 Immunosuppressants FK506 and Rapamycin Function as Reversal Agents of the Multidrug Resistance Phenotype

By Robert J. Arceci, Kimberly Stieglitz, and Barbara E. Bierer

The multidrug-resistant (MDR) phenotype is characterized in vitro by the resistance displayed by cell lines to a broad spectrum of natural product cytotoxic agents. This high level of cross-resistance is due to the increased expression of a membrane glycoprotein termed P-glycoprotein. Encoded in humans by the mdr1 gene, P-glycoprotein functions as an energy-dependent efflux pump of these cytotoxic agents. In this report, we demonstrate that the newly characterized FK506 and its structural analogue, rapamycin, are capable of functioning as MDR reversal agents.

FK506 and rapamycin increase both intracellular, cytotoxic drug (daunomycin) accumulation, and the cytotoxicity of chemotherapeutic agents in multidrug-resistant cells. The increase in cytotoxic drug accumulation is observed at concentrations of FK506 and rapamycin 1,000-fold greater than the concentrations required for FK506 and rapamycin to inhibit T-lymphocyte activation and similar to those shown to be effective for other MDR reversal agents such as cyclosporine A (CsA) and verapamil. The effect of FK506 or rapamycin on both intracellular accumulation and cytotoxicity of daunomycin is additive. This is supported by the ability of FK506 and rapamycin to directly compete the binding of the photoaffinity analogue [125I]-idoaryl azidoprazosin to the P-glycoprotein. The data demonstrate that FK506 and rapamycin represent a new class of structurally distinct molecules that can function as MDR reversal agents and suggest a previously unidentified, potential clinical role for these compounds.

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RESISTANCE of malignant tumors to chemotherapeutic agents remains the major cause of failure in cancer therapy. Some tumors are resistant at the onset of therapy to many of the most active cytotoxic agents, while others that are initially responsive develop resistance to a broad range of agents during the course of therapy. Understanding the intrinsic and acquired resistance of malignant tumors to chemotherapeutic agents remains one of the major challenges in developing successful strategies for the treatment of patients with cancer.1,3

The analysis of cell lines displaying resistance to multiple drugs in vitro has provided significant insight into the mechanisms of multidrug resistance. These studies have identified a multigene family, designated mdr for multidrug resistance, which encodes a family of membrane glycoproteins, termed P-glycoproteins.2-6 The mdr1 gene is amplified and overexpressed in multidrug-resistant cell lines which, although selected for resistance to a single cytotoxic agent, display cross-resistance to a broad spectrum of structurally and functionally unrelated compounds.2,6 The P-glycoprotein appears to function as a transmembrane energy-dependent transporter capable of eflluxing and thus decreasing the intracellular concentration of a variety of cytotoxic agents.7,8 These natural product agents, which are all hydrophobic, low molecular weight molecules, include many of the most potent compounds currently used in cancer chemotherapy—the anthracyclines, actinomycin D,2-6 cyclosporine A,2-6 and various natural product alkaloids, epipolyolactones, and certain protein synthesis inhibitors such as actinomycin D.2,6

The mdr multigene family is differentially expressed in both normal and malignant tissues.9,10 The tumors that appear to express the highest levels of the P-glycoprotein are often derived from the tissues that also express the highest levels. In addition, numerous clinical cases have been documented in which the level of P-glycoprotein expression increases in a tumor after exposure to chemotherapeutic agents.9,11,12 Although the role of mdr gene expression in the development of clinical chemotherapeutic drug resistance remains to be clarified, several studies have demonstrated that the response to therapy may be correlated with the level of P-glycoprotein expressed in tumor cells.13-22 The development of effective means to circumvent multidrug resistance in the clinical setting would provide a major advance in the successful treatment of cancer.23,24

A variety of compounds have now been identified that are capable of reversing the multidrug-resistant (MDR) phenotype in drug-resistant cells.2,6 These agents, which include verapamil, quinidine, progesterone, tamoxifen, and other related compounds, and the immunosuppressant cyclosporine A (CsA), are capable of increasing the intracellular accumulation and cytotoxicity of chemotherapeutic agents in MDR cells.3,4 Clinical trials with several of these compounds have been initiated to evaluate their effect in patients with resistant tumors.25-31

We have investigated the ability of two newly characterized immunosuppressants, FK506 and rapamycin, to function as MDR reversal agents. Both FK506 and rapamycin are structurally distinct from CsA. FK506, a macrolide antibiotic, has recently been shown to inhibit T-cell function at concentrations 10 to 100 times lower than, but by a mechanism similar to, CsA.32,33 A structural analogue of FK506, rapamycin also potently inhibits T-cell prolifera-

From the Division of Pediatric Hematology/Oncology, Dana-Farber Cancer Institute and the Children's Hospital, the Hematology/Oncology Division, Brigham and Women's Hospital; and the Department of Pediatrics and Medicine, Harvard Medical School, Boston, MA.


Supported by National Institutes of Health (NIH) Grants No. CA48162 and GM38156 and the Gaffen Foundation (R.J.A.), as well as NIH Grant No. CA39542 and the Dyson Foundation (B.E.B.). B.E.B. is the recipient of a Clinician-Scientist Award from the American Heart Association and a McDonnell Scholar Award from the James S. McDonnell Foundation.

Address reprint requests to Robert J. Arceci, MD, PhD, Dana-Farber Cancer Institute, Division of Pediatric Oncology, 44 Binney St, Boston, MA 02115.

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FK506 AND RAPAMYCIN REVERSE MDR PHENOTYPE

1529

tion, but by a different mechanism from that of CsA or FK506. FK506 and rapamycin, but not CsA, are capable of inhibiting each other's actions in a number of different assays of T-cell activation, suggesting that they bind to a common receptor binding site. Biologic roles for these agents distinct from T-cell immunosuppression have not been defined.

In this report, we demonstrate that FK506 and rapamycin are capable of reversing the MDR phenotype by increasing the intracellular accumulation and cytotoxicity of chemotherapeutic agents. The concentrations of FK506 and rapamycin required to reverse MDR are substantially higher than those that are immunosuppressive, but similar to the concentration required to reverse MDR by either CsA or verapamil. In addition, both FK506 and rapamycin are capable of inhibiting the binding of [125I]-iodoaryl azidoprazosin to P-glycoprotein, suggesting that their reversal of the MDR phenotype is due to direct interactions with P-glycoprotein. The results demonstrate that FK506 and rapamycin may be useful compounds in the treatment of chemotherapeutic drug resistance in patients and define a new biologic action for these agents unrelated to immunosuppression.

MATERIALS AND METHODS

Drugs. CsA (Sandoz, Basel, Switzerland), FK506 (a gift of Dr Stuart Schreiber, Department of Chemistry, Harvard University, Boston, MA), and rapamycin (Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD) were dissolved in 100% ethanol. Verapamil (Sigma, St Louis, MO) was dissolved in distilled water. Daunomycin was obtained from Wyeth Laboratories (Philadelphia, PA) and its tritium radiolabeled form from DuPont (New England Nuclear, Boston, MA), [125I]-iodoaryl azidoprazosin was from DuPont.

Cell lines. The human T-cell lymphoblastic leukemia cell line, CEM, and its MDR derivative, CEM/VBL 100, were kindly provided by Dr William Beck (St Jude's Hospital for Sick Children, Memphis, TN). The CEM/VBL 250 and CEM/VBL 500 MDR cell lines, which show increased levels of the P-glycoprotein and MDR compared with CEM/VBL 100 (unpublished data), were derived by stepwise selection in increasing amounts of vinblastine of CEM/VBL 100 to a final concentration of 250 ng/mL and 500 ng/mL, respectively.

Drug accumulation studies. Intracellular accumulation of daunomycin was performed using modifications of previously described procedures. Cells were collected, washed three times in phosphate-buffered saline (PBS), and counted. The cells were resuspended at 1 x 10^6 cells/mL RPMI medium containing 10% fetal bovine serum (FBS) and 10 mM/L HEPES. Aliquots of 0.25 mL were dispensed to 75-mm round-bottom plastic tubes and mdr reversal agents added. The tubes were incubated in a 37°C shaking water bath for 60 minutes. At the end of this incubation, the entire contents of each tube were centrifuged at 10,000 g at 4°C for 1 minute to separate the cells from drug-containing medium. The medium and oil mixture were removed by aspiration and the cell pellets solubilized in 1 mL of 1 mol/L NaOH at 60°C overnight. Glacial acetic acid (0.2 mL) was added to each tube and the contents thoroughly mix before transfer into 10 mL of Biofluor Scintillation Cocktail (DuPont). The radioactivity was quantitated in a Beckman Scintillation Counter (Beekman Instruments, Fullerton, CA) with greater than 10,000 cpm detected per sample and an error value of less than 5%. The “fold increase” in daunomycin accumulation was calculated by dividing the amount of radioactivity in the control tube (ie, the solvent-containing tube) into the amount of radioactivity in the tubes containing different concentrations of MDR inhibitors. Values reported are the average of at least three experiments with each experiment being performed in duplicate.

Growth inhibition studies. The inhibition of cell growth was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] dye assay as described. Cells were plated in 96-well plates in RPMI containing 5% FBS and incubated at 37°C for approximately 1 hour. MDR inhibitors, control solvents, and varying concentrations of daunomycin were diluted into the medium and added to the cell suspension (final volume, 200 mL). The cells were incubated for 4 days at 37°C at 5% CO2, after which time 50 mL of 2 mg/mL MTT dye (Sigma) in PBS was added to the wells. The plates were incubated for 4 hours at 37°C and then centrifuged at 450g for 5 minutes. The supernatant (225 mL) was carefully aspirated and 150 mL of dimethylsulfoxide (DMSO) was added and mixed on a plate shaker for 10 minutes. The optical density of the color reaction in each well was determined by absorbance spectrophotometry at 550 nm and 490 nm using a Whitaker EIA Model MA 310 plate reader (Whitaker, Walkersville, MD) with control, negative wells serving as a baseline value. All points represent the average of at least three assays, each performed in duplicate, with standard deviations less than 20%.

Competitive binding studies with [125I]-iodoaryl azidoprazosin. The ability of FK506 and rapamycin to compete for binding with the photoaffinity analogue for the mdr P-glycoprotein, [125I]-iodoaryl azidoprazosin, was performed as described by Greenberger et al. Plasma membrane–enriched fractions of MDR and drug-sensitive cells were prepared by resuspending cells at approximately 2 x 10^6 cells/mL in 10 mmol/L HEPES, pH 7.3, 0.25 mol/L sucrose, and allowing the cells to swell for 10 minutes on ice. The cells were then subjected to 20 to 30 strokes of a Type B Dounce homogenizer, and the nuclei and unbroken cells pelleted at 1,000 rpm for 9 minutes at 4°C in an RC3B centrifuge. The postnuclear supernatant was then centrifuged at 75,000 rpm for 15 minutes at 4°C in a Sorvall RT108 AT4-230 rotor (Sorvall, Wilmington, DE). The plasma membrane–enriched pellet was resuspended in PBS and the protein concentration determined with the Bio-Rad Protein Assay System (Richmond, CA). Labeling with the azidoprazosin analogue was accomplished by incubating 50 mg of the plasma membrane fraction in 50 mmol/L Tris, pH 7.4, with 2.5 mmol/L [125I]-iodoaryl azidoprazosin and varying concentrations of inhibitors in 1.5-mL Eppendorf polypropylene tubes. The mixture was then incubated for 1 hour at 25°C in the dark. After this incubation, the contents of the tubes were exposed at a distance of 5 cm to a long-wave (366 nm) UV lamp for 20 minutes. One-fifth volume of Laemmli sample buffer was then added and the proteins loaded without any heating onto a 10% polyacrylamide gel and electrophoresed. The polyacrylamide gel was fixed for 10 minutes at 10% acetic acid, soaked in 2% glycerol in water for 30 minutes, dried, and exposed to XAR-3 film (Eastman Kodak, Rochester, NY) with an intensifying screen.

Flow cytometry. Surface staining of cells for P-glycoprotein expression was accomplished using 4E3, a mdr1-specific anti-P-glycoprotein monoclonal antibody. Cells were collected and washed twice in cold PBS before resuspending 1 x 10^6 cells in 100 mL PBS containing a 1:1 dilution of human serum with PBS. The
cells were then incubated at 4°C for 30 minutes to block any Fc receptors. Two milliliters of PBS was then added to the cells, which were collected by centrifugation at 600g for 3 minutes. Pelleted cells were resuspended in 100 μL of PBS containing 2% goat serum and 10 μg/mL of the anti-P-glycoprotein antibody 4E3.16 or a mouse IgG2a isotype-matched control antibody. This mixture was incubated for 30 minutes at 4°C and cells were washed twice with cold PBS followed by resuspension in 100 μL of PBS containing 2% goat serum and fluorescein isothiocyanate (FITC)-labeled goat antimouse immunoglobulin (Fab')2 fragment (TAGO, Burlingame, CA) at a 1:30 dilution. Cells were incubated with the second antibody for 30 minutes at 4°C in the dark, followed by two washes in cold PBS and fixation in 2% paraformaldehyde before analysis. The level of P-glycoprotein expression was then determined using a Becton Dickinson FACSCAN (Mountain View, CA) and the LYSYS software application.

RESULTS

P-glycoprotein expression and drug accumulation in CEM and CEM/VBL 250. The CEM/VBL 250 MDR cell line was generated by stepwise selection of the CEM/VBL 100 MDR cell line using increasing amounts of vinblastine. As shown in Fig 1A and B, CEM/VBL 250 expresses approximately 150 times more P-glycoprotein than the CEM parental cell line as determined by immunofluorescent staining using a mouse monoclonal antibody (4E3) directed to an external epitope of the mdr1 P-glycoprotein. This is consistent with low-level mdr1 mRNA in CEM detected by polymerase chain reaction (PCR). Figure 1C demonstrates a marked difference of intracellular daunomycin accumulation between CEM and CEM/VBL 250. Plateau levels of accumulated drug are achieved by 20 minutes; all further drug accumulation studies were performed at 60 minutes.

FK506 and rapamycin increase daunomycin accumulation in MDR cells. The effects of FK506 and rapamycin on daunomycin accumulation in CEM and CEM/VBL 250 were assessed. No significant effect of FK506, rapamycin, CsA, or verapamil on intracellular daunomycin accumulation was observed in this assay using the drug-sensitive parent cell line, CEM (Fig 2). However, both 1 μmol/L FK506 (Fig 2A) and 1 μmol/L rapamycin (Fig 2B) are capable of significantly increasing the intracellular accumulation of daunomycin in the CEM/VBL 250 MDR cell line. Plateau levels of increased daunomycin accumulation are achieved at 2.5 μmol/L FK506 and at 5 μmol/L rapamycin (Fig 2A and B). The level of increased daunomycin accumulation was similar to that observed at the same concentrations of either CsA (Fig 2C) or verapamil (Fig 2D). Several other MDR cell lines, including a human lung adenocarcinoma, SW1573/500,43 a hamster fibroblast, LZ,44 and a mouse leukemia, L1210DN,45 demonstrated similar sensitivity to FK506 and rapamycin (data not shown).

The different reversal compounds were next tested in combination at concentrations (1.25 μmol/L) that alone would result in a subplateau level of daunomycin accumulation. The effect on cytotoxic drug accumulation by FK506 and rapamycin is additive when these two agents are used in combination either with each other or with CsA or verapamil (Fig 3). No increase above the plateau levels was
FK506 AND RAPAMYCIN REVERSE MDR PHENOTYPE

Fig 2. Both FK506 and rapamycin cause an increase in \(^{3}H\)-daunomycin intracellular accumulation in multidrug-resistant cells similar to CsA and verapamil. The data are shown as the fold increase in the intracellular accumulation of radiolabeled daunomycin in the presence of an mdr reversal agent relative to the level observed in the absence of the reversal agent. CEM (B), CEM/VBL 250 (C).

observed when higher concentrations of the reversal agents were used in combination (data not shown).

**FK506 and rapamycin potentiate the cytotoxicity of daunomycin in MDR cells.** To test whether the effect of increasing daunomycin accumulation in CEM/VBL 250 by FK506 and rapamycin resulted in the reversal of the MDR phenotype in vitro, cytotoxicity assays were performed at varying concentrations of these two compounds. Both FK506 and rapamycin, like CsA and verapamil, are capable of reversing the MDR phenotype by increasing the cytotoxicity of daunomycin in CEM/VBL 250 (Fig 4). An effect of increasing the cytotoxicity of daunomycin in CEM/VBL 250 was observed at an inhibitor concentration of 1 \(\mu\)mol/L (Fig 4C) and complete reversal observed at an inhibitor concentration of 5.0 \(\mu\)mol/L (Fig 4E). A detectable but less pronounced effect of these agents on CEM growth inhibition in the presence of daunomycin was also observed (Fig 4). Similar observations demonstrating low levels of MDR reversal on parental cell lines have been previously observed. This may be due to very low levels of expression of the P-glycoprotein \(^{22,43,45,49}\) (Fig 1). In addition, an additive potentiation of daunomycin cytotoxicity was observed on CEM/VBL 500 using FK506 or rapamycin in combination with each other or CsA (Fig 5).

The ability of FK506 to increase the cytotoxicity of daunomycin in CEM/VBL 250 was not due to direct toxicity (Fig 6). The effect of FK506 on cell growth was
photoaffinity analogue 125I-iodoaryl azidoprazosin to P-glycoprotein. Many MDR reversal agents, including CsA and verapamil, have been shown to bind to P-glycoprotein and effectively compete binding of the photoaffinity analogue \(^{125}\)I-iodoaryl azidoprazosin or \(^3\)H-azidopamine.\(^{41,51}\) To determine whether FK506 and rapamycin were also capable of interacting directly with the P-glycoprotein, their ability to displace \(^{125}\)I-iodoaryl azidoprazosin was tested. In a concentration-dependent manner, both FK506 and rapamycin effectively inhibited the binding of \(^{125}\)I-iodoaryl azidoprazosin to the P-glycoprotein (Fig 7). No binding of \(^{125}\)I-iodoaryl azidoprazosin to P-glycoprotein was observed in the drug-sensitive parental cell line (Fig 7, lane 1) or to the \(mdr\) cell line in the absence of UV cross-linking (Fig 7, lane 11). These results demonstrate that the MDR P-glycoprotein is another FK506 and rapamycin cellular binding target that is not a member of the FKBP family.

**DISCUSSION**

The discovery of P-glycoprotein as a mediator of multidrug resistance offers a potentially important therapeutic target.\(^{1,5,23}\) A variety of structurally dissimilar compounds have been shown to be able to inhibit P-glycoprotein transport of cytotoxic agents and thereby reverse the MDR phenotype.\(^{1,5,35}\) These MDR reversal agents include several calcium channel blockers, phenothiazines, progesterone, tamoxifen, CsA, and some of its chemically modified derivatives.

In this report, we show that the immunosuppressive agents FK506 and rapamycin are capable of reversing the MDR phenotype. Both FK506 and rapamycin, which are structurally distinct from other known MDR reversal agents, are able to increase the intracellular accumulation of cytotoxic drug in MDR cells. Increasing intracellular daunomycin accumulation potentiates its cytotoxicity in MDR cells as determined by the MTT growth inhibition assay. In addition, the ability of FK506 and rapamycin to increase cytotoxic drug accumulation and cytotoxicity in MDR cells is additive when used in combination with each other or with other known MDR reversal agents such as CsA and verapamil. No increase in drug accumulation beyond the
FK506 AND RAPAMYCIN REVERSE MDR PHENOTYPE

Fig 5. MTT assays demonstrating that the effect of combinations of FK506 and rapamycin with each other or with CsA are additive on increasing daunomycin cytotoxicity. Open symbols represent values for CEM and filled symbols show values for CEM/VBL 500. The percent survival is determined relative to that observed in control samples (ie, no inhibitor). Control (C), FK506 1.25 μmol/L (●), rapamycin 1.25 μmol/L (○), CsA 1.25 μmol/L (□), FK506 1.25 μmol/L plus CsA 1.25 μmol/L (△), rapamycin 1.25 μmol/L plus CsA 1.25 μmol/L (■), FK506 1.25 μmol/L plus rapamycin 1.25 μmol/L (□).

plateau levels achievable with maximum amounts of the individual reversal agents was observed, suggesting that the effect was additive and not synergistic.

The concentration of either FK506 or rapamycin required to increase cytotoxic drug accumulation in MDR cells is similar to that for CsA and verapamil and 1,000-fold higher than the concentrations which result in T-cell immunosuppression. Recently, two other reports have also demonstrated that FK506 can reverse the MDR phenotype at concentrations similar to those used for CsA and verapamil. While FK506 shares no structural similarity with CsA, both drugs appear to inhibit T-cell signaling pathways by similar mechanisms involving the calcium-dependent serine/threonine phosphatase calcineurin. Although FK506 is 10- to 100-fold more effective than CsA in the inhibition of T-cell activation, it appears to be equipotent with CsA in the reversal of the MDR phenotype. Rapamycin, a structural analogue of FK506, is also capable of inhibiting T-cell proliferation. However, it inhibits T cells by inhibiting the later signals transmitted by growth factors binding to their receptors. We have previously shown that rapamycin and FK506 are able to inhibit each other's actions, suggesting that they both bind to the same receptor binding site. In contrast to their effects on T lymphocytes, the observation that the effects of FK506 and rapamycin on reversing the MDR phenotype are additive and not antagonistic further suggests that the mechanism of MDR reversal is different from that of T-cell immunosuppression. CsA binds to a family of intracellular receptors, cyclophilins, while FK506 and rapamycin bind to a structurally distinct family of intracellular receptors, FK506-binding proteins or FKBPs. Both cyclophilins and FKBPs share the capacity to catalyze, in vitro, the cis-trans intraconversion of peptide-prolyl bonds (rotamase activity). Reversal of the MDR phenotype may therefore involve inhibition of rotamase activity in the cell. However, the data presented here that both FK506 and rapamycin are able to inhibit the photoaffinity analogue 125I-iodoaryl azidoprazosin binding to P-glycoprotein demonstrate that the mechanism of MDR reversal may be unrelated to rotamase inhibition. The data therefore demonstrate that P-glycoprotein can directly interact and bind both FK506 and rapamycin.

Ongoing clinical trials with MDR reversal compounds in combination with cytotoxic drugs have been reported and many more trials are currently underway. Several of the first reported trials using verapamil were hindered by the cardiotoxicity associated with high serum levels of this MDR reversal agent. However, serum levels (2 to 4 μmol/L) of other reversal agents, such as CsA, capable of reversing the MDR phenotype, have been achieved without excessive toxicities (Dahl et al, in preparation). In all of these clinical trials, the MDR reversal agent is given intermittently and only briefly concurrent with chemotherapy administration. Therefore, immunosuppression or other
potential toxicities resulting from prolonged exposure of the reversal agent would be less of a clinical concern.

The identification of FK506 and rapamycin as a new structurally distinct class of MDR reversal agents should prove useful in studying P-glycoprotein/drug interactions. The structure of FK506 and rapamycin further suggests that this class of reversal agents may serve as lead compounds, and may be excellent substrates for chemical modifications. In addition, our preliminary results demonstrate that FK506 is able to increase intracellular cytotoxic drug accumulation in P-glycoprotein–positive human leukemic blasts (unpublished data) and therefore suggest a potential clinical role for FK506 in the treatment of chemotherapy-resistant malignancies. We are currently attempting to test this possibility using tumor xenograft animal models.

ACKNOWLEDGMENT

We thank Kimberly Basu for help with performing some of the cytotoxicity assays and Martha Mahoney for help in manuscript preparation. We also thank Dr James Croop, Dr Steven Burakoff, and David Fruman for critically reviewing the manuscript.

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RJ Arceci, K Stieglitz and BE Bierer