to drug hydrophobicity, because cyclosporin A is substantially more hydrophilic than verapamil, as judged by the octanol/water partition coefficients of these compounds (data not shown). Moreover, valinomycin, which has an octanol/water partition coefficient similar to that of cyclosporin A, is a potent Pgp-ATPase activator (data not shown). Thus, in any search for high affinity chemosensitizers using this or any other drug-stimulated Pgp-ATPase assay system, it is necessary to look not only for the ATPase-stimulatory action of a given drug but also for the inhibition of verapamil stimulation by that drug. Importantly, either Pgp-ATPase stimulators like the macrolides or competitive inhibitors like the cyclosporins can be expected to exert a chemosensitizing action, because both types of Pgp substrates can effectively interfere with cytotoxic drug transport catalyzed by the Pgp. It is conceivable, however, that ATPase stimulators might be even more effective chemosensitizers than are competitive inhibitors, because the former could elicit depletion of cellular ATP stores in multidrug-resistant cells, as well as interfering with cytotoxic drug extrusion.

The results presented herein also bear in an important way on the issue of immunosuppressant drug transport catalyzed by the Pgp. In a recent report by Saeki *et al.* (18), the results of transcellular transport experiments with polarized cultured epithelial cell layers were interpreted to mean that both FK506 and cyclosporin A are transported by the human Pgp. Whereas the measured transepithelial fluxes of FK506 strongly supported this contention, in our view the cyclosporin A fluxes were far less convincing, being very near background levels. The results presented here strongly suggest that cyclosporin A cannot be actively transported by the Pgp. Simply stated, if cyclosporin A does not elicit ATP hydrolysis by the Pgp, then there is no obvious source of energy for the active transport process. Thus, it seems unlikely that cyclosporin A can be actively transported by the Pgp.

These experiments could also be of value in the search for better immunosuppressant drugs. The direct demonstration that two of the most clinically useful immunosuppressants, cyclosporin A and FK506, are high affinity Pgp substrates raises the distinct possibility that the unwanted side effects of

these immunosuppressants are due to interference with the normal function of the Pgp, whatever that may be. Drug structure-activity studies combining conventional assays for immunosuppressant activity with the Pgp-ATPase activity for Pgp function could lead to the discovery of new immunosuppressants with little or no affinity for the Pgp, which would eliminate Pgp-related side effects in immunosuppressant therapies.

Finally, the results of these studies may be pertinent to considerations regarding the molecular mechanism of the immunosuppressant action of cyclosporin A and FK506. It is generally thought that these immunosuppressants exert their action in combination with a drug-specific binding protein, or immunophilin, by binding to the protein phosphatase calcineurin (19, 20). However, the validity of this model for immunosuppressant action has been questioned (21), and it remains possible that the story is as yet incomplete. The demonstration herein that both cyclosporin A and FK506 are potent substrates of the Pgp raises the possibility that the immunosuppressant action of these drugs may somehow be related to their effects on the Pgp. Interestingly, long-sought membrane transporters that carry antigenic peptides across the endoplasmic reticulum membrane for binding to the class I and II major histocompatibility molecules on the surface of antigen-presenting cells have recently been identified and shown to belong to the Pgp family of membrane transporters (22–26). It has also been suggested that certain interleukins important in the immune response may be exported from cells by as yet unidentified Pgp-like transporters (27). The possibility that another aspect of the immunosuppressant action of cyclosporin A and FK506 involves interference with antigenic peptide or interleukin transport catalyzed by these newly recognized and hypothesized Pgp homologues may therefore be worth considering.

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