The Immune Modulator FTY720 Inhibits Sphingosine-1-phosphate Lyase Activity*

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Padmavathi Bandhuvula, Yuen Yee Tam, Babak Oskouian, and Julie D. Saba

From the Children’s Hospital Oakland Research Institute, Oakland, California 94609-1673

FTY720 is a novel immunomodulatory agent that inhibits lymphocyte trafficking and prevents allograft rejection. FTY720 is phosphorylated in vivo, and the phosphorylated drug acts as agonist for a family of G protein-coupled receptors that recognize sphingosine 1-phosphate. Evidence suggests that FTY720-phosphate-induced activation of S1P, is responsible for its mechanism of action. FTY720 was rationally designed by modification of myriocin, a naturally occurring sphingoid base analog that causes immunosuppression by interrupting sphingolipid metabolism. In this study, we examined interactions between FTY720, FTY720-phosphate, and sphingosine-1-phosphate lyase, the enzyme responsible for irreversible sphingosine 1-phosphate degradation. FTY720-phosphate was stable in the presence of active sphingosine-1-phosphate lyase, demonstrating that the lyase does not contribute to FTY720 catabolism. Conversely, FTY720 inhibited sphingosine-1-phosphate lyase activity in vitro. Treatment of mice with FTY720 inhibited tissue sphingosine-1-phosphate lyase activity within 12 h, whereas lyase gene and protein expression were not significantly affected. Tissue sphingosine 1-phosphate levels remained stable or increased throughout treatment. These studies raise the possibility that disruption of sphingosine 1-phosphate metabolism may account for some effects of FTY720 on immune function and that sphingosine-1-phosphate lyase may be a potential target for immunomodulatory therapy.

EXPERIMENTAL PROCEDURES

Materials—FTY720 and FTY720-P were kindly provided by Volker Brinkmann of Novartis (Basel, Switzerland). The human SPL was kindly provided by Timothy Hla (University of Connecticut Health Center). C17 and C18 sphingosines, Pso, and S1P were obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). [4,5-3H]-erythro-DHS1-P was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All other chemicals including 1-butanol and o-phthalaldehyde (OPA) were purchased from Sigma.

FTY720-P Metabolism—Whole cell extracts of HEK293 cells expressing human SPL via an adenoviral expression system were prepared as described previously (21). These extracts contain ~1430 pmol/mg of protein/mn of SPL activity, based upon activity against DHS1-P substrate. HEK293 cells expressing GFP via the adenoviral expression system were employed as controls. Twenty nmol of FTY720-P were suspended in SPL reaction buffer (21), and 25 μl of whole cell extract plus 25 μl of extraction buffer (0.5 mM potassium phosphate buffer, pH 7.4, 2 mM EDTA, 2 mM mercaptoethanol, 11% glycerol, 0.2 mM pyridoxal 5’-phosphate) were added to initiate the reaction. At various time points, the reaction was stopped by addition of 0.3 ml of concentrated HCl, 1 ml of 1 M KCl, and 1.5 ml of chloroform to the tube. The sample was mixed by vortexing and centrifuged at 1200 rpm for 5 min. Organic phase was collected, dried down using a speed vac and resuspended in methanol. After derivatization with OPA, the sample was injected onto the HPLC for quantification. The HPLC analysis was conducted using a Beckman system Gold 125 solvent module with a Spectra-Physics SP8410 fluorescence detector and a C18 (2) Luna 3-m, 75 × 4.6-mm column from Phenomenex (CA). The mobile phase was methanol:10 mM potassium phosphate buffer, pH 7.2, 1 mM TBAP in water = 83:16:1, v/v/v, and the flow rate was 1 ml/min. The calculation of the FTY720-P in each sample was based on the integration of the peaks of interest. For comparison, 20 nmol of S1P was incubated with extracts from HEK293 cells overexpressing SPL or control HEK293 cells, and extraction of S1P was performed as for FTY720. Recovery of S1P was determined by HPLC quantification, as described below for S1P measurements in mouse tissues.

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1 To whom correspondence should be addressed: Children’s Hospital Oakland Research Institute (CHORI), 5700 Martin Luther King Jr. Way, Oakland, CA 94609-1673. Tel: 510-450-7690; Fax: 510-450-7910; E-mail: jsaba@chori.org.

2 The abbreviations used are: S1P, sphingosine 1-phosphate; DHS1-P, dihydrosphingosine 1-phosphate; FTY720-P, FTY720-phosphate; HPLC, high performance liquid chromatography; OPA, o-phthalaldehyde; Pso, phytosphingosine; Pso-P, phytosphingosine 1-phosphate; 5K, sphingosine kinase; SPL, sphingosine-1-phosphate lyase; TBAP, tetrabutylammonium dihydrogen phosphate; LCMS, liquid chromatography mass spectrometry; GFP, green fluorescent protein.
Confirmation of FTY720-P by LCMS—Samples corresponding to FTY720-P peak identified on HPLC were evaluated by LCMS to confirm FTY720-P by mass analysis. Twenty microliters of sample were injected into the LCMS, and mass spectrum scans from 300 to 500 m/z and specific ions m/z 388 and 410 were quantified for FTY720-P. A reverse phase C18 column from Phenomenex (2.1 mm × 15 cm) was used. Two mobile phases were used: solution A is water: methanol:acetic acid (69:30:1) containing 5 mM ammonium acetate, and solution B is methanol:acetic acid (99:1) containing 5 mM ammonium acetate. The program for those two mobile phases was started with 50% solutions A and B, continued for 2 min, and then solution B was increased to 100% in 6 min, continued for 5 min in 100% solution B, and finally solution B was decreased back to 50% in 2 min. The flow rate was 0.3 ml/min. The retention time is 11.2 min.

SPL Enzyme Assay—Tissues were homogenized by sonication in 9 volumes of ice-cold homogenization buffer (5 mM MOPS, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 0.25 M sucrose, and 5 μg/ml each chymostatin, leupeptin, pepstatin A and antipain). SPL assays were conducted using 100 μg of protein per assay and radioactive [4,5-3H]DHS1-P substrate, as described previously (20). For in vitro enzyme assays, whole cell extracts were prepared from adenoirally infected HEK293 cells expressing SPL or GFP control and assayed as described above. For in vitro SPL FTY720 and FTY720-P experiments, the drug was incubated with whole cell extracts prepared from HEK293 cells infected with an adenoiviral vector expressing human SPL. The drug was incubated with extracts for 30 min, followed by addition of extracts to reaction buffer and substrate to initiate the reaction. Competition assays were performed using 100 μg of protein from thymic extracts of mice treated with 1 mg/kg body weight of FTY720 and euthanized at 24 h after drug administration. Assays were conducted using increasing concentrations of cold DHS1-P substrate, from 10–80 nmol.

Drug Treatments and Leukocyte/Lymphocyte Counts—Four-week-old FVB mice were obtained from Charles River Laboratories (Wilmington, MA). Animals were maintained in a pathogen-free facility in microisolator cages. A single dose of FTY720 at 1 mg/kg in sterile water was injected intraperitoneally at time 0. Mice were euthanized by CO2 inhalation at various time points post-injection, followed by tissue harvest. Blood counts were determined by Coulter and manual differential. All animal studies were performed in accordance with the approved Children’s Hospital Oakland Research Institute Institutional Animal Care and Use Committee protocols.

SIP Measurements—SIP was isolated by two-phase lipid extraction, derivatized with OPA, and quantitated by HPLC essentially as described (22–24). All tissues were weighed prior to snap-freezing in liquid nitrogen and were stored in −100°C until processing. One nmol of P50 and P50-P were added as internal standards before homogenization of tissues. Lipids were extracted with a 1-ml mixture of chloroform and methanol (1:2 ratio). Samples were incubated at room temperature with sonication for 3 h and then dried by SpeedVac. One ml of 0.1 N NaOH in methanol was added for 1 h at 37°C to achieve hydrolysis. After addition of 4 ml of chloroform and 4 ml of 1 M KCl, samples were mixed and phases separated by centrifugation. The aqueous phase was isolated and mixed with 0.3 ml of concentrated HCl and 6 ml of chloroform. The phases were separated by centrifugation, and the organic phase was collected and dried for SIP determination. All samples for SIP determination were re-dissolved in methanol. After derivatization with OPA, samples were injected onto HPLC for quantification. The mobile phase was methanol:10 mM phosphate buffer, pH 7.21:1 ml TBAP in water = 83:16:1, v/v/v, and the flow rate was 1 ml/min.

SPL Expression—To evaluate the effect of FTY720 and FTY720-P on SPL gene expression, HEK293 cells were transfected with a reporter construct containing 7.8 kb of sequence upstream of the SPL ATG start codon. The transfected cells were treated for 24 h with 3 μM FTY720 or FTY720-P (the maximal dose at which no cytotoxicity was appreciated by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-tetrazolium bromide (MTT) assay), at which time luciferase activity was determined.

RESULTS AND DISCUSSION

SPL Does Not Catabolize FTY720—FTY720 demonstrates a long elimination half-life after single dose administration (2). Known routes of drug metabolism include conversion to FTY720-P by SK, and ω oxidation of the octyl side chain, followed by β oxidation. We were interested in determining whether FTY720 might function as a substrate for SPL, which recognizes phosphorylated long chain bases varying in chain length, hydroxylating, and saturation. To test this possibility, FTY720-P was incubated under standard SPL assay conditions with HEK293 cells demonstrating high levels of SPL activity by virtue of an adenoiviral SPL expression system. Incubation proceeded for various lengths of time, after which FTY720-P was recovered by two-phase lipid extraction, derivatized with the fluorescent compound OPA, and quantitated by HPLC. As shown in Fig. 1a, FTY720-P remained stable for up to 24 h in the presence of SPL. In contrast, SIP levels diminished under the same conditions in cells expressing high levels of SPL but not in control cells expressing GFP (Fig. 1b). SIP degradation correlated with an increase in SPL reaction products, as determined using a standard SPL assay (Fig. 1c). Our results indicate that, under these conditions, SIP is degraded in an SPL-dependent fashion, whereas FTY720-P does not serve as a substrate for SPL.

Inhibition of SPL Activity by FTY720 in Vitro—We considered it likely that the structural features that allow FTY720 to be recognized by SK, FTY720-P to be recognized by SIP receptors, and both phosphorylated and unphosphorylated forms of the drug to be effectively translocated by long chain base transporters, as well as the access of FTY720 to intracellular compartments, would support interactions with other enzymes of long chain base metabolism (6, 9, 15, 16). To evalu-

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**FIGURE 2.** FTY720 inhibits SPL activity in vitro. Various concentrations of FTY720 or its phosphorylated derivative were incubated for 30 min on ice with whole cell extracts containing high SPL activity by virtue of a human SPL adenoviral expression construct. SPL activity was determined as described under “Experimental Procedures.” This experiment was performed in triplicate and is representative of three experiments. a, FTY720; †, p = 0.05 (not significant); ††, p < 0.005; †††, p < 0.002. b, FTY720-P; †, p < 0.003.

**FIGURE 3.** FTY720 inhibits SPL activity in vivo. a, one mg/kg FTY720 was administered by peritoneal injection to FVB mice at time 0. Mice were euthanized at the indicated times, and SPL activity in thymic tissue was determined as described under “Experimental Procedures.” This experiment was performed in quadruplicate and is representative of two experiments. †, p < 0.02; ††, p < 0.005; †††, p < 0.002. b, SPL activity in tissue homogenates of animals treated with FTY720 for 24 h (gray bars) and untreated controls (black bars) was measured in the presence of increasing concentrations of cold DHS1-P substrate. Actual SPL activity in each extract is shown. It should be noted that total activity decreases in both experimental and control tissue homogenates as the amount of cold substrate increases, due to reduction in specific activity of the substrate. FTY720-mediated inhibition in extracts from treated animals was determined as percent of control and is 21% in the presence of 10 nmol of substrate, 14% in the presence of 20 nmol of substrate, and 25% in the presence of 40 nmol of substrate.

**TABLE ONE**

| Time in hours | Lymphocytes (1000/μl)a | S.D. |
|---------------|------------------------|------|
| 0             | 7.71                   | 0.96 |
| 12            | 1.44                   | 0.15 |
| 24            | 3.23                   | 0.14 |
| 36            | 2.07                   | 0.74 |
| 48            | 2.83                   | 0.26 |

*a n = 4 animals per time point.

**FIGURE 4.** Gene expression as determined by an SPL-luciferase reporter system (Fig. 4a) nor tissue SPL protein expression as determined by immunoblotting (Fig. 4b) were significantly affected by FTY720 treatment at doses that inhibited enzyme activity. Although small changes in protein expression may not be detectable by immunoblotting, these studies suggest that the major effect of the drug on SPL activity is not by altering SPL expression.

**TABLE TWO**

| Time in hours | S1P lyase activity (pmoles/mg/min) |
|---------------|-----------------------------------|
| 0             | 3 nmol                             |
| 12            | 30 nmol                            |
| 24            | 100 nmol                           |

gene expression as determined by an SPL-luciferase reporter system (Fig. 4a) nor tissue SPL protein expression as determined by immunoblotting (Fig. 4b) were significantly affected by FTY720 treatment at doses that inhibited enzyme activity. Although small changes in protein expression may not be detectable by immunoblotting, these studies suggest that the major effect of the drug on SPL activity is not by altering SPL expression.

FTY720 Administration Does Not Reduce Tissue S1P Levels—SK is responsible for S1P synthesis, whereas SPL and S1P phosphatases are responsible for S1P catabolism. Since FTY720 interacts with both SK and SPL and may potentially influence the efficiency of both S1P synthesis and degradation, we were interested to determine the sum effect of FTY720 administration on tissue S1P levels. Interestingly, FTY720 treatment was associated with a slight increase in thymic tissue S1P at 36 h, followed by a return to baseline levels by 48 h (TABLE TWO). S1P levels increased somewhat more dramatically in splenic tissue after FTY720 treatment. DHS1-P levels remained stable throughout 48 h of treatment (data not shown). These findings indicate that, despite potential competition with sphingosine by FTY720 as a substrate for SK, tissue S1P levels were not significantly affected by FTY720 treatment.

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3 B. Oskouian and J. D. Saba, unpublished observations.
levels do not decrease and may even increase transiently during FTY720 administration, consistent with FTY720-mediated inhibition of SPL.

In summary, these studies demonstrate that FTY720 inhibits the activity of SPL, the major enzyme responsible for irreversibly removing S1P from the sphingosine-S1P cycle. This effect is observed in vitro and in vivo at therapeutic doses of the drug and occurs concomitant with drug-induced lymphopenia. These studies suggest that FTY720, in addition to interacting with SK and S1P receptors, also interacts with SPL and that inhibition of SPL by FTY720 may potentially contribute to some aspects of drug function (Fig. 5). This notion is consistent with the observation that deoxypyridoxine, a competitive inhibitor of pyridoxal 5′-phosphate-dependent enzymes including SPL, is a potent immunosuppressant and prolongs allograft survival (26, 27). Loss of SPL expression and concomitant changes in long chain base metabolism produce significant phenotypes in several developmental model systems (28–30). However, these simple metazoan organisms do not express recognizable S1P receptors and lack well-developed immune systems. Whether loss of SPL expression in mammals has any effect on lymphocyte trafficking or immunity should be clarified with the generation and characterization of mouse SPL knock-out models.

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