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ORIGINAL ARTICLE

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Immunosuppressant inhibition of P-glycoprotein function is independent of drug-induced suppression of peptide-prolyl isomerase and calcineurin activity

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Abstract *Purpose*: P-glycoprotein is a 170-kDa plasma membrane multidrug transporter that actively exports cytotoxic substances from cells. Overexpression of P-glycoprotein by tumor cells is associated with a multidrug-resistant phenotype. Immunosuppressive agents such as cyclosporins and macrolides, have been shown to attenuate P-glycoprotein activity. However, the mechanism by which some immunosuppressants inhibit P-glycoprotein function has not been determined. Since cyclosporin and macrolide immunosuppressants inhibit calcineurin (CaN) phosphatase and FKBP12 peptide-prolyl isomerase (FKBP12 PPI) activity, studies were conducted to determine if these effects are directly related to the inhibitory effects these immunosuppressants have on P-glycoprotein function. *Methods*: Western blot analysis was performed to assess CaN and FKBP12 protein levels in P-glycoprotein-negative (MCF-7) and -positive (MCF-7/Adr) breast cancer cell lines. P-glycoprotein function was determined by intracellular doxorubicin accumulation and/or cytotoxicity assays before and after CaN and FKBP12 were independently inhibited by pharmacological antagonists. *Results*: CaN and FKBP12 levels were similar in MCF-7 and MCF-7/Adr cells. P-glycoprotein function was not affected by treatment of P-glycoprotein-expressing MCF-7/Adr cells with CaN and FKBP12 antagonists. *Conclusions*: These results demonstrate that the inhibitory effects of immunosuppressive agents on P-glycoprotein function are independent of CaN or FKBP12 PPI activity.

Key words P-glycoprotein · Calcineurin · Cyclosporin · Ascomycin · Multidrug resistance

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Introduction

Tumor cell multidrug resistance (MDR) remains a major cause of therapeutic failure in cancer treatment. Some tumors are intrinsically resistant to many antineoplastic agents, while others that are initially responsive acquire resistance to a broad range of agents during the course of therapy. An important cause of MDR is overexpression of P-glycoprotein, the product of the *MDR1* gene [1–3]. P-glycoprotein is a 170-kDa plasma membrane transporter that functions as an energy-dependent drug efflux pump [4, 5]. Cells expressing P-glycoprotein are characterized by decreased intracellular drug accumulation and reduced sensitivity to cytotoxic agents [6, 7].

A variety of compounds are effective in increasing intracellular accumulation and enhancing cytotoxicity of chemotherapeutic agents in MDR tumor cells that overexpress P-glycoprotein [8–10]. Immunosuppressive agents such as cyclosporin A (CsA) and ascomycin improve the responsiveness of MDR cells to chemotherapeutic agents both in vitro and in vivo, but their clinical application has been hampered by undesirable side effects [11–13]. Analogs of cyclosporin without immunosuppressive side effects have been developed [31, 32]. Identifying the mechanisms responsible for mediating their ability to inhibit P-glycoprotein function may

provide insights for the development of additional MDR reversal agents with minimal adverse effects.

CsA and ascomycin differ significantly in structure, yet both inhibit the same intracellular pathway responsible for mediating their immunosuppressive actions, suggesting that this common pathway is involved in regulating P-glycoprotein function. Specifically, these agents form a heterodimeric complex consisting of drug bound to a cytoplasmic receptor protein called an immunophilin (cyclophilin in the case of CsA, and FK506 binding protein in the case of ascomycin) [29, 30]. The resulting complex then binds to and inhibits calcineurin [29, 30]. Calcineurin is a serine/threonine phosphatase that is responsible for dephosphorylation of a number of cytosolic regulatory proteins [29, 30]. In addition to their effects on calcineurin, immunophilin proteins also function as *cis-trans*-peptidyl-prolyl isomerases (PPIases) [29, 30]. Folding, assembly and trafficking of proteins in the cellular milieu are regulated by PPIases. Binding of CsA or ascomycin to their respective immunophilin inhibits PPIase activity [30].

Several models can be proposed to explain the P-glycoprotein-inhibiting effects of CsA and ascomycin. One possibility is that calcineurin positively regulates P-glycoprotein function, and immunosuppressants reverse P-glycoprotein activity by inhibiting calcineurin. Evidence in support of this model includes the fact that active P-glycoprotein undergoes rapid cycles of phosphorylation/dephosphorylation, and that phosphorylation of P-glycoprotein enhances its transport function [21–24]. Phosphorylation of P-glycoprotein occurs exclusively on serine residues [21], is mediated by protein kinase C, and is isozyme specific [22]. The phosphatase responsible for P-glycoprotein dephosphorylation has not been established. Calcineurin is a likely candidate when one considers that immunosuppressive agents are capable of inhibiting P-glycoprotein function, and that their pharmacological activity is mediated, in part, through calcineurin.

A second potential mechanism is that immunophilin-P-glycoprotein interaction is required for P-glycoprotein processing and function. Because immunophilins are important for proper trafficking of many other large, multimeric integral membrane proteins such as rhodopsin, and the receptors ryanodine and inositol 1,4,5-triphosphate [33–36], it is reasonable to speculate that immonophilins play a role in processing P-glycoprotein as well. The immunophilin FKBP12 is required for murine P-glycoprotein function in transformed yeast, as demonstrated by the fact that FKBP12 mutants show greater sensitivity to chemotherapeutic agents than do FKBP12 wildtype strains [28].

A final possibility is that cyclosporin and macrolide immunosuppressants interact directly with P-glycoprotein, and that their actions on calcineurin and immunophilin proteins are unrelated to P-glycoprotein inhibition. Several P-glycoprotein modulating agents have been shown to bind, compete for binding sites, and/or be transported by P-glycoprotein, suggesting that chemosensitizers may act simply as competitive inhibitors of P-glycoprotein [26, 27].

Regulation of P-glycoprotein function by immunosuppressive agents has been investigated in a *Saccharomyces cerevisiae* model system [28], but not in mammalian cells. Therefore, studies were conducted to determine if CsA- and ascomycin-mediated suppression of P-glycoprotein function is directly related to their inhibitory effects on calcineurin (CaN) phosphatase or FKBP12 peptide-prolyl isomerase (FKBP12 PPI) activity. Utilizing a pharmacological approach in human breast carcinoma (MCF-7/Adr) cells, we independently inhibited the immunosuppressant target proteins CaN and FKBP12 and determined the effects on P-glycoprotein function.

Materials and methods

Drugs and chemicals

Deltamethrin, cypermethrin, and fenvalerate were purchased from Calbiochem (San Diego, Calif.). Doxorubicin was purchased from Sigma Chemical Company (St. Louis, Mo.). Cyclosporin A was kindly provided by Sandoz Pharmaceuticals (East Hanover, N.J.). Ascomycin and 18(OH)-ascomycin were kindly provided by Abbott Pharmaceuticals (Abbott Park, Ill.).

Cell lines and culture conditions

MCF-7 and MCF-7/Adr cell lines were a generous gift from Dr. Kenneth Cowan of the National Cancer Institute (Bethesda, Md.) and were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere comprising 5% CO_2/95% air. Using an ELISA/PCR system obtained from Boehringer Mannheim (Indianapolis, Ind.), cells were found to be free of contamination by *Mycoplasma*.

Determination of intracellular doxorubicin concentration

Doxorubicin enters cells by passive diffusion through the cell membrane and can be visualized by fluorescence microscopy. Cells with functional P-glycoprotein are demonstrated by reduced fluorescence, whereas treatment with drugs that inhibit P-glycoprotein function results in intracellular accumulation of doxorubicin and enhanced fluorescence. Cells were maintained in drug-free medium for 48 h prior to doxorubicin accumulation studies, then harvested from 25-cm tissue culture flasks by trypsinization. Cells were initially plated at a density of 1×10^5 cells/well in chambered borosilicate coverglass slides (Nunc, Naperville, Ill.) and placed in the incubator for 36 h to allow cells to adhere to the chamber slides. Doxorubicin (5 μM) or doxorubicin plus experimental drug were added to the chamber slides. Chamber slides containing cells but no drug were used as negative controls. MCF-7/Adr cells incubated with either doxorubicin alone, or with the well-characterized P-glycoprotein reversal agent, verapamil, were used as positive controls for P-glycoprotein function and inhibition, respectively. MCF-7 parental cells were subjected to the same treatments to ensure that chemosensitizing effects were indeed attributable to P-glycoprotein inhibition (data not shown).

Cells were incubated with drug for approximately 18 h, washed once with phosphate-buffered saline, and examined immediately by argon-ion laser cytometry (Meridian Ultima Workstation, Meridian Instruments, Okemos, Mich.) to quantitate intracellular fluorescence intensity. In order to relate fluorescence intensities of doxorubicin obtained by laser cytometry

from different treatments with intracellular doxorubicin concentration, excitation and detection parameters were kept constant, and a suspension calibration curve was generated with graded concentrations (0–2 m*M*) of doxorubicin in suspension. Correction for differences in optical thickness between suspension analysis and intracellular doxorubicin concentration was accomplished according to previously described methods [14]. The laser cytometer was set at an excitation wavelength of 488 nm, and the emitted fluorescence was detected with a barrier filter (BP 530/30). Ten microscopic fields, each containing aggregates of 10–15 cells, were analyzed for each treatment. Two experiments on different days were performed.

SDS-PAGE and Western blot analysis

Cells were harvested by trypsinization, pelleted, rinsed in phosphate-buffered saline, and lysed with 1 ml Laemmli sample buffer (62.5 m*M* Tris, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol; pH 6.8). Protein concentrations were determined using a Bio-Rad protein assay kit (BioRad, Hercules, Calif.) according to the manufacturer's directions. Equal amounts of protein (50 μg/lane) were loaded on a polyacrylamide gel and electrophoresed through a 14% resolving gel. Proteins were transblotted on to Immobilon-P membrane (Millipore Corporation, Bedford, Mass.). The membrane was blocked with blotto buffer (50 m*M* Tris HCl, 2 m*M* $CaCl_2$, 80 m*M* NaCl, 5% nonfat dry milk, 0.2% Nonidet P-40, 0.03% sodium azide) for 1 h at 25 °C, then incubated (25 °C for 16 h) with an anticalcineurin monoclonal antibody, raised against the beta subunit (Upstate Biotechnology, Lake Placid, N.Y.). Additionally, C219 anti-P-gp monoclonal antibody obtained from Signet (Dedham, Mass.), and a rabbit polyclonal anti-FKBP12 antibody obtained from Affinity Bioreagents (Golden, Colo.) were used as indicated. Membranes were washed in fresh blotto buffer and incubated with the appropriate alkaline phosphatase-labeled or horseradish peroxidase-conjugated secondary antibody. Membranes were washed with buffer A (50 m*M* Tris HCl, 2 m*M* $CaCl_2$, 80 m*M* NaCl) and developed using either nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate with an alkaline phosphatase conjugate substrate kit obtained from BioRad (Hercules, Calif.) or chemiluminescence detection kit (Amersham Life Sciences, Arlington, Ill.).

Cytotoxicity assay

For cytotoxicity assays, cells were plated in 24-well plates at 1×10^5 cells per well, and then placed in an incubator overnight to allow cells to adhere to the plate. Cells were treated for 24 h with the indicated concentrations of experimental drugs. Corresponding controls were treated with vehicle only. Doxorubicin-induced cytotoxicity was assessed using the formazan salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay, in which viable cells reduce MTT to a blue, formazan product [19]. On the day of assay, the treatment medium was replaced with fresh medium containing 0.83 mg/ml MTT and incubated for 4 h. The medium was then aspirated off, and 250 μl DMSO was added to the wells to solubilize the crystals. The optical density of each sample was read on a microplate reader (model 7520 Cambridge Technology, Watertown, Mass.) at 570 nm against a blank prepared from cell-free wells. Cell survival was expressed as a fraction of vehicle-treated controls.

Statistical analysis

Statistical analysis was performed using SAS/STAT (SAS Institute, Cary, N.C.). Dunnett's Procedure was used to compare differences between treatment and control means. The level of significance was $P < 0.05$.

Results

Functional expression of P-glycoprotein in MCF-7/Adr cells

Western blot analysis with the P-glycoprotein-specific monoclonal antibody C219 confirmed that MCF-7/Adr cells expressed high levels of P-glycoprotein. In contrast, P-glycoprotein was undetectable in parental cells (Fig. 1A). P-glycoprotein function was determined by assessing intracellullar doxorubicin concentration in MCF-7/Adr cells as compared to P-glycoprotein-negative MCF-7 cells. After a 16-h incubation with doxorubicin, MCF-7 cells exhibited a significantly higher intracellular concentration of doxorubicin when compared with MCF-7/Adr cells (Fig. 1B). Collectively, these results confirm the presence and functionality of P-glycoprotein in MCF-7/Adr cells, but not in MCF-7 cells.

Expression of calcineurin and FKBP12 in drug-sensitive and drug-resistant MCF-7 breast carcinoma cells

Western blot analysis of MCF-7 and MCF-7/Adr whole cell lysate showed that both CaN and FKBP12 levels

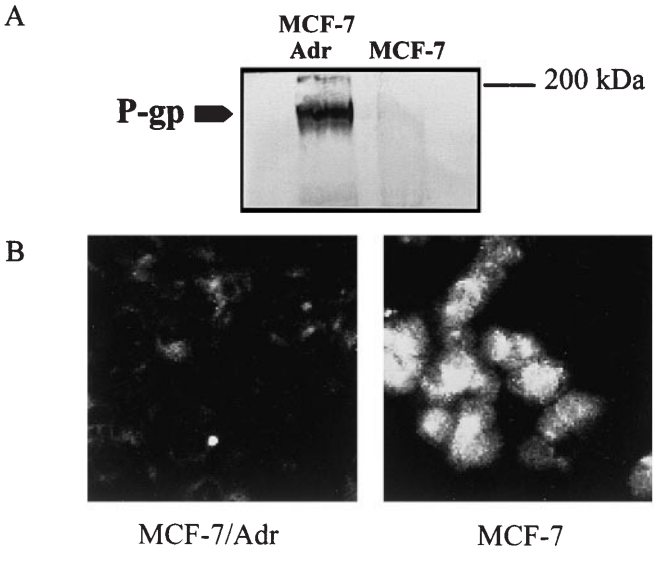

Fig. 1 **A** Immunoblot analysis of P-glycoprotein expression in MCF-7 and MCF-7/Adr cell lines. Cell extracts, each containing 50 μg of protein, were electrophoresed on an 8% polyacrylamide gel, transferred and immunoblotted with monoclonal (C219) anti-P-glycoprotein antibody. A molecular weight standard is indicated on the right. The *arrow* on the left indicates the position of P-glycoprotein. **B** Digital images of doxorubicin accumulation in MCF-7/Adr cells (*left*), and MCF-7 cells (*right*), representing cells treated with 5 μ*M* doxorubicin for 18 h, and then visualized using argon-ion laser cytometry. The doxorubicin molecule is inherently fluorescent, therefore intracellular fluorescence intensity is directly related to doxorubicin concentration. Cells expressing functional P-glycoprotein actively extrude doxorubicin, resulting in lower intracellular fluorescence than cells that do not express P-glycoprotein

were similar in P-glycoprotein-positive (MCF-7/Adr) and -negative (MCF-7) cells (Fig. 2).

Effect of experimental drugs on P-glycoprotein function

P-glycoprotein function was determined by evaluating intracellular doxorubicin fluorescence in MCF-7/Adr cells treated with doxorubicin alone or in combination with either CsA or ascomycin. Both CsA and ascomycin significantly increased intracellular doxorubicin concentration, and serve as positive controls (Fig. 3). Additionally, experiments were performed to determine whether suppression of CaN activity was associated with CsA- or ascomycin-mediated inhibition of P-glycoprotein function. Three synthetic pyrethroids (fenvalerate, cypermethrin, and deltamethrin) have been shown to potently inhibit CaN activity independently of immunophilin proteins (IC_{50} values for CaN inhibition are 2×10^{-8} *M*, 2×10^{-9} *M*, and 3×10^{-10} *M* for fenvalerate, deltamethrin, and cypermethrin, respectively) [15]. These CaN inhibitors had no effect on P-glycoprotein function as determined by intracellular doxorubicin accumulation, even at concentrations greatly exceeding the IC_{50} values (Fig. 3). To determine whether FKBP12 PPI activity is required for P-glycoprotein function, an ascomycin analog was utilized. This analog (18(OH)-ascomycin) binds to FKBP12 and inhibits PPI activity, but, in contrast to ascomycin, does not inhibit CaN [16–18]. P-glycoprotein function was not found to be affected by 18(OH)-ascomycin as determined by assessing intracellular doxorubicin concentration (Fig. 3). These results indicate that FKBP12 PPI activity is not required for function of P-glycoprotein in mammalian cells.

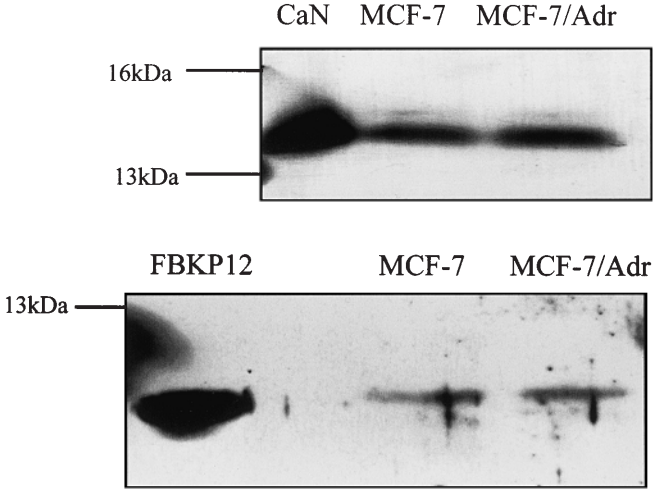

Fig. 2 Immunoblot analysis of calcineurin and FKBP12 expression in MCF-7 and MCF-7/Adr cell lines. Cell extracts, each containing 50 µg of protein, were electrophoresed on a 14% polyacrylamide gel, transferred and immunoblotted with either an anti-calcineurin monoclonal antibody, raised against the beta subunit, or a rabbit polyclonal anti-FKBP12. Molecular weight standards are indicated on the left. Purified bovine calcineurin or human recombinant FKBP12 were used as positive controls

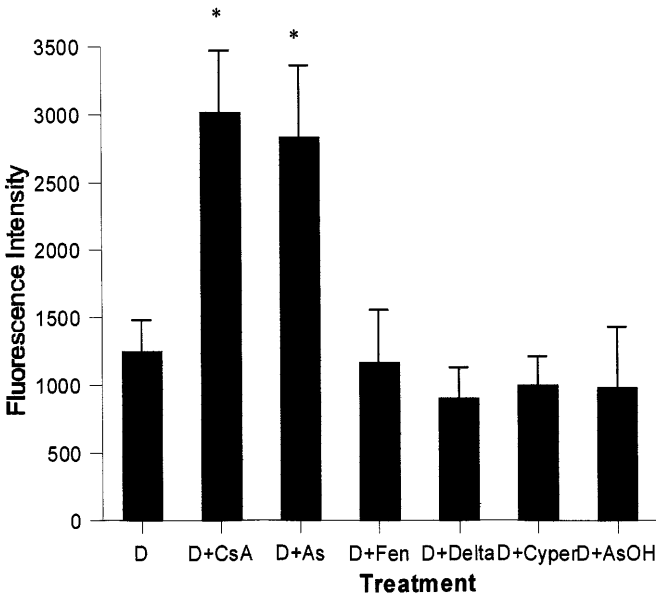

Fig. 3 Mean (+/− SD) fluorescence intensities of MCF-7/Adr cells incubated with doxorubicin alone or doxorubicin in combination with experimental drugs (*D* doxorubicin, 5 µ*M*; *CsA* cyclosporin A, 10 µ*M*; *As* ascomycin, 10 µ*M*; *Fen* fenvalerate, 1 µ*M*; *Delta* deltamethrin, 1 µ*M*; *Cyper* cypermethrin, 1 µ*M*; *AsOH* 18-hydroxy-ascomycin, 10 µ*M*). Cells were incubated in the presence of drug for 18 h, and examined by argon-ion laser cytometry

Effect of experimental drugs on doxorubicin-induced cytotoxicity

MCF-7 and MCF-7/Adr cells in culture were incubated with doxorubicin alone and in combination with experimental drugs for 24 h. Cell survival values are expressed as a fraction of vehicle-treated control (Fig. 4). Concentrations of drug used were identical to those used in the doxorubicin accumulation studies. P-glycoprotein-negative cells (MCF-7) were extremely sensitive to doxorubicin, and were not affected by drug treatment. By comparison, P-glycoprotein-positive cells (MCF-7/Adr) were resistant to doxorubicin alone, but treatment with either of the known P-glycoprotein inhibitors, CsA or ascomycin, completely abolished this resistance. Treatment with the CaN inhibitors cypermethrin, deltamethrin, and fenvalerate did not affect resistance of MCF-7/Adr cells to doxorubicin, consistent with doxorubicin accumulation data (Fig. 3). Because the FKBP12 PPI inhibitor 18(OH)-ascomycin was no longer available, we were unable to determine its effect on doxorubicin-induced cytotoxicity.

Discussion

Because of its role in chemotherapeutic drug resistance, pharmacological inhibition of P-glycoprotein function

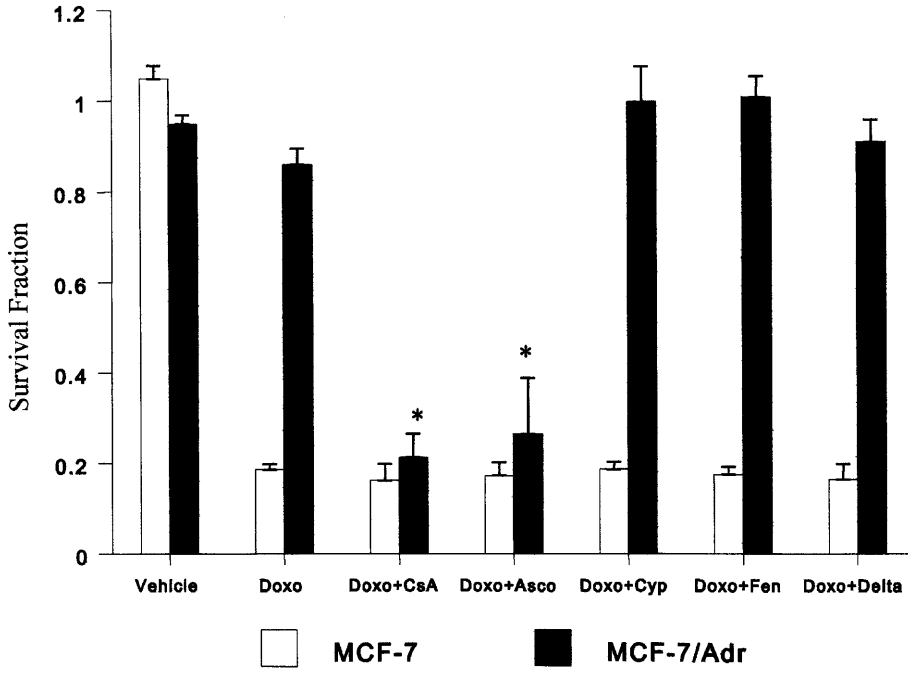

Fig. 4 Effect of experimental drugs on the sensitivity of parental (MCF-7) and P-glycoprotein-expressing, resistant (MCF-7/Adr) cells to doxorubicin (5 μ*M*). Cells were incubated with doxorubicin alone or doxorubicin in combination with experimental drugs (*Doxo* doxorubicin, 5 μ*M*; *CsA* cyclosporin A, 10 μ*M*; *Asco* ascomycin, 10 μ*M*; *Fen* fenvalerate, 1 μ*M*; *Delta* deltamethrin, 1 μ*M*; *Cyper* cypermethrin, 1 μ*M*). Cells were incubated in the presence of drug for 24 h and cell survival determined by MTT metabolism. Cell survival is expressed as a fraction of vehicle (DMSO)-treated control. Results are expressed as mean (+/− SD)

has been extensively investigated. Although CsA and macrolide immunosuppressive agents are among the most clinically useful P-glycoprotein inhibitors, the mechanism by which they inhibit P-glycoprotein function is unknown. Several models have been proposed to explain the P-glycoprotein-inhibitory effects of immunosuppressive agents, including direct interactions of these agents with P-glycoprotein, or interactions with various downstream, intracellular molecules. The experiments reported here examined potential roles of two immunosuppressant target proteins, CaN and the immunophilin FKBP12, in abrogating P-glycoprotein function. Independent inhibition of CaN phosphatase activity or FKBP12 PPI activity had no effect on P-glycoprotein function, indicating that the mechanism by which CsA and ascomycin inhibit P-glycoprotein is independent of their actions on these intracellular targets. Although further research is required to determine how immunosuppressive agents inhibit P-glycoprotein, these results support the model that these drugs interact directly with P-glycoprotein.

Protein kinase C positively regulates P-glycoprotein function [20–22], and pharmacological agents that inhibit protein kinase C also inhibit P-glycoprotein [23, 24]. Furthermore, cells overexpressing P-glycoprotein also express high levels of protein kinase C as compared to P-glycoprotein-negative cells, suggesting that proteins involved in regulating P-glycoprotein function may be induced in conjunction with P-glycoprotein expression [25]. Based on these observations, western blot analysis was performed to determine the relative levels of expression of CaN and FKBP12 in P-glycoprotein-negative (MCF-7) and -positive (MCF-7/Adr) cells. Overexpression of either CaN or FKBP12 in P-glycoprotein-positive cells as compared with -negative cells would have supported a potential role for one of these proteins in regulating P-glycoprotein function. However, CaN and FKBP12 levels were similar in MCF-7 and MCF-7/Adr cell lysates.

Utilizing laser cytology, P-glycoprotein function was assessed by evaluating relative intracellular concentrations of doxorubicin, a P-glycoprotein substrate. Both ascomycin and CsA increased intracellular doxorubicin accumulation in MCF-7/Adr cells to the same level as cells that did not express P-glycoprotein (data not shown). The intracellular concentration of doxorubicin in MCF-7/Adr cells treated with synthetic pyrethroids, agents that inhibit CaN independently of immunophilin proteins, was not different from that in cells treated with doxorubicin alone, indicating that these agents do not alter P-glycoprotein function. Therefore, ascomycin and CsA inhibit P-glycoprotein function by a mechanism independent of CaN. Similarly, FKBP12 PPI activity was inhibited by treating MCF-7/Adr cells with the ascomycin analog 18(OH)-ascomycin. P-glycoprotein function was not altered under these conditions, indicating that FKBP12 PPIase activity is not required for P-glycoprotein function in MCF-7/Adr cells. Collectively, these results provide further evidence that the mechanism by which immunosuppressive agents inhibit P-glycoprotein function is not related to CaN phosphatase or FKBP12 PPI activity.

A second method of assessing drug-induced changes in P-glycoprotein phenotype was utilized because the cytotoxic effects of some P-glycoprotein modulating agents (including CsA) are unrelated to intracellular drug accumulation [26, 27]. In this investigation, experimental drugs that inhibited P-glycoprotein-mediated

doxorubicin efflux significantly enhanced doxorubicin-induced cytotoxicity (i.e. reversed the drug-resistant phenotype). Conversely, experimental drugs that failed to inhibit P-glycoprotein-mediated doxorubicin efflux, were not effective in enhancing doxorubicin-induced cytotoxicity. These findings indicate that for this particular system, doxorubicin accumulation does correspond to cytotoxicity.

The results of the present investigation extend previous findings performed in a yeast model system. Utilizing yeast mutants lacking CaN, both murine P-glycoprotein and the yeast P-glycoprotein homolog STE6 function normally, indicating that CaN is not required. However, in contrast to results observed in mammalian cells, murine P-glycoprotein, but not STE6, function is severely compromised in yeast mutants lacking FKBP12 [28], demonstrating that FKBP12 is required for P-glycoprotein function in yeast. Because a mutant FKBP12 with reduced PPI activity restored P-glycoprotein function in yeast, it was concluded that FKBP12 PPI activity is not required for P-glycoprotein function, but that an alternate function of FKBP12 may be involved [28].

In summary, the results presented here demonstrate that the inhibitory effects of the immunosuppressive agents CsA and ascomycin on P-glycoprotein function are independent of their actions on CaN phosphatase and FKBP12 PPI.

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