SCY-635, a Novel Nonimmunosuppressive Analog of Cyclosporine That Exhibits Potent Inhibition of Hepatitis C Virus RNA Replication *In Vitro*[†]

Sam Hopkins,^{1*} Bernard Scorneaux,¹ Zhuhui Huang,³ Michael G. Murray,³ Stephen Wring,¹ Craig Smitley,¹ Richard Harris,¹ Frank Erdmann,² Gunter Fischer,² and Yves Ribeill¹

Scynexis, Inc., Durham, North Carolina 27713¹; Max-Planck Research Unit for Enzymology of Protein Folding, Weinbergweg 22, Halle/Saale D-06120, Germany²; and Southern Research Institute, 431 Aviation Way, Frederick, Maryland 21701³

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SCY-635 is a novel nonimmunosuppressive cyclosporine-based analog that exhibits potent suppression of hepatitis C virus (HCV) replication *in vitro*. SCY-635 inhibited the peptidyl prolyl isomerase activity of cyclophilin A at nanomolar concentrations but showed no detectable inhibition of calcineurin phosphatase activity at concentrations up to 2 μ M. Metabolic studies indicated that SCY-635 did not induce the major cytochrome P450 enzymes 1A2, 2B6, and 3A4. SCY-635 was a weak inhibitor and a poor substrate for P-glycoprotein. Functional assays with stimulated Jurkat cells and stimulated human peripheral blood mononuclear cells indicated that SCY-635 is a weaker inhibitor of interleukin-2 secretion than cyclosporine. A series of two-drug combination studies was performed *in vitro*. SCY-635 exhibited synergistic antiviral activity with alpha interferon 2b and additive antiviral activity with ribavirin. SCY-635 was shown to be orally bioavailable in multiple animal species and produced blood and liver concentrations of parent drug that exceeded the 50% effective dose determined in the bicistronic con1b-derived replicon assay. These results suggest that SCY-635 warrants further investigation as a novel therapeutic agent for the treatment of individuals who are chronically infected with HCV.

Hepatitis C virus (HCV) is a member of the *Flaviviridae* family, which comprises three distinct genera, including the flaviviruses (such as yellow fever virus, dengue virus, West Nile virus, and Japanese encephalitis virus), the pestiviruses (bovine viral diarrhea virus and classical swine fever virus), and the hepaciviruses (of which HCV is the only member) (16). HCV is highly polymorphic, and current taxonomic schemes recognize six major genotypes and several subtypes. Although no strict relationship exists between the genotype and the severity of HCV disease or the clinical outcome, numerous clinical studies indicate that patients who are infected with genotype 1 viruses are less responsive to antiviral therapy than individuals who are infected with genotypes 2 through 6 (10, 11). Chronic infection with HCV now represents a major global health problem, with approximately 170 million people worldwide being infected (26). The current standard of care for chronic hepatitis C virus infection involves treatment for up to 1 year with combination chemotherapy of pegylated alpha interferon coadministered with ribavirin. At this time, there are no approved drugs specifically indicated for the treatment of patients who do not respond to first-line therapy. Complete clearance of the virus is achieved in approximately 50% of all HCV-infected patients who initiate therapy (10, 11), and the response rates are related to viral factors (the genotype and the viral load), as well as

multiple host factors (the presence of liver fibrosis, cirrhosis, ethnicity, coinfection with HIV type 1 [HIV-1], alcohol consumption, and metabolic disorders).

At this time, the combined action of interferon and ribavirin against HCV infection is poorly understood. The exogenous administration of type 1 alpha interferon confers a nonspecific antiviral state which is characterized by the induction of a broad array of interferon-stimulated genes (ISGs). The principal actions of the ISGs are to block the initiation of viral protein synthesis and to decrease the stability of viral RNA, as well as to stimulate both the adaptive and the innate immune responses to infection (6). Clinically, interferon (most notably, its pegylated derivatives) has been demonstrated to induce multi-log-unit declines in the levels of plasma viremia. Ribavirin undergoes intracellular phosphorylation to its mono-, di-, and triphosphate derivatives. Ribavirin triphosphate is a low-affinity inhibitor of the viral NS5B polymerase and a substrate for incorporation into nascent genomic RNA. The utilization of ribavirin triphosphate as a substrate for RNA synthesis may ultimately inhibit viral RNA replication through error catastrophe. Ribavirin monophosphate competitively inhibits IMP dehydrogenase, which could deplete intracellular GTP levels, further augmenting the inhibitory effects of ribavirin triphosphate (6). Studies of ribavirin monotherapy indicate that it results in the transient and modest suppression of plasma viremia in some but not all patients (20); however, its greatest treatment benefit may be in suppressing the rebound of viremia following the completion of combination therapy with pegylated interferon. Treatment with pegylated alpha interferon and ribavirin is associated with a wide range of severe toxicities, including neuropsychiatric events, bone marrow tox-

* Corresponding author. Mailing address: Scynexis, Inc., 3501C Tri-center Boulevard, Durham, NC 27713. Phone: (919) 544-8640. Fax: (919) 544-8697. E-mail: Sam.Hopkins@scynexis.com.

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iciencies, endocrine disorders, cardiovascular events, and anemia (25). In addition, ribavirin is teratogenic in multiple animal species. These observations underscore the need to discover and develop potent and specific antiviral agents that can augment the clinical anti-HCV activity of the current standard of care without increasing toxicity. Ultimately, it is envisioned that highly specific orally bioavailable antiviral agents could form the cornerstones of new treatment regimens that do not contain interferon and ribavirin.

Several groups have confirmed that the replication of subgenomic and full-length HCV genomes depends on the expression of the host protein cyclophilin A (CyPA) (4, 14, 34). The levels of HCV RNA replication and viral protein production were reduced up to 1,000-fold in cell lines harboring the stable knockdown of CyPA expression. The knockdown of cyclophilin B, C, and D expression had no effect on HCV-specific RNA replication or protein production. The inhibition of replication was rescued by the overexpression of a wild-type CyPA escape mutant; however, the overexpression of a mutant CyPA containing the H126Q substitution (which abolishes peptidyl prolyl isomerase [PPIase] activity) did not rescue replication. These results further suggest that HCV RNA replication requires PPIase catalytic activity. The *in vitro* anti-HCV activity of cyclosporine (CsA) has been reported, thus confirming that the cyclophilins are potential drug targets (28). The potential clinical utility of cyclosporine-based inhibitors has been suggested by the introduction of nonimmunosuppressive analogs of CsA that retain cyclophilin binding activity. NIM 811 and Debio-025 contain modifications at the three sarcosine and the four methyl leucine residues. Both compounds inhibit HCV-specific RNA replication *in vitro* (17, 19). Clinical studies indicate that Debio-025 suppresses HCV plasma viremia when it is given as monotherapy for 14 days to patients coinfecting with HIV-1 and HCV and when it is given as a component of a two-drug combination regimen with pegylated interferon for 28 days to HCV-monoinfected patients (8, 9).

This report describes the preclinical profile of SCY-635, a 3,4-disubstituted nonimmunosuppressive CsA analog that exhibits potent suppression of HCV RNA replication *in vitro*. SCY-635 is a reversible, nanomolar inhibitor of the PPIase activity expressed by CyPA. SCY-635 is orally bioavailable in multiple species and distributes extensively to hepatocytes. Metabolic studies indicate that the administration of SCY-635 is unlikely to result in adverse pharmacological interactions. Two-drug synergy studies indicate that SCY-635 exhibits additive to synergistic antiviral activity when it is tested *in vitro* with alpha interferon 2b (IFN α -2b) or ribavirin without increasing cell cytotoxicity. These results are consistent with the recent observation of the potent clinical antiviral activity of SCY-635 (13) and suggest that further clinical studies assessing the safety and antiviral activity of SCY-635 in combination with interferon and ribavirin are warranted.

MATERIALS AND METHODS

Purification of CyPA. CyPA was purified by a methodology published previously (22). Recombinant CyPA protein without N- or C-terminal extensions was overexpressed in *Escherichia coli* M15 and was induced with 1 mM isopropyl-beta-D-thiogalactopyranoside for 5 h at 37°C. Cells were lysed in 20 mM Tricine buffer (pH 8.0), and the lysates were purified on an anion-exchange column [Fractogel EMD DEAE-650(M); Merck, Darmstadt, Ger-

many]. The flowthrough was collected and applied to an affinity column (Fractogel TSK AF-Blue; Merck). CyPA was eluted with a gradient of 0 to 3 M KCl in 20 mM Tricine buffer (pH 8.0). The eluted fractions were collected and dialyzed twice against 10 mM HEPES (pH 7.0). CyPA was further purified by a Fractogel 6/27-R1 SO-3 exchange column (Merck), and the protein was eluted with a 0 to 1 M gradient of NaCl. Fractions were examined for purity by SDS-PAGE and high-pressure liquid chromatography (HPLC). By using the appropriate extinction coefficient, the preparation was demonstrated to be 94% pure.

MDCKII-hMDR1 cells. MDCKII-hMDR1 cells were generously provided by Pieter Borst at The Netherlands Cancer Institute. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) with the dipeptide form of L-glutamine (Glutamax), 10% (vol/vol) fetal bovine serum, and 1% (vol/vol) penicillin-streptomycin at 10,000 units/ml. The cell monolayers were fed the cell culture medium 24 h after seeding and were used for the permeability studies 2 days later.

HCV replicon cell line. The ET cell line was kindly provided by Ralf Bartenschlager at the University of Heidelberg. The ET cell line is a human hepatoma cell line (Huh-7) that contains a con1 (genotype 1b) bicistronic subgenomic replicon and is described further in supplement S1 in the supplemental material.

Antiviral activity and cytotoxicity assay. Assessments of antiviral activity and cytotoxicity are described in supplement S1 in the supplemental material.

Effects of human serum on the anti-HCV activity of SCY-635. The subgenomic replicon system was used to assess the effects of increasing concentrations of human serum on the anti-HCV activity of SCY-635. The standard cell culture conditions used for the replicon system are described in supplement S1 in the supplemental material. Culture medium was supplemented either with 5% fetal bovine serum (FBS) (0% human serum) or with human serum to achieve final concentrations of 10%, 20%, and 40%. Anti-HCV activity (expressed as the 50% effective concentration [EC₅₀]) was assessed by using the luciferase end point following 72 h of incubation with SCY-635 at concentrations ranging from 0.05 to 5 μ M.

PPIase inhibition assay. PPIase activity was determined by using the peptide N-succinyl-Ala-Ala-Pro-Phe-4-nitroanilide (L-1400; Bachem, Torrance, CA) in a protease-coupled assay, according to published procedures (21). Changes in the absorbance at 390 nm were monitored for 240 s. Inhibition constants were determined as previously described for tightly binding reversible enzyme inhibitors (18). PPIase experiments were performed with in-house-prepared human recombinant CyPA at concentrations in the low-nanomolar range (0.5 to 2.0 nM).

Indirect inhibition of calcineurin phosphatase activity. The level of inhibition of calcineurin phosphatase activity was determined by measuring the dephosphorylation of *p*-nitrophenyl phosphate, according to published procedures (2). Briefly, CsA or SCY-635 was incubated in a 1:1 molar ratio with CyPA in the presence of calcineurin (1.32 nM) and calmodulin (50 nM) at 22°C for 30 min. All determinations were performed in triplicate. Mean values were expressed as the percentage of calcineurin activity calculated relative to that for an inhibitor-free control.

***In vitro* immunosuppression assays.** The effects of SCY-635 and CsA on cellular proliferation and interleukin-2 (IL-2) secretion were evaluated by using Jurkat cells and human peripheral blood mononuclear cells (PBMCs; institutional review board approval was obtained). A detailed description of the methods used in these studies is presented in supplement S2 in the supplemental material.

Human plasma protein binding. Binding of SCY-635 to human plasma proteins was determined by ultrafiltration by methods adapted to reduce nonspecific binding (27). CsA, SCY-635, or a control compound (warfarin, imipramine, or carbamazepine) was added to pooled fresh human plasma (Bioreclamation, Liverpool, NY), and the mixture was incubated at 37°C for 30 min before centrifugation. The amounts of unbound (free) and bound compound in the ultrafiltrate and retentate, respectively, were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Briefly, chromatographic separation was achieved by gradient elution from a Luna C₈ 3- μ m-particle-size HPLC column (50 by 2 mm; Phenomenex, Torrance, CA). The starting gradient conditions were 80:20 water-methanol (5 mM ammonium formate and 0.1% formic acid) for 15 s, followed by a linear gradient to 5:95 water-methanol over 1.75 min and a hold for 1 min. SCY-635 eluted at 2.3 min. An Applied Biosystems (Foster City, CA) API 4000 tandem mass spectrometer with turbo ion spray ionization was operated in the multiple-reaction-monitoring mode to monitor the precursor-to-product ion transition resulting from the doubly charged ion (*m/z* 661.5/156.0).

P-glycoprotein (P-gp)-mediated transport. MDCKII-hMDR1 cell monolayers were grown to confluence in 12-well Costar Transwell plates (Corning, Corning,

NY). Digoxin (Sigma-Aldrich, St. Louis, MO) was used as a high-permeation marker. Digoxin was prepared in permeation assay buffer (Hanks balanced salt solution containing 10 mM HEPES and 15 mM glucose at pH 7.4; Sigma-Aldrich) and was then added to either the apical (A) or the basolateral (B) compartment at a concentration of 10 μ M. Cell monolayers were incubated with and without the test article at 37°C for up to 2 h. The effect of the test compound on the permeation of digoxin was evaluated by adding SCY-635 at concentrations of 1, 5, and 15 μ M or CsA at concentrations of 5 and 10 μ M. Duplicate samples were removed from the apical and basolateral compartments at 1 and 2 h after the addition of the test article. Samples were assayed by HPLC with tandem mass spectrometry for the concentrations of digoxin. Values for apparent permeability (P_{app}) and percent recovery were calculated. An efflux ratio ($P_{app} B \rightarrow A / P_{app} A \rightarrow B$) was calculated for digoxin.

In a separate experiment with MDCKII-hMDR1 cell monolayers, the permeation of SCY-635 and CsA was evaluated when the compounds were added to either the apical or the basolateral compartment at a concentration of 3 μ M. Cell monolayers were incubated with the test article in triplicate for 1 h at 37°C. Samples were removed from the apical and basolateral compartments after incubation and were assayed for test compound concentrations by LC-MS/MS with electrospray ionization. Values for $P_{app} A \rightarrow B$, $P_{app} B \rightarrow A$, and the efflux ratio were calculated for each compound.

In vitro microsomal metabolism and CYP450 inhibition. The metabolic stabilities of CsA and SCY-635 were evaluated with CD-1 mouse and mixed-gender human liver microsomal fractions (XenoTech, Lenexa, KS). CsA and SCY-635 (1 μ M) were incubated with pooled liver microsomes (1.05 mg protein/ml) for 0, 5, 15, and 30 min at 37°C in an oxygen-enriched environment in the presence of NADPH. At the end of each incubation, the reactions were stopped by the addition of 3 volumes of ice-cold acetonitrile. The incubation mixtures were analyzed for the parent compound by HPLC with tandem mass spectrometry. The metabolic competencies of the microsome preparations were evaluated by using the control compounds 7-ethoxycoumarin, propranolol, and verapamil. Intrinsic clearance (CL_{int}) and half-life ($t_{1/2}$) values were determined for CsA and SCY-635.

The potential for CsA and SCY-635 to inhibit cytochrome P450 (CYP) enzyme activities was assessed by using P450-Glo assay kits (Promega, Madison, WI). Assays for the cytochrome P450 enzymes 3A4, 2C19, 2C9, and 2D6 were performed according to the manufacturer's instructions. Briefly, CsA and SCY-635 were added to membrane preparations containing recombinant human CYP enzymes at concentrations ranging from 1 to 100 μ M, together with luminogenic substrates specific for each enzyme. Specific inhibitors for each CYP enzyme were included as controls. The reactions were initiated by the addition of an NADPH-regenerating system and were allowed to proceed for up to 30 min. CYP enzyme activity was determined following the addition of a luciferin detection reagent and the subsequent measurement of luminescence. The 50% inhibitory concentrations (IC_{50} s) of SCY-635 and CsA were determined for each CYP enzyme. The potential for drug-drug interactions was characterized as high ($IC_{50} < 1 \mu$ M), moderate (1μ M $< IC_{50} < 10 \mu$ M), or low ($IC_{50} > 10 \mu$ M), according to the level of enzyme inhibition observed (15).

Induction of oxidative metabolism in primary human hepatocytes. Primary cultures of human hepatocytes were used to evaluate the potential of SCY-635 to induce the major liver microsomal cytochrome P450 enzymes CYP1A2, CYP2B6, and CYP3A4. A detailed description of the methods is provided in supplement S3 in the supplemental material.

Drug combination assays. The HCV subgenomic replicon was used to assess the effect of SCY-635 in combination with recombinant IFN α -2b (rIFN α -2b) or ribavirin. (The culture conditions for the replicon cells together with the methods describing antiviral activity determinations in the subgenomic and full-length replicon assays are presented in supplement S1 in the supplemental material.) The cells were plated at 5×10^3 cells per well. Plates for antiviral activity and cytotoxicity determinations were prepared in parallel. On the following day, the test articles were diluted and added to the plates to create 40 to 45 discrete two-drug combinations. SCY-635 was tested at nine concentrations with rIFN α -2b prepared at five concentrations. SCY-635 was serially diluted twofold with concentrations ranging from 0.008 to 2.0 μ M. rIFN α -2b was prepared as half-log₁₀ dilutions, producing concentrations of 0.005 to 0.5 IU/ml. SCY-635 was tested at eight concentrations with ribavirin tested at five concentrations. SCY-635 was prepared by the use of twofold dilutions, with the resulting concentrations ranging from 3.91 to 500 nM. Ribavirin was also serially diluted twofold to concentrations ranging from 1.25 to 20 μ g/ml. After 72 h of incubation, the cells were processed to determine the antiviral activity (luciferase) or cytotoxicity (lactate dehydrogenase release).

To determine whether the effects of the two-drug combinations were either

synergistic, additive, or antagonistic, the antiviral activity data were analyzed by using the Prichard and Shipman MacSynergy II data analysis program (23).

PK studies with rats and monkeys. The pharmacokinetics (PKs) and bioavailability of SCY-635 were evaluated in rats and monkeys following intravenous and oral administration. A detailed description of the methods employed for the PK studies is provided in supplement S4 in the supplemental material. The values of the PK parameters were determined from composite mean whole-blood concentration-time data for rats and individual whole-blood concentration-time data for monkeys by using the noncompartmental modeling program in WinNonlin Professional software (version 5.1; Pharsight Corp., Mountain View, CA).

SCY-635 distribution into liver and whole blood. The biological distribution of SCY-635 in male Sprague-Dawley rats was assessed following the intravenous injection of 10 mg/kg of body weight and following oral gavage with 10 and 30 mg/kg. Fifteen rats were used per group; three animals were used per time point. Whole-blood samples for pharmacokinetic analysis were obtained prior to dosing and at 0.5, 1, 2, 4, 6, 8, 12, and 24 h after administration. Terminal liver tissue samples were obtained at 2, 4, 8, 12, and 24 h after dosing. The animals were anesthetized, and their livers were perfused with normal saline prior to removal. The concentration of SCY-635 in the liver was determined from whole-tissue homogenates from the left lateral lobe. Blood and liver samples were stored at -70°C prior to analysis. Samples were assayed for the concentration of SCY-635 by nonvalidated LC-MS/MS methods. PK analysis was performed with WinNonlin Professional software.

Cellular distribution in human hepatocytes and NPCs. Liver cell suspensions were purchased from Celsis (Baltimore, MD). Briefly, whole liver tissue was perfused with physiological saline prior to perfusion with collagenase to disperse the liver cells. The cell suspension was centrifuged at $20 \times g$ for 2 min at 21°C to pellet the hepatocytes. The supernatant, which contained nonparenchymal cells (NPCs), was decanted and centrifuged at $600 \times g$ for 10 min. The pellet of NPCs was resuspended in 30 to 40 ml of Hanks' buffer containing 2.5 mM Ca^{2+} , 11 mM glucose, and 0.5% bovine serum albumin. The NPC fraction was carefully placed on a double layer of Percoll (50% and 25%) and centrifuged at $200 \times g$ for 15 min at 4°C. The middle layers enriched for NPCs (Kupffer and endothelial cells) were collected, centrifuged at $650 \times g$ for 7 min at 4°C, and resuspended in 5 ml of Hanks buffer. The cells were seeded onto sterile petri dishes in medium supplemented with 10% FBS at 37°C.

The hepatocytes were seeded in the bottom of a 24-well Costar plate (Fisher Scientific, Pittsburgh, PA) at a density of 2×10^5 cells/well. A Transwell insert plate was carefully added and the NPCs were seeded in each insert at a density of 1×10^5 cells per well. SCY-635 was added at a final concentration of 500 or 3,000 ng/ml. The plates were incubated for 1 h at 37°C in a humidified incubator with 5% CO₂. The cells were collected at the end of the incubation. The protein content was measured as described by Lowry (Calbiochem, Gibbstown, NJ). The concentration of SCY-635 in the hepatocytes and nonparenchymal cells was determined by LC-MS/MS. The SCY-635 concentration was expressed as the ng of SCY-635 per μ g of protein.

RESULTS

Structure of SCY-635. Figure 1 contains the structural formulas of CsA and SCY-635. Within CsA, residues 9, 10, 11, 1, 2, and 3 form a continuous surface that constitutes the CyPA binding domain, whereas residues 4 through 7, on the opposing face of the molecule, comprise the calcineurin binding domain. SCY-635 differs from CsA at the 3 and 4 positions. SCY-635 contains a dimethylamino-ethylthio substituent at the 3 sarcosine alpha carbon atom and a hydroxyl substituent at the gamma carbon of the 4 *N*-methyl leucine residue. Substitution at the sarcosine position introduces chirality at the alpha carbon atom. The absolute configuration at the sarcosine alpha carbon atom is *R*.

SCY-635 suppresses HCV replication in a time- and a dose-dependent manner. SCY-635 exhibited potent activity in the HCV subgenomic replicon system. The EC₅₀s determined by using the luciferase end point following incubation for 24, 48, 72, and 120 h were 0.20, 0.07, 0.08, and 0.15 μ M, respectively (Fig. 2). The experimental methods and the replicon cell culture conditions used are described in detail in supplement S1

FIG. 1. Chemical structures of CsA (I) and SCY-635 (II). SCY-635 differs from CsA at two positions. The alpha carbon atom of sarcosine at position 3 contains the dimethylamino-ethylthio substituent. The gamma carbon atom of the *N*-methyl leucine at position 4 contains a hydroxyl group.

in the supplemental material. Complete inhibition of HCV replication was not observed at the 24-h (~70% maximal observed suppression) or the 48-h (~96% maximal observed suppression) time points. Greater than 99% inhibition of HCV replication was observed at 72 and 120 h of incubation. Al-

though the EC_{50} s remained relatively constant, these results demonstrate that the inhibitory activity of SCY-635 increased as the time of incubation in culture increased.

The average $EC_{50} \pm$ standard deviation (SD) was determined in a series of 23 subsequent assessments by using the luciferase end point and a 72-h incubation period. The EC_{50} and the EC_{90} were determined to be $0.10 \pm 0.02 \mu\text{M}$ and $0.35 \pm 0.07 \mu\text{M}$, respectively, under these conditions. No significant cytotoxicity for replicon cells was observed following 72-h incubations with SCY-635 at concentrations up to $5 \mu\text{M}$.

Effects of human serum on anti-HCV activity of SCY-635.

There was no evidence of cell cytotoxicity with any of the combinations of SCY-635 and added human serum. In the presence of 0%, 10%, 20%, and 40% human serum, SCY-635 exhibited EC_{50} s of $0.08 \mu\text{M}$, $0.09 \mu\text{M}$, $0.11 \mu\text{M}$, and $0.12 \mu\text{M}$, respectively. Although the EC_{50} s tended to increase slightly with the increasing proportions of added human serum, all values obtained in this study were within the range of values determined for SCY-635 with the subgenomic replicon system under standard conditions. These results suggest that the antiviral activity of SCY-635 is not affected by the addition of human serum.

SCY-635 inhibits the peptidyl prolyl *cis/trans*-isomerase activity of cyclophilin A. Cyclophilins are expressed throughout human tissues. Cyclophilins catalyze the *cis/trans* isomerization of the peptide bond that directly precedes proline in a polypeptide chain (5). The catalytic domains that contain the PPIase activity of all cyclophilins share a high degree of sequence homology with CyPA (CyP18). CyPA has a molecular mass of approximately 18 kDa and is the prototypic member of this family of proteins.

The ability of SCY-635 to inhibit the PPIase activity of CyPA was assessed in order to establish a correlation between cyclo-

FIG. 2. Suppression of HCV replication *in vitro*. The anti-HCV activity of SCY-635 was assessed by using the HCV subgenomic replicon system. HCV RNA replication was assessed by quantifying HCV replicon-derived luciferase activity. The antiviral activity of SCY-635 was evaluated at 24, 48, 72, and 120 h after drug treatment. Exposure of replicon cells to SCY-635 reduced the level of HCV replication in a time- and a dose-dependent manner.

FIG. 3. Inhibition of PPIase activity of CyPA by CsA and by SCY-635. PPIase activity was determined in a coupled assay with chymotrypsin by using *N*-succinyl-Ala-Ala-Pro-Phe-4-nitroanilide as the substrate. Determinations were performed in duplicate. Inhibition constants were determined by fitting the data to a model for tightly binding reversible enzyme inhibitors.

philin binding and the inhibition of HCV replication (Fig. 3). PPIase activity was determined in a coupled assay with chymotrypsin by using the model substrate *cis*-succinyl-Ala-Ala-Pro-Phe-4-nitroanilide (7).

The rate of product formation catalyzed by the PPIase activity of CyPA was reduced in the presence of increasing concentrations of CsA or SCY-635. K_i values of 0.00264 ± 0.00056 μM and 0.00184 ± 0.00033 μM were determined for CsA and SCY-635, respectively. These results suggest that SCY-635 is a tightly binding inhibitor of the PPIase activity and that the 3 and 4 positions are not critical determinants for the binding of SCY-635 to the active site of CyPA.

Reduced calcineurin binding activity of the CyPA-SCY-635 binary complex. X-ray crystallographic studies indicate that calcineurin, CyPA, and CsA combine to form a stable ternary complex with a 1:1:1 stoichiometry (15). Residues 9 through 11 and residues 1 through 3 of CsA form a continuous surface that fits into the PPIase active site of CyPA. Residues 4 through 7, on the opposing face of CsA, form the primary interface with calcineurin and make bridging contacts with both the alpha and the beta subunits. The side chains of the 4-methyl leucine and the 6-methyl leucine residues comprise the primary interface with calcineurin. Secondary contacts with calcineurin are made through residues 8, 9, and 1 (through the 2-butenyl substituent).

Preincubation studies were performed with the binary complexes formed between CyPA and either CsA or SCY-635 in order to determine if the substitutions at the 3 and 4 positions in the cyclosporine ring of SCY-635 altered the affinity of the CyPA-SCY-635 binary complex for calcineurin. Formation of the ternary complex was measured by determining the level of inhibition of calcineurin phosphatase activity in the presence of a fixed concentration of calcineurin and various concentra-

tions of each respective drug-CyPA binary complex by published methods (2).

The dose-dependent inhibition of calcineurin phosphatase activity was observed in the presence of the CyPA-CsA binary complex over the range of 0.01 to 10 μM (Fig. 4). The complete inhibition of phosphatase activity was observed at 5 μM , whereas 50% inhibition was observed at 0.12 μM . No inhibition of calcineurin phosphatase activity was observed in the presence of the CyPA-SCY-635 binary complex at concentrations up to 2 μM , which represented the highest concentration tested. These results suggest that the CyPA-SCY-635 binary complex exhibits a relatively low binding affinity for calcineurin.

SCY-635 is a weak inhibitor of IL-2 production in Jurkat cells and in freshly isolated human PBMCs. SCY-635 and CsA were compared for their abilities to inhibit IL-2 production in immortalized T lymphocytes and in freshly isolated human PBMCs. CsA was a potent inhibitor of IL-2 production and exhibited an EC_{50} of 0.005 μM in Jurkat cells (Fig. 5). No cytotoxicity was observed for CsA at the highest concentration tested (0.416 μM). SCY-635 was a weak inhibitor of IL-2 production and exhibited an EC_{50} of 9.9 μM in Jurkat cells. SCY-635 exhibited 40% cell cytotoxicity at this concentration. Comparison of the EC_{50} s indicates that SCY-635 is 1,980-fold less active than CsA in this system; however, this comparison may underestimate the relative difference in IL-2 suppression due to the moderate degree of cytotoxicity observed for SCY-635 at its EC_{50} .

When CsA was evaluated with human PBMCs, it was a potent inhibitor of IL-2 secretion and exhibited an average EC_{50} of 0.0031 μM , in the absence of a significant effect on cellular proliferation. A significant inhibition (>50%) of proliferation was observed for CsA at concentrations greater than

FIG. 4. Inhibition of calcineurin phosphatase activity by the binary complex formed between CsA and human CyPA and by the binary complex formed between SCY-635 and human CyPA. Calcineurin phosphatase activity was determined by measuring the dephosphorylation of *p*-nitrophenyl phosphate. CsA or SCY-635 was incubated in a 1:1 molar ratio with CyPA in the presence of calcineurin (0.00132 μM) and calmodulin (0.050 μM) at 22°C for 30 min. All determinations were performed in triplicate. Mean values were expressed as the percentage of calcineurin activity calculated relative to that for an inhibitor-free control.

0.3 μM . SCY-635 inhibited IL-2 secretion at an average EC_{50} of 5.3 μM ; however, a sharp decline in cellular proliferation was observed at 10 μM . The levels of cell viability with 10 μM ranged from 10.9 to 37.5% of that for the control. These data indicate that SCY-635 is approximately 1,767-fold less potent than CsA with respect to the suppression of IL-2 secretion from stimulated human PBMCs. This difference should be

considered an underestimate due to the observation that the suppression of IL-2 secretion and the inhibition of cellular proliferation are both observed over a relatively narrow nominal concentration range of 3 to 10 μM .

Human plasma protein binding. The binding of SCY-635 to human plasma proteins was determined by ultrafiltration. The mean $\pm$ SD values for the degree of binding for the control

FIG. 5. SCY-635 is a weaker inhibitor of IL-2 secretion than CsA. Jurkat cells (2×10^5 cells/well) were pretreated for 1 h at 37°C with either SCY-635 (concentration range, 0.0015 μM to 10 μM) or CsA (concentration range, 0.000033 μM to 0.416 μM). Cells were induced with a mixture of anti-CD3 and anti-CD28 antibodies at 0.4 μg /well and 2 μg /well, respectively. Sample supernatants were collected for measurement of the level of IL-2 production at the end of a 6-h incubation.

TABLE 1. Inhibition of P-gp-mediated transport of digoxin by CsA and SCY-635 and the permeation and efflux ratios for SCY-635 and CsA

Study	Compound	Concn (μM)	P_{app} A $\rightarrow$ B ^a (nm/s)	P_{app} B $\rightarrow$ A ^a (nm/s)	Efflux ratio
1	Control	0	1.5	99.7	64.3
	SCY-635	1	1.9	98.5	53.2
	SCY-635	5	6.5	33.9	5.2
	SCY-635	15	8.2	12.2	1.5
	CsA	5	5.7	9.3	1.6
2	SCY-635	3	1.86 $\pm$ 0.45*	30.7 $\pm$ 3.1*	16.5
	CsA	3	1.60 $\pm$ 0.44*	446 ^b	279

^a Values represent the means from duplicate determinations, except as noted by as asterisk, in which case the values represent the means from triplicate determinations presented with the SDs.

^b The SD is not reported; the mean was determined from two replicate values.

compounds warfarin, imipramine, and carbamazepine were 98% $\pm$ 1%, 93% $\pm$ 2.5%, and 68% $\pm$ 7.7%, respectively, and were consistent with previous findings in our laboratories. In contrast to CsA, which was >99.6% bound, SCY-635 exhibited a modest degree of binding to plasma proteins (77% $\pm$ 4.5% bound).

SCY-635 is a weak inhibitor of P-gp-mediated transport. MDCKII-hMDR1 cell monolayers were used to assess the ability of SCY-635 to inhibit the P-gp-mediated transport of digoxin (31). In the first study, the values for P_{app} A $\rightarrow$ B, P_{app} B $\rightarrow$ A, and the efflux ratio (P_{app} B $\rightarrow$ A/ P_{app} A $\rightarrow$ B) were determined for digoxin in the presence of increasing concentrations of SCY-635 (Table 1). In the absence of SCY-635, the efflux ratio for digoxin was 64.3, consistent with its recognition as a known substrate for P-gp. In the presence of SCY-635, dose-related changes in P_{app} A $\rightarrow$ B, P_{app} B $\rightarrow$ A, and the efflux ratio were observed. The values for P_{app} A $\rightarrow$ B increased over the nominal SCY-635 concentration range of 0 μM , 1 μM , 5 μM , and 15 μM and were 1.5, 1.9, 6.5, and 8.2 nm/s, respectively, whereas the values for P_{app} B $\rightarrow$ A decreased and were 99.7, 98.5, 33.9, and 12.2 nm/s at the same nominal concentrations of SCY-635, respectively. The corresponding values for the efflux ratio were 64.3, 53.2, 5.2, and 1.5, respectively. These results indicate that the nearly complete inhibition of the P-gp-mediated efflux of digoxin was achieved with SCY-635 at 15 μM (the highest concentration tested). In comparison, nearly complete inhibition of digoxin efflux (efflux ratio = 1.6) was achieved with CsA at 5 μM (the lowest concentration tested), suggesting that SCY-635 is a less potent P-gp inhibitor.

A second study examined the *in vitro* permeation and the P-gp interaction of CsA and SCY-635 (Table 1). When they were added to MDCKII-hMDR1 cell monolayers at a concentration of 3 μM , CsA and SCY-635 showed mean P_{app} A $\rightarrow$ B values of 1.60 and 1.86 nm/s, respectively, and mean P_{app} B $\rightarrow$ A values of 446 and 30.7 nm/s, respectively. The corresponding efflux ratios for CsA and SCY-635 were 279 and 16.5, respectively, indicating that SCY-635 is less efficiently recognized and transported by P-gp than CsA.

SCY-635 is a compound with low clearance when it is incubated with human or mouse liver microsomes and does not inhibit cytochrome P450 enzymes. The values of CL_{int} and $t_{1/2}$

TABLE 2. Comparison of the *in vitro* microsomal metabolism of CsA and SCY-635

Compound	CL_{int} ^a (ml/min/mg)		$t_{1/2}$ ^a (min)	
	Mouse	Human	Mouse	Human
SCY-635	9	14	93	63
CsA	77	29	11	29

^a The values for CL_{int} and $t_{1/2}$ represent the means from duplicate determinations.

were determined for CsA and SCY-635 following incubation with CD-1 mouse and mixed-gender human liver microsomes (Table 2). The clearance values for CsA in CD-1 mouse liver microsomes and in human liver microsomes were 77 $\mu\text{l}/\text{min}/\text{mg}$ and 29 $\mu\text{l}/\text{min}/\text{mg}$, respectively. The corresponding clearance values for SCY-635 were 9 $\mu\text{l}/\text{min}/\text{mg}$ and 14 $\mu\text{l}/\text{min}/\text{mg}$, respectively. The half-lives for CsA in CD-1 mouse and human liver microsomes were 11 min and 29 min, respectively. The corresponding values for SCY-635 were 93 min and 63 min, respectively. These results indicate that SCY-635 exhibits intrinsic clearance values that are approximately 2.1- to 8.6-fold lower than the values calculated for CsA. In each species, the half-lives for SCY-635 were greater than those for CsA and increased inversely with the change in clearance.

The potential for CsA and SCY-635 to inhibit cytochrome P450 enzyme activities was assessed by using P450-Glo assay kits from Promega. The potential for drug-drug interactions which are mediated through the inhibition of cytochrome P450 activities was classified as high ($\text{IC}_{50} < 1 \mu\text{M}$), moderate ($1 \mu\text{M} < \text{IC}_{50} < 10 \mu\text{M}$), or low ($\text{IC}_{50} > 10 \mu\text{M}$), according to the observed level of enzyme inhibition (15). CsA yielded IC_{50} s of 6.6 μM and 7.0 μM for CYP3A4 and CYP2C19, respectively, indicating moderate risk, whereas the IC_{50} s were >10 μM for CYP2C9 and CYP2D6 (Table 3). In contrast, SCY-635 exhibited IC_{50} s that were >10 μM for all CYP isoforms studied, indicating a relatively low potential for CYP450-based adverse drug-drug interactions.

Exposure of primary human hepatocytes to SCY-635 does not induce cytochrome P450 isoform 1A2, 2B6, or 3A4. Human hepatocytes were used to evaluate the potential of SCY-635 to induce the expression of the major cytochrome P450 isozymes, CYP1A2, CYP2B6, and CYP3A4. Four concentrations of SCY-635 (0.1, 1, 10, and 15 μM) together with positive controls, including the known cytochrome P450 inducers 3-meth-

TABLE 3. Potential for CsA and SCY-635 to inhibit cytochrome P450 enzyme activities

CYP isoform	IC_{50} (μM) ^a	
	CsA	SCY-635
3A4	6.6 $\pm$ 0.86	58.5 $\pm$ 0.94
2C19	7.0 $\pm$ 0.94	12.4 $\pm$ 0.80
2C9	38.8 $\pm$ 0.61	13.4 $\pm$ 0.99
1A2	>100	>100
2D6	>100	>100

^a The potential for drug-drug interactions was classified as high ($\text{IC}_{50} < 1 \mu\text{M}$), moderate ($1 \mu\text{M} < \text{IC}_{50} < 10 \mu\text{M}$), or low ($\text{IC}_{50} > 10 \mu\text{M}$), according to the level of enzyme inhibition observed. The values are the means $\pm$ SDs of triplicate determinations.

TABLE 4. Induction of CYP enzyme activity in primary human hepatocytes

Treatment (concn [μ M])	Fold induction of enzyme activity relative to that by 0.1% DMSO control ^a		
	CYP1A2	CYP2B6	CYP3A4
3-Methylcholanthrene (2)	21.23 $\pm$ 0.38	1.33 $\pm$ 0.40	0.97 $\pm$ 0.30
Phenobarbital (1,000)	1.67 $\pm$ 0.40	9.10 $\pm$ 5.55	6.73 $\pm$ 2.92
Rifampin (10)	1.37 $\pm$ 0.30	3.60 $\pm$ 0.69	6.63 $\pm$ 1.42
SCY-635 (0.1)	1.50 $\pm$ 0.10	1.07 $\pm$ 0.15	1.03 $\pm$ 0.32
SCY-635 (1)	1.13 $\pm$ 0.11	1.00 $\pm$ 0.10	0.93 $\pm$ 0.06
SCY-635 (10)	1.40 $\pm$ 0.46	1.10 $\pm$ 0.26	0.93 $\pm$ 0.11
SCY-635 (15)	1.07 $\pm$ 0.15	0.87 $\pm$ 0.15	1.00 $\pm$ 0.36

^a The values represent the mean fold induction of enzyme activity $\pm$ SD determined from hepatocyte preparations from three separate donors. Boldface values represent the induction responses of the positive controls.

ylcholanthrene (CYP1A2), phenobarbital (CYP2B6), and rifampin (CYP3A4), were incubated in cultures of human hepatocytes prepared from three separate donors. At the end of 72 h of incubation, microsomes were isolated and the enzyme activities for CYP1A2, CYP2B6, and CYP3A4 were determined by using selective substrates (Table 4). The mRNA levels for each of the cytochrome P450 isozymes were analyzed by quantitative reverse transcription-PCR.

Increases in marker enzyme activities and in the mRNA levels for all of the positive control compounds were observed relative to the activities and mRNA levels for the control incubations containing 0.1% dimethyl sulfoxide (DMSO). In the triplicate cultures, exposure to 3-methylcholanthrene was associated with 21.23 ± 0.38 -fold increases in the CYP1A2-catalyzed conversion of phenacetin to acetaminophen. The corresponding mRNA levels increased from 33- to 84-fold. Exposure to phenobarbital was associated with 9.1 ± 5.55 -fold increases in the CYP2B6-catalyzed conversion of bupropion to hydroxybupropion. The corresponding mRNA levels increased from 6- to 29-fold. Exposure to rifampin was associated with 6.63 ± 1.42 -fold increases in the CYP3A4-catalyzed conversion of testosterone to 6- β -hydroxytestosterone. The corresponding mRNA levels increased from 11- to 30-fold. Exposure to SCY-635 at all concentrations tested was associated with less than 6% increases in marker enzyme activities and no detectable increases in the levels of mRNA expression. The results, as summarized in Table 4, indicate that SCY-635 is not an inducer of the CYP1A2, CYP2B6, or CYP3A4 enzyme, suggesting that there is a low potential for drug-drug interactions with SCY-635.

The two-drug combination of SCY-635 and interferon alpha-2b exhibits synergistic antiviral activity; SCY-635 in combination with ribavirin exhibits additive antiviral activity. The two-drug combination of SCY-635 with rIFN α -2b was evaluated in the subgenomic replicon assay by using a 72-h incubation period. Antiviral results were quantified by using luciferase activity as the end point. Various concentrations of SCY-635 and rIFN α -2b were tested either alone or in combination. Luciferase activity was reduced in a concentration-dependent manner in the presence of rIFN α -2b and SCY-635 and yielded EC₅₀s that were consistent with previous determinations in the subgenomic replicon system. Combinations of rIFN α -2b and SCY-635 reduced HCV-specific RNA replica-

tion, as measured by the determination of luciferase activity, in a manner greater than expected.

The two-drug combination of SCY-635 and ribavirin was evaluated in the subgenomic replicon assay by using a 72-h incubation period. Antiviral results were quantified by using the replicon-derived luciferase activity. Various concentrations of SCY-635 and ribavirin were tested either alone or in combination. It is important to recognize that in the replicon assay, ribavirin exhibits no appreciable antiviral activity in the absence of cell cytotoxicity. At the highest concentration tested, 20 μ g/ml (82 μ M), exposure to ribavirin was associated with approximately 30 to 60% cell cytotoxicity. The data collected under these conditions were used to assess the potential cytoprotective effects of the combination of SCY-635 and ribavirin. Luciferase activity was reduced in a concentration-dependent manner in the presence of SCY-635 and yielded EC₅₀s that were consistent with previous determinations in the subgenomic replicon system. Combinations of ribavirin and SCY-635 reduced HCV-specific RNA replication in a manner greater than expected.

To determine whether the effects of the two-drug combinations were synergistic, additive, or antagonistic, the antiviral activity data were analyzed by using the MacSynergy II program. The data from a dilution scheme that comprised 40 or 45 discrete two-drug combinations were analyzed. The results are presented in a three-dimensional Cartesian coordinate system to yield surfaces of activity that can fall above (indicating synergy), below (indicating antagonism), or in the plane of (indicating additive interactions) the central x - y axis. A synergy volume of 122 μ M $\cdot$ IU/ml % was achieved at the 95% confidence interval for the combination of SCY-635 and rIFN α -2b, indicating antiviral synergy for the two-drug combination (Fig. 6). The effect of the combination of SCY-635 and rIFN α -2b on cell viability was assessed by using the release of lactate dehydrogenase as the end point. A reduction in cell viability of greater than 10% was observed only with the highest concentration of SCY-635 tested, 2.0 μ M. The results indicate that the two-drug combination resulted in no change in cytotoxicity with the concentrations tested. For the combination of ribavirin and SCY-635 at the 95% confidence interval, synergy volumes equaling 68.3, 26.3, and 34.0 μ g/ml $\cdot$ nM % (mean = 42.9 μ g/ml $\cdot$ nM %) were calculated from three independent experiments. Exclusion of the antiviral synergy volumes at concentrations of ribavirin where the cytotoxicity exceeded 30% resulted in synergy volumes of 44.0, 26.3, and 26.7 μ g/ml $\cdot$ nM % (mean = 32.3 μ g/ml $\cdot$ nM %), suggesting additive effects for the two-drug combination of SCY-635 and ribavirin. The effect of SCY-635 in combination with ribavirin on cell viability was assessed by using lactate dehydrogenase as the end point. The results indicate that the two-drug combination exhibited cell cytotoxicity less than expected, suggesting that the combination had reduced cytotoxic effects.

Pharmacokinetics following oral and intravenous administration to rodents and primates. Tables 5 and 6 contain summaries of the values of the pharmacokinetic parameters determined after the administration of single doses of SCY-635 to rats and cynomolgus monkeys by intravenous and oral administration. The experimental methodology is described in detail in supplement S4 in the supplemental material.

CsA and other macrolide immunosuppressive agents distrib-

FIG. 6. SCY-635 exhibits synergistic antiviral activity when it is used in combination with rIFN α -2b. SCY-635 was tested at nine concentrations (range, 0.008 to 2.0 μ M) with rIFN α -2b at five concentrations (range, 0.005 to 0.5 μ M). After a 72-h incubation period, the cells were processed to determine antiviral activity (luciferase) and the data were analyzed using the Prichard and Shipman MacSynergy II data analysis program.

ute predominantly between plasma and erythrocytes (24). The pharmacokinetic analyses therefore focused on quantifying the concentration of parent compound in whole blood in order to more accurately reflect the total systemic exposure of animals to SCY-635.

Following intravenous administration at doses of 2 and 5 mg/kg in rats, SCY-635 demonstrated values for the apparent elimination $t_{1/2}$ of 26.3 and 22.2 h, respectively; systemic clearance values of 88.8 and 134 ml/h/kg, respectively; and volume of distribution (V_z) values of 3.37 and 4.30 liters/kg, respectively (Table 5). Following the intravenous administration of doses of 1.4 and 1 mg/kg in monkeys, SCY-635 demonstrated apparent elimination $t_{1/2}$ values of 42.2 ± 14.4 and 22.3 ± 1.01 h, respectively; systemic clearance values of 28.2 ± 2.6 and 31.7 ± 1.21 ml/h/kg, respectively; and V_z values of 1.69 ± 0.45 and 1.02 ± 0.02 liters/kg, respectively. SCY-635 administered intravenously showed a low level of clearance in rats and mon-

keys that approached 10% of the hepatic blood flow. The volume of distribution of SCY-635 was moderate to high in both species, suggesting good distribution into tissues.

Following oral administration, SCY-635 was well absorbed into the systemic circulation (Table 6). Maximum blood concentrations (C_{max}) were observed at 4 to 8 h in rats and at 2 to 3 h in monkeys. The values of C_{max} and the area under the concentration-time curve (AUC) to infinity ($AUC_{0-\infty}$) generally increased in a dose-related fashion in both species. In rats, the composite mean values for C_{max} increased in a slightly less than proportional fashion compared to the mean values attained with the nominal dose. $AUC_{0-\infty}$ increased in direct proportion to the dose between the 5- and 10-mg/kg dose groups; however, a slightly less than proportional increase was observed between the 10- and 20-mg/kg dose groups. The oral bioavailability of SCY-635 in rats ranged from 18.9 to 23.1%. In monkeys, C_{max} increased between the 5-, 7.5-, and 15-mg/kg

TABLE 5. Pharmacokinetic parameters for SCY-635 in whole blood following intravenous administration^a

Species	Dose (mg/kg)	C_{max} (ng/ml)	$AUC_{0-\infty}$ (ng · h/ml)	$t_{1/2}$ (h)	CL (ml/h/kg)	V_z (liters/kg)
Rat	2	1,510	22,500	26.3	88.8	3.37
	5	2,990	37,300	22.2	134	4.30
Monkey	1.4	$2,310 \pm 165$	$49,900 \pm 4,390$	42.2 ± 14.4	28.2 ± 2.6	1.69 ± 0.45
	1	$2,560 \pm 50$	$31,500 \pm 1,230$	22.3 ± 1.01	31.7 ± 1.21	1.02 ± 0.02

^a The values of the pharmacokinetic parameters in whole blood were determined from one composite mean profile for each dose group in rats and from three individual animal profiles per dose group in monkeys $\pm$ SDs. All doses were administered by the intravenous route.

TABLE 6. Pharmacokinetic parameters for SCY-635 in whole blood following oral administration^a

Species	Dose (mg/kg)	C _{max} (ng/ml)	T _{max} (h)	AUC _{0-∞} (ng · h/ml)	t _{1/2} (h)	F (%)
Rat	5	900	4.0	13,000	19.2	23.1
	10	1,400	8.0	26,500	21.4	23.6
	20	1,760	4.0	42,500	23.8	18.9
Monkey	5	1,810	2.0	27,900	24.6	17.7
	7.5	1,930	2.7	42,000	33.1	15.7
	15	2,430	2.0	59,200	43.3	11.1

^a The pharmacokinetic parameters in whole blood were determined from one composite mean profile for each dose group for rats and from a minimum of three individual profiles per dose group for monkeys. All doses were administered by the oral route. T_{max}, time to C_{max}; F, oral bioavailability.

dose groups in a less than proportional fashion. AUC_{0-∞} increased in a proportional fashion between the 5- and 7.5-mg/kg dose groups but increased in a slightly less than proportional fashion between the 7.5- and 15-mg/kg dose groups. The oral bioavailability of SCY-635 in monkeys ranged from 11.1 to 17.7%. The values for the apparent elimination half-life of SCY-635 following oral administration ranged from 19.2 to 23.8 h in rats and from 24.6 to 43.3 h in monkeys.

Biological distribution and bioavailability following intravenous and oral administration to rats. A comparison of the SCY-635 exposures achieved in liver and whole blood is shown in Table 7. The biological distribution of SCY-635 was examined in rats following the intravenous administration of a single dose of SCY-635 at 10 mg/kg and after the administration of single oral doses of 10 and 30 mg/kg. The concentrations of SCY-635 in whole blood and liver homogenates were measured by nonvalidated LC-MS/MS methods. Within each treatment group, higher concentrations of SCY-635 were observed in liver tissue homogenates than in whole blood at each time point (2, 4, 8, 12, and 24 h after dosing). The average concentration of SCY-635 (2 to 24 h) in the liver was approximately 4.5- to 6.7-fold greater than the corresponding concentration in whole blood in each treatment group, under the assumption that the density of liver tissue is approximately equal to that of the whole-blood fraction (1).

Cellular distribution of SCY-635 in human hepatocytes *in vitro*. The cellular distribution of SCY-635 was examined *in vitro* by using purified preparations of human hepatocytes and nonparenchymal cells and a Transwell culture system. SCY-635 was added at two concentrations, 500 and 3,000 ng/ml (0.378 μM and 2.27 μM, respectively). At the completion of a 1-h incubation, cells were recovered from each reservoir and the content of SCY-635 in each cellular fraction was determined. At the starting concentration of 500 ng/ml of SCY-635, 0.55 ± 0.04 ng SCY-635/μg of cellular protein was associated with the nonparenchymal fraction, whereas 1.03 ± 0.03 ng SCY-635/μg of cellular protein was associated with the hepatocyte fraction. At the starting concentration of 3,000 ng/ml of SCY-635, 3.16 ± 0.27 ng SCY-635/μg of cellular protein was associated with the nonparenchymal fraction, whereas 6.05 ± 0.21 ng SCY-635/μg of cellular protein was associated with the hepatocyte fraction. These data indicate that SCY-635 preferentially distributes into the hepatocyte fraction. The concentration of SCY-635 associated with each respective cellular fraction increased in direct proportion to the nominal concentration of drug, indicating no evidence of saturation at the doses tested.

DISCUSSION

The currently approved options that are available for the treatment of individuals who are chronically infected with hepatitis C virus include combination therapy with pegylated interferons and ribavirin (25). It is well documented that these treatment options are successful in conferring sustained virological responses in approximately 50% of all patients who are indicated for therapy. The wide range of clinical toxicities, warnings, and contraindications for the currently approved medications underscores the need to discover and to develop safer, mechanistically based antiviral agents that can be used to improve the rates of sustained virological responses and to provide treatment options for patients for whom treatment is currently contraindicated.

Twenty years ago it was reported that the intracellular ligand for CsA was involved in the replication of non-A non-B hepatitis virus (28). Those studies demonstrated that 28 days of

TABLE 7. Comparison of SCY-635 exposures in liver and whole blood in rats^a

Organ	Route	Dose (mg/kg)	AUC ₂₋₂₄ (ng · h/ml) ^b	C _{max} (ng/ml)	C _{min} (ng/ml)	C _{avg} (ng/ml) ^c
Liver ^d	i.v.	10	139,000	13,500	2,110	6,300
	p.o.	10	120,000	11,100	844	5,440
	p.o.	30	141,000	16,800	1,520	6,430
Blood	i.v.	10	30,500	3,360	703	1,390
	p.o.	10	18,000	1,870	138	818
	p.o.	30	30,200	2,220	600	1,370
Blood/liver ratio	i.v.	10	4.6	4.0	3.0	4.5
	p.o.	10	6.7	5.9	6.1	6.7
	p.o.	30	4.7	7.6	2.5	4.7

^a The pharmacokinetic parameters in whole blood were determined from one single composite mean profile for each dose group. AUC₂₋₂₄, AUC from 2 to 24 h; C_{min}, minimum concentration in plasma; C_{avg}, average concentration in plasma; i.v., intravenous; p.o., oral.

^b The AUC was calculated from 2 through 24 h to reflect common sampling time frames for blood and liver specimens.

^c C_{avg} was calculated as AUC₂₋₂₄ divided by 22 h.

^d The liver concentrations were measured as ng/g of tissue homogenate; the density of the liver homogenate was assumed to be 1.0 g/ml.

intravenous administration of CsA at a dose of 20 mg/kg/day to chronically infected chimpanzees was associated with improvement in liver histometric scores. The authors concluded that CsA inhibited the proliferation of non-A non-B hepatitis virus, albeit through an unknown mechanism of action. The authors speculated that if it were possible to separate the immunosuppressive properties of CsA from its antiviral activity, then "there may be some possibilities of a clinical application of CsA to certain viral diseases" (28). Several groups have now reported that CsA (and nonimmunosuppressive derivatives of the cyclosporine type) suppresses HCV genomic replication *in vitro* (17, 19, 22, 32) and that the observed anti-HCV activity correlates with the cyclophilin binding and PPIase inhibition properties of CsA and not its calcineurin or P-gp binding and inhibitory activities (3, 19, 32). Further studies have confirmed that the efficient replication of HCV RNA depends on the expression of CyPA (4, 14, 34).

SCY-635 is a novel disubstituted analog of CsA that contains the cyclic undecapeptide core structure of CsA but differs from the parent molecule at the 3- sarcosine and 4-*N*-methyl leucine positions. SCY-635 contains a dimethylamino-ethylthio substituent at the 3-sarcosine alpha carbon atom and a hydroxyl substituent at the gamma carbon of the 4-*N*-methyl leucine residue (22). Enzyme inhibition studies indicate that CsA and SCY-635 are equipotent inhibitors of the PPIase activity expressed by CyPA. These results indicate that the substitutions at the 3-sarcosine and 4-*N*-methyl leucine residues do not alter the recognition of SCY-635 as a competitive, active site-directed inhibitor of CyPA. The disubstitution at positions 3 and 4 has profound consequences for the potential immunosuppressive activity of SCY-635. CsA and SCY-635 both readily form binary complexes when either molecule is incubated in the presence of CyPA; however, only the binary complex formed between CyPA and CsA is capable of forming a ternary complex with calcineurin, ultimately resulting in the potent inhibition of the phosphatase activity expressed by calcineurin. No inhibition of calcineurin phosphatase activity was observed at SCY-635 and CyPA concentrations up to 2 μ M. The results of binding studies are consistent with the results of functional assays, which indicate that SCY-635 is approximately 2,000-fold less potent than CsA with respect to the inhibition of IL-2 production from either stimulated Jurkat cells or freshly isolated human PBMCs. CsA is a potent substrate for P-gp (efflux ratio = 279) in the MDCKII-hMDR1 cell line, whereas SCY-635 exhibits an efflux ratio of 16.5, demonstrating that SCY-635 is less efficiently transported by P-gp. SCY-635 exhibits potent suppression of HCV-specific RNA replication in replicon cells. The inhibition of RNA replication was time and dose dependent. A 72-h incubation was required in order to achieve greater than 98% suppression of RNA replication. These results are consistent with previously published observations which indicate that the *in vitro* anti-HCV activity of CsA and its nonimmunosuppressive analogs coincides with cyclophilin binding and PPIase inhibition activity and is independent of either calcineurin binding and phosphatase inhibition or substrate recognition by P-gp (3, 19, 32).

SCY-635 exhibited additive to synergistic antiviral activity when it was tested in two-drug combinations *in vitro* with either rIFN α -2b or ribavirin. Interestingly, the two-drug combination of SCY-635 and ribavirin exhibited cell cytotoxicity that was

less than expected. A growing body of evidence now indicates that the mitochondrial permeability transition (MPT) plays a central role in the pathogenesis of the necrotic as well as the apoptotic processes associated with ischemic/reperfusion injury. NIM811 has been demonstrated to decrease storage/reperfusion injury after rat liver transplantation by inhibiting the onset of the MPT (29), the effects of which are largely mediated through its interaction with cyclophilin D (30). SCY-635 binds to cyclophilin D and is capable of inhibiting calcium-induced MPT-dependent swelling in isolated mitochondria (R. Harris, unpublished data). These results suggest that the observation of the diminished cytotoxicity of SCY-635 when it is tested in combination with ribavirin may be due to modulation of the MPT by SCY-635 at the level of mitochondrial cyclophilin D.

It is well established that the oral bioavailability of CsA in humans is highly variable. CsA undergoes extensive hepatic and intestinal metabolism predominantly through the activity of CYP3A (33). Intestinally expressed CYP3A may account for as much as 50% of the drug metabolism following oral administration (12). The intestinal expression of P-gp may also contribute to the variability in the absorption of CsA following oral administration (12). P-gp functions as an efflux transporter pump which inhibits the absorption of CsA from the intestinal lumen to the plasma compartment. The activity of P-gp may therefore have the compound effect of reducing the absorbed fraction of parent drug and making it more accessible to oxidative metabolism at the intestinal lumen. The expression of CYP3A and P-gp has been reported to vary by as much as 10-fold when it is measured with intestinal biopsy specimens obtained from healthy volunteers and may therefore account for the highly variable oral bioavailability of CsA in humans. As discussed above, SCY-635 exhibits a markedly lower efflux ratio in the MDCKII-h MDR1 cell line, suggesting that it is a lower-affinity substrate for transport by P-gp. Incubations performed with human liver microsomes demonstrated that the intrinsic clearance of SCY-635 is approximately 2- to 8.5-fold lower than that of CsA, suggesting the decreased substrate recognition and turnover of SCY-635 by cytochrome P450 enzymes. Inhibition studies suggest that SCY-635 has a low potential for drug-drug interactions with the major cytochrome P450 enzymes. Induction studies with freshly isolated human hepatocytes indicate that SCY-635, like CsA, is not an inducer of cytochrome 1A2, 2B6, or 3A4. Collectively, these observations suggest that the oral administration of SCY-635 could result in greater bioavailability, more consistent absorption, a lower potential for adverse pharmacological interactions, and a lower degree of interindividual variation in comparison to the results achieved after CsA administration.

SCY-635 is well absorbed and is orally bioavailable in multiple animal species (oral bioavailability range, 14.8 to 21.9%). At the highest doses tested, the $C_{\max}$ s in whole blood were approximately 13-fold greater than the replicon-derived EC_{50} . Estimates for terminal elimination-phase half-lives range from 22 h in rodents to 32 h in primates. Biological distribution studies demonstrate that liver concentrations exceed the corresponding whole-blood concentrations by a factor of 5, indicating that relatively high target-tissue concentrations of drug can be maintained over time. SCY-635 distributes predominantly to hepatic parenchymal cells rather than Kupffer cells by

approximately twofold. Given that the liver comprises approximately 70% parenchymal cells, these data indicate that the majority of the SCY-635 detected in whole liver tissue is associated with the primary cellular reservoir for HCV infection and replication.

These studies demonstrate that SCY-635 exerts anti-HCV activity by acting at a unique biological target, host CyPA, which is critical for supporting HCV RNA replication. CyPA has not been implicated in the mechanism of action of either interferon or ribavirin (6); therefore, the *in vitro* observations of additive to synergistic anti-HCV activity for SCY-635 are expected when it is tested in combination with a novel inhibitor that acts at an orthogonal target. Drug metabolism studies suggest that the inclusion of SCY-635 as a component of a combination regimen has a low potential to cause adverse pharmacological interactions when the combination is evaluated in clinical tests either with the currently approved therapies or with investigational agents such as protease or polymerase inhibitors. The demonstration that SCY-635 does not inhibit or induce oxidative metabolism and only weakly inhibits P-gp activity suggests that SCY-635 may constitute a fixed component of combination chemotherapy to which other investigational agents could then be added. The reduced immunosuppressive activity (relative to that of the parent compound, CsA) may mitigate potential dose-limiting immunosuppressive side effects and diminish the potential for SCY-635 to antagonize the clinical anti-HCV activity of interferon. SCY-635 is well absorbed in multiple animal species. In rodent models, the concentrations detected in liver tissue exceed those detected in the whole-blood fraction. The pattern of cellular distribution into hepatocytes further suggests that SCY-635 specifically penetrates a key cellular reservoir for inhibition of the replication of HCV.

Recently, the results of a 15-day assessment of SCY-635 administered as monotherapy to patients with chronic hepatitis C virus infection (genotype 1 only) were reported (13). SCY-635 was safe and well tolerated at all dose levels. No evidence of a dose-limiting toxicity was observed. At the highest dose tested (900 mg per day, 300 mg three times daily), a mean maximal decline in the level of plasma viremia of 2.3 log₁₀ was observed on study day 15 (13). One subject exhibited a 5.3-log₁₀ reduction in the level of plasma viremia, and the level reached the lower limit of quantitation (10 IU/ml) on study day 15. These results confirm the safety and clinical antiviral activity of SCY-635 and provide a proof of principle for SCY-635 as a novel antiviral agent. Overall, these data suggest that SCY-635 represents a novel antiviral agent that warrants further clinical evaluation as a potential new treatment for individuals who are chronically infected with hepatitis C virus.

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