The Farnesyl Protein Transferase Inhibitor SCH66336 Is a Potent Inhibitor of MDR1 Product P-glycoprotein

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ABSTRACT

P-glycoprotein (Pgp)-mediated drug efflux is a major factor contributing to the variance of absorption and distribution of many drugs, particularly cancer chemotherapeutics. Multidrug resistance (MDR) is caused largely by the efflux of therapeutics out of the tumor cell by Pgp, resulting in reduced efficacy of chemotherapy. SCH66336, a farnesyl transferase inhibitor in development for cancer therapy, was examined in the present study for its ability to inhibit Pgp. In a test system consisting of a NIH-G185 cell line presenting an overexpressed amount of the human transporter Pgp, known Pgp inhibitors, such as cyclosporin A, paclitaxel, verapamil, tamoxifen, and vinblastine, were demonstrated to inhibit the Pgp-mediated efflux of daunorubicin. SCH66336 significantly inhibited daunorubicin transport with an IC_{50} of about 3 μM and similarly affected the transport of rhodamine 123 with a potency similar to cyclosporin A. Additionally, by an ATP-hydrolysis assay, SCH66336 was shown to decrease Pgp-mediated ATP hydrolysis by >70% with a K_{m} of 3 μM. This observation indicates that SCH66336 directly interacts with the substrate binding site of Pgp, a quality unique to SCH66336 and its analogues, although not inherent to farnesyl transferase inhibitors in general. Moreover, low concentrations of SCH66336 exhibit synergy with the Pgp substrate/inhibitors paclitaxel, tamoxifen, and vinblastine respectively by significantly potentiating their inhibition of Pgp. Treatment with SCH66336 would be predicted to be synergistic with coadministered cancer therapies that are substrates of Pgp. A further benefit of coadministration of SCH66336 could be reduced chemotherapy dosage, hence, lower exposure to normal cells and, therefore, less undesired toxicity.

INTRODUCTION

Although it is one of the major ways of treating cancer, chemotherapy can be impeded dramatically by the development of MDR to a variety of structurally unrelated cytotoxic drugs. Overexpression of Pgp (M, 170,000–180,000), the product of the mdr1 gene, is the most commonly observed characteristic of multidrug resistant cells grown in vitro (1–3) and in a number of tumors (4, 5). Pgp, an ATP-dependent multidrug pump belonging to the ABC superfamily of proteins (6), protects cells from cytotoxic compounds by transporting them out of the cells and reducing the intracellular levels below their effective concentrations. This transport enzyme uses ATP (via hydrolysis) in two homologous halves as the source of energy for “translocating” various substrates (7–9). Substrates enter from the lipid bilayer and bind to two (or more) nonidentical sites (10, 11) at the cytoplasmic leaflet (12). Allosteric and perhaps synergistic effects have been observed for certain substrate combinations and conditions (10, 13). Physiologically, Pgp is widely expressed in the epithelial cells of the intestine, liver, kidney, and placenta, and in the endothelial cells of the brain. With its broad substrate specificity and distinctive expression locations suggesting that Pgp may have a direct role in modulating the absorption and disposition of drugs or xenobiotics, this membrane transporter has become recognized as a critical factor in the exposure to cancer therapeutics (14). Consequently, the inhibition of Pgp could result in far-reaching implications, particularly overcoming MDR in tumor cells.

A large number of compounds that interact with the Pgp efflux pump have been identified, and some are under development as drugs (14, 15). These compounds have no common chemical structural features except for hydrophobicity. Some of them are positively charged at physiological pH. The early generation of modulators of Pgp, such as cyclosporin A (16), verapamil (17), and quinidine (18), failed to show clinical utility because of inadequate efficacy, their own dose-related toxicity, or profound alterations in pharmacokinetics when used in combination with anticancer drugs. Although the more recently developed modulators possess a higher affinity for Pgp, their efficacy is still under clinical evaluation. Examples of this class include the cyclosporin A analogue PSC 833 (19); the acridinocarbocyclic compound SCH66336, which has been reported in preclinical cancer models when SCH66336 is combined with cyclophosphamide, 5-fluorouracil, or vincristine (20, 21), the triazinoaminopiperidine derivative S9788 (22); a yohimbine analogue, trimethoxybenzoylhemin (23); and other compounds, including MS-073 (24) and R-isomer of verapamil (25).

SCH66336 is an orally active, potent, and selective inhibitor of the FPT enzyme (26). This novel therapeutic agent has activity against a wide variety of human tumor xenografts and also causes regression of tumors in wap-H-ras transgenic mice. Enhanced antitumor activity has been reported in preclinical cancer models when SCH66336 is combined with cyclophosphamide, 5-fluorouracil, or vincristine (20, 21), all of which are substrates of Pgp. Furthermore, a synergistic effect on the antitumor activity of SCH66336 and taxanes (also Pgp substrates) has recently been described (28). Therefore, the purpose of these evaluations was to quantify and characterize any interaction of SCH66336 with Pgp, and a potent inhibition is described.

MATERIALS AND METHODS

Chemicals. SCH66336 was obtained from Schering-Plough compound resources. DNR, verapamil, tamoxifen, paclitaxel, vinblastine, colchicine, cyclosporin A, marnitom DTT, ATP disodium, ammonium molybdate, ascorbic acid, sodium meta-arsenite, aprotinin, leupeptin, EGTA, EDTA, HEPES, ouabain, phenylmethylsulfonyl fluoride, and TRIZMA base were purchased from Sigma Chemical Co. (St. Louis, MO). HBSS, α-MEM, DMEM, penicillin/streptomycin, PBS, and trypsin/EDTA were obtained from Life Technologies, Inc. (Rockville, MD). Sodium orthovanadate was purchased from Pfanstald & Bauer Inc. (Waterbury, CT). Microplates (Costar 96-well), plastic tubes, and cell culture flasks (75-cm²) were purchased from Corning Inc. (Corning, NY). All other reagents were of the highest grade commercially available.

Cell Lines. The NIH-G185 cell line presenting the gene product of MDR1 was licensed from Dr. Michael Gottesman at NIH and maintained in DMEM supplemented with 10% FBS, penicillin/streptomycin (50 units/50 μg/ml) in a 5% CO₂-95% air atmosphere at 37°C. Colchicine (60 ng/ml) was added to the culture medium. CR1/R12 cell line (pressure selected from CHO cells, hamster ovary tumor), provided by Dr. Alan Senior (University of Rochester, Rochester, NY), was maintained in complete α-MEM supplemented with 10% FBS, penicillin/streptomycin (50 units/50 μg/ml) in a 5% CO₂-95% air atmosphere at 37°C. Colchicine (0.5 μg/ml) was added to the

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The abbreviations used are: MDR, multidrug resistance; Pgp, P-glycoprotein; ABC, ATP-binding cassette; DNR, daunorubicin; Rho, rhodamine 123; FPT, farnesyl protein transferase; FBS, fetal bovine serum.

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culture medium. Cells were grown to 80–90% confluence and treated with trypsin-EDTA before subculturing.

Fluorescence-activated Cell Sorter Flow Cytometry. Fluorescence measurements of individual cells were performed using a Becton Dickinson FACScalibur fluorescence-activated cell sorter (San Jose, CA), equipped with an UV argon laser (excitation at 488 nm, emission at 530/30 and 570/30 nm band-pass filters). Analysis was gated to include single cells on the basis of forward and side light-scatter and was based on acquisition of data from 10,000 cells. Log fluorescence was collected and displayed as single-parameter histograms. A direct functional assay for the Pgp efflux pump in viable cells was performed with the flow cytometer (29).

Cell Viability Test. Cell viability was assessed using exclusion of 0.4% trypan blue as well as propidium iodide staining. Dead cells in which propidium iodide was bound to double strands of DNA were detected in certain regions of the cytometry dot plots and not included in the final data calculations.

Calculation of Relative Fluorescence. The DNR fluorescence intensity of individual cells was recorded as histograms. The mean fluorescence intensity of 10,000 cells was used for comparison among different experimental conditions. Verapamil and ciclosporin A were selected as positive controls to normalize the measurements because each can maximally inactivate the Pgp efflux pump. Relative fluorescence was used for quantitation and comparison of the effects of different compounds on transport. The relative fluorescence (% inhibition) represents a ratio obtained through the following formula: the geometric mean fluorescence of a discrete sample divided by the geometric mean fluorescence in the presence of 100 μM verapamil, times 100, or expressed as:

Relative fluorescence = 

\[
\frac{\text{Fluorescence of sample geometric mean}}{\text{Fluorescence of reference standard geometric mean}} \times 100
\]

Membrane Microsome Preparations. CR1R12 cell membranes enriched with the hamster MDR1 gene product transport enzyme was used for preparation of membrane microsomes. Cells were washed with complete Hanks’ buffer before being resuspended in 10 ml of lysis buffer [Tris-HCl, 50 mM; mannitol, 50 mM; EGTA, 2 mM; and DTT, 2 mM (pH 7.0) at 25°C] containing protease inhibitors (phenylmethylsulfonyl fluoride, 1 mM; aprotinin, 10 μg/ml; leupeptin, 10 μg/ml). All of the subsequent steps were performed at 4°C. The cells were lysed by nitrogen cavitation (Parr Instrument Co., Moline, IL) at 500 psi for 15 min twice. Nuclei and mitochondria were sedimented by centrifugation at 4000 × g for 10 min. The microsomal membrane fraction was then sedimented by centrifugation at 100,000 × g for 60 min. The pellet was resuspended in 0.25 M sucrose buffer (10 mM Tris-HCl, 1 mM EDTA (pH 7.5)) and homogenized using a Potter-Elvehjem homogenizer. Aliquots of membrane microsomes were rapidly frozen and stored at −80°C until analysis.

ATP Hydrolysis and Phosphate Release. The consumption of ATP was determined by the liberated Pi, which forms a color complex with molybdate (30). We have developed an ATP hydrolysis assay based on phosphate-release determination using membrane microsome preparations (31–33). The method was modified to be carried out in a 96-well microplate. The microsomes were thawed on ice prior to diluting to 3.5 μg protein per well in ice-cold ATPase buffer [sodium ATP, 3 mM; KCl, 10 mM; MgSO4, 10 mM; DTT, 3 mM; Tris-HCl, 50 mM (pH 7.0)] containing 0.5 mM EGTA (to inhibit Ca-ATPase), 0.5 mM ouabain (to inhibit the Na/K-ATPase), and 3 mM sodium azide (to inhibit the mitochondrial ATPase). The total incubation volume including the various inhibitors was 100 μl. The incubation reaction was initiated by transferring the plate from ice to 37°C and incubating for 30 min; the reaction was terminated by the addition of 50 μl of 12% SDS solution at room temperature, followed by the addition of 50 μl of a mixture solution of (equal volumes) 18% fresh ascorbic acid in 1 N HCl and 3% ammonium molybdate in 1 N HCl. After 4 min, 100 μl of a solution of 2% sodium citrate and 2% sodium meta-arsenite in 2% acetic acid were added to fix the color formation. After 30-min incubation at room temperature, the fixed released phosphate was quantitated colorimetrically in a microplate reader (Bio-Tek FL600, Winooski, VT) at 750 nm. The respective values for background with ATPase assay buffer alone were obtained in parallel and subtracted from the values for experimental samples. By comparison to a standard curve, the amount of phosphate released, and hence ATP consumed, was quantified. Water-insoluble drugs were dissolved in methanol; the maximum methanol concentration (2% v/v) was shown not to affect the ATPase activity and was included as controls.

RESULTS

As fluorescent substrates transported by Pgp, DNR and Rho serve as markers for active transport function simply by measurement of retained fluorescence per cell (29). Herein we show that the FPT inhibitor SCH66336 effectively inhibited the Pgp-mediated transport of DNR or Rho, yet did not effect the parent nontransfected NIH-3T3 cell line. The IC90 (concentration at half-maximum inhibition) can be determined from a simple function, as shown in Fig. 1, where the retained fluorescence is measured for samples of viable cells (i.e., stable human MDR1 transfectant NIH3T3-G185) by a flow cytometer at varying concentrations of SCH6636. The concentration dependency of inhibition displayed a sigmoidal response curve (Fig. 1A), a consequence of cooperativity (13), with the Hill equation for allosteric interaction enzymes, therefore, being the appropriate function for fitting to the data: v = Vmax Sn/(K + Sn), where v = inhibition, Vmax = maximum inhibition, and S = substrate/inhibitor concentration. The IC50 of DNR transport in the CR1R12 cell line was ~9 μM, and SCH66336 achieved an extent of inhibition exceeding that of verapamil. As shown in Fig. 1B, the IC90 for SCH66336 in the NIH3T3-G185 cells (which express the gene product of human MDR1) is ~3 μM, which is three times as potent as the inhibition observed in the CR1R12 cell line overexpressing the rodent enzyme (IC50 ~9 μM; Fig. 1A). Because these cell lines overexpress the Pgp transporter enzyme, the IC50 would be expected to be lower under in vivo conditions, under which far fewer copies of the enzyme would be
contained per cell. The other fluorescent marker, Rho, is also significantly retained in the presence of SCH66336 with an IC_{50} of ~11 μM (Fig. 2; NIH-G185 cells; or about 9 μM in CR1R12 cells). Table 1 shows the results from similar experiments with other well-known inhibitors of Pgp. Significantly, SCH 66336 is similar in potency to cyclosporin A, the most potent of the representative sample of well-known Pgp inhibitors shown (Table 1).

When SCH66336 is combined with tamoxifen, paclitaxel, or vinblastine, a synergistic interaction is observed (Fig. 3). By adding 0.5, 1, or 2 μM of SCH66336, respectively, to each of these chemotherapeutics, the potency (quantified by IC_{50}) of inhibition is increased. The effect of SCH66336 on the Pgp binding qualities of these compounds is not only additive but also synergistic because it increases their apparent affinity as inhibitors, thus potentiating their respective inhibition.

**ATP Hydrolysis.** As ATP is consumed at a purported rate of about one or two per transport event, the hydrolysis of ATP represents transport function turnover rate or activity assay (34–38, 11). In the absence of exogenous substrate, the enzyme is still able to hydrolyze ATP to produce a basal level of activity, which is probably attributable to the transport of endogenous substrates. Therefore, activity data are presented as a percentage of the basal or control activity, and examples of results from various known Pgp substrates are shown in Table 2 (hamster Pgp). Any change in the rate of ATP hydrolysis represents the sum of the basal activity and the contribution of the exogenous substrate to ATP hydrolysis. Therefore, a substrate with a rate similar to basal activity may not exhibit altered ATP hydrolysis activity as a result of masking by the basal activity. The presence of SCH66336 causes a concentration-dependent decrease in the rate of ATP hydrolysis relative to baseline rate, a result that identifies SCH66336 as a comparatively slow substrate (the standard being the putative endogenous substrate causing the baseline ATP hydrolysis) for Pgp (Ref. 39; Fig. 4). This effect is ostensibly attributable to

### Table 1. Pgp inhibition parameters of several example compounds

Data are nonlinear regression solutions to Hill function followed by SE. I_{max} is a percentage of inhibition by verapamil. Inhibition of DNR transport function was evaluated using NIH3T3-G185 cell line.

| Compound    | IC_{50} ,
<table>
<thead>
<tr>
<th></th>
<th>μM</th>
<th>I_{max} , %</th>
<th>n (Hill coefficient)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCH66336</td>
<td>2.7 ± 0.1</td>
<td>101 ± 2</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>1.4 ± 0.1</td>
<td>112 ± 4</td>
<td>3.6 ± 1.0</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>54 ± 1.3</td>
<td>54 ± 0.6</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>Verapamil</td>
<td>4.2 ± 1.1</td>
<td>100</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>18 ± 0.3</td>
<td>90 ± 0.4</td>
<td>4.9 ± 0.2</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>7.1 ± 0.3</td>
<td>73 ± 1.4</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>7.2 ± 0.2</td>
<td>76 ± 1</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>5.6 ± 0.4</td>
<td>112 ± 2</td>
<td>1.6 ± 0.2</td>
</tr>
</tbody>
</table>

### Table 2. Kinetic parameters of ATP hydrolysis for various substrates

Microsomes were from CR1R12 cells overexpressing the hamster Pgp.

<table>
<thead>
<tr>
<th>Compound</th>
<th>V_{max} , % control</th>
<th>SD</th>
<th>K_{m} , μM</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCH66336</td>
<td>28</td>
<td>4</td>
<td>3.2</td>
<td>1.7</td>
</tr>
<tr>
<td>Verapamil</td>
<td>194</td>
<td>7</td>
<td>22</td>
<td>6</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>180</td>
<td>7</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>220</td>
<td>18</td>
<td>2.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Quinidine</td>
<td>239</td>
<td>9</td>
<td>8</td>
<td>1.6</td>
</tr>
<tr>
<td>Progesterone</td>
<td>236</td>
<td>8</td>
<td>7.4</td>
<td>2</td>
</tr>
<tr>
<td>DNR</td>
<td>170</td>
<td>17</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>TPP{a}</td>
<td>365</td>
<td>30</td>
<td>41</td>
<td>17</td>
</tr>
</tbody>
</table>

{a} TPP, tetraphenyl phosphonium.
competition with an endogenous substrate with a rate of transport approximately that of the baseline ATP hydrolysis rate. The $K_m$ is $\sim 3 \, \mu M$, and the $V_{\text{max}}$ is $\sim 28\%$ of baseline. The ATP hydrolysis activity assay results are consonant with the transport function inhibition assays (described above).

**DISCUSSION**

SCH66336 is an orally active, FPT inhibitor that suppresses tumor growth in human xenograft and transgenic mouse cancer models *in vivo* (26). In addition to its FPT inhibitor function, the cancer therapeutic SCH66336 demonstrates, as our results show, dramatic inhibition of Pgp transport in a concentration-dependent manner with an IC$_{50}$ of $<3 \, \mu M$. The effect on rhodamine transport function is slightly less potent; however, differences in the binding and transport between the two binding sites has often been reported (10, 11, 13) and the more sensitive marker substrate (DNR) is more relevant to performance. These experiments use the NIH-G185 transfected cell line expressing supraphysiologic quantities of the Pgp transporter enzyme. Therefore, SCH66336 inhibition of Pgp *in vivo* should be significantly more potent. ATP hydrolysis kinetics with microsomes from CR1R12 cells show that SCH66336 interacts with Pgp with a $K_m$ of about $3 \, \mu M$. Moreover, the kinetic results indicate that SCH66336 is also a very slow substrate of Pgp and, hence, a superior inhibitor of Pgp, because it should have a prolonged residence inside the cell. In other words, this potent Pgp inhibitor is apparently removed from membrane inner leaflet (or cytosol) relatively slower than the commonly faster substrates. The molecular structure of SCH66336 typifies the “type II unit” of 3-electron donor groups with a spatial separation of $4.6 \pm 0.6 \, \AA$ suggested by Seelig (40) as one of two general patterns for substrate recognition by Pgp (constructed from a structure activity relationship study of known substrates of Pgp).

Many cancer therapeutics are actively removed from tumors by Pgp, and in many tumor cells, the amount of Pgp presented at the surface of the cell is significantly elevated above that of normal cells. This active efflux renders many tumors uniquely resistant and requires elevated dosages of chemotherapy, which in turn produces increased toxicity in normal cells. Because SCH66336 is a potent inhibitor as well as a remarkably slow substrate of Pgp, it can obstruct a major form of drug resistance. The close analogues to SCH66336 also exhibit similar effects, as expected, particularly because the Pgp substrate recognition site is well known for tolerance to a wide variety of amphipathic and/or lipophilic compounds (data not shown). The inhibition of Pgp by the FPT inhibitor SCH66336 is serendipitous, because this binding is apparently not a fundamentally shared quality.

There is no rationale to hypothesize that there is a concerted architecture between the active sites of FPT and Pgp, nor a mechanistic or pharmacological linkage. FPT catalyzes the transfer of isopenoids (farnesyl) to a transmembrane protein, whereas Pgp moves various structurally unrelated amphipathic and/or lipophilic compounds from the membrane inner leaflet to the exterior of the cell. Treatment with the potent Pgp inhibitor SCH66336 would be expected to be synergistic with coadministered cancer therapeutics that are substrates of Pgp. Indeed, SCH66336 exhibits enhanced antitumor activity when combined with vincristine (26, 27), an efficient substrate of Pgp. Moreover, a recent report shows a synergy with coadministration of paclitaxel or docetaxel (two known substrates of Pgp) *in vitro* and *in vivo* (28). SCH66336 significantly enhanced the effect of paclitaxel in the NCI-H460 lung cancer xenograft model and was able to sensitize wap-ras/F mammary tumors as well as tumor cell lines to paclitaxel (28). We have directly characterized and quantified a specific synergy on Pgp function between SCH66336 and tamoxifen, paclitaxel, or vinblastine. Relatively small concentrations of SCH66336 can increase the affinity (potency) of these additional compounds as inhibitors of Pgp function. The synergy provided by SCH66336 on other Pgp inhibitors may contribute to the observed enhanced efficacy *in vivo* by these pairs, but it is difficult to exclude a role for other mechanisms of interaction. Moreover, SCH66336 may inhibit other export transporters in this ABC transporter superfamily, and we will pursue this question experimentally. We are currently unaware of another chemical series of FPT inhibitors that directly affects Pgp function. Because the testing of a FPT inhibitor that is not a Pgp inhibitor may result in some similar *in vivo* enhancements via another ABC transporter, such studies would reveal little about mechanism.

Several MDR reversing agents are in various stages of clinical development to enhance and increase drug absorption and retention, particularly in tumor cells. These clinical synergies or enhancements of chemotherapeutic effects can be because of inhibition of Pgp by SCH66336, which, when coadministered with the many therapies that are substrates of Pgp, increases drug retention in tumor cells. A further benefit of coadministration of SCH66336 could be reduced chemotherapy dosage, hence less assault to normal cells and therefore lower undesired toxicity.

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**REFERENCES**


![Fig. 4. Pgp-mediated ATP hydrolysis rates in the presence of SCH 66336. The data are fit to a hyperbola and the $V_{\text{max}} = 28\%$ of control with a $K_m = 3.2 \pm 1 \, \mu M$. Enzyme activity from microsomes of CR1R12 cells overexpressing hamster Pgp.](image-url)


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