Pharmacologic Inhibition of Site 1 Protease Activity Inhibits Sterol Regulatory Element-Binding Protein Processing and Reduces Lipogenic Enzyme Gene Expression and Lipid Synthesis in Cultured Cells and Experimental Animals


ABSTRACT
Sterol regulatory element-binding proteins (SREBPs) are major transcriptional regulators of cholesterol, fatty acid, and glucose metabolism. Genetic disruption of SREBP activity reduces plasma and liver levels of cholesterol and triglycerides and insulin-stimulated lipogenesis, suggesting that SREBP is a viable target for pharmacological intervention. The proprotein convertase SREBP site 1 protease (S1P) is an important post-transcriptional regulator of SREBP activation. This report demonstrates that 10 μM PF-429242 (Bioorg Med Chem Lett 17:4411–4414, 2007), a recently described reversible, competitive aminopyrrolidineamide inhibitor of S1P, inhibits endogenous SREBP processing in Chinese hamster ovary cells. The same compound also down-regulates the signal from an SRE-luciferase reporter gene in human embryonic kidney 293 cells and the expression of endogenous SREBP target genes in cultured HepG2 cells. In HepG2 cells, PF-429242 inhibited cholesterol synthesis, with an IC50 of 0.5 μM. In mice treated with PF-429242 for 24 h, the expression of hepatic SREBP target genes was suppressed, and the hepatic rates of cholesterol and fatty acid synthesis were reduced. Taken together, these data establish that small-molecule S1P inhibitors are capable of reducing cholesterol and fatty acid synthesis in vivo and, therefore, represent a potential new class of therapeutic agents for dyslipidemia and for a variety of cardiometabolic risk factors associated with diabetes, obesity, and the metabolic syndrome.

Sterol regulatory element-binding proteins (SREBPs) are major transcriptional regulators of cholesterol, fatty acid, and glucose biosynthesis. There are three major forms of SREBP. SREBP-1a and SREBP-1c preferentially regulate fatty acid synthesis, whereas SREBP-2 preferentially regulates sterol synthesis (Horton et al., 2002). All SREBPs are transcriptionally autoregulated. SREBP-1a and SREBP-1c are encoded by a single gene, but the expression of each is regulated by a different promoter, and they differ in their N-terminal regions as a result of alternative processing (Shimomura et al., 1997). SREBP-1c is also transcriptionally regulated by insulin and liver X receptor (Repa et al., 2000).

Each SREBP is synthesized as an endoplasmic reticulum integral membrane protein with two transmembrane domains, one ER luminal loop, and cytosolic C- and N-terminal domains (Brown and Goldstein, 1997). Translocation of membrane-bound SREBP from the ER to the cis-Golgi and the subsequent cleavage events in the Golgi comprise major transcriptionally autoregulated. SREBP-1a and SREBP-1c are encoded by a single gene, but the expression of each is regulated by a different promoter, and they differ in their N-terminal regions as a result of alternative processing (Shimomura et al., 1997). SREBP-1c is also transcriptionally regulated by insulin and liver X receptor (Repa et al., 2000).

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points of regulation in the generation of active SREBP, requiring SREBP cleavage-activating protein (SCAP), the recently characterized factors Insig-1 and Insig-2 (Engelking et al., 2005), and two processing enzymes, SREBP site 1 protease (S1P) and SREBP site 2 protease (S2P) (Brown and Goldstein, 1997).

SREBP processing is regulated intracellularly by cholesterol and oxysterols. When the sterol content is high, cholesterol-bound SCAP associates with the SREBP C-terminal cytoplasmic domain to form a complex that binds to oxysterol-bound Insig (Radhakrishnan et al., 2004). This association with Insig blocks the transport of SCAP/SREBP from the ER to the Golgi, where the SREBP-processing enzymes, S1P and S2P, reside (Sun et al., 2005, 2007). In low-sterol conditions, the SREBP-SCAP complex is released from Insig, and SREBP is escorted by SCAP, sorted into coat complex II vesicles, and transported to the Golgi, where the two proteases effect two sequential cleavages of SREBP. S1P cleaves SREBP at the loop inside the lumen of the Golgi, and this cleavage must occur before the second cleavage, which is catalyzed by S2P within the first transmembrane domain. This event releases active SREBP, which subsequently migrates to the nucleus, binds to sterol response elements (SREs) of cholesterol and fatty acid biosynthetic target genes, and activates their transcription.

The SREBPs, especially SREBP-1c, are up-regulated in response to many pharmacological and physiological stimuli. Increased SREBP-1c contributes to the elevated rates of hepatic fatty acid synthesis that leads to steatosis in diabetic mice (Browning and Horton, 2004). Inhibition of SREBP processing can overcome up-regulation of SREBP expression, leading to down-regulation of cholesterol and fatty acid synthesis (Engelking et al., 2004). Currently, there is no evidence for differential regulation of the three SREBPs through the proteolytic activation process. Overexpression of Insig and deletion of SCAP or S1P result in down-regulation of all SREBP target genes (Matsuda et al., 2001; Yang et al., 2001; Engelking et al., 2004), including the SREBP genes themselves (Yang et al., 2001). In S1P and SCAP liver-specific knockout mice, expression levels of SREBP target genes were decreased, and the animals had lower levels of liver and plasma cholesterol and triglyceride compared with wild-type mice (Matsuda et al., 2001; Yang et al., 2001). These results suggested that inhibitors of SREBP processing could be effective agents to reduce hepatic and plasma cholesterol and triglyceride levels in vivo.

SIP is a serine protease that belongs to the proprotein convertase family (Sakai et al., 1998) but is a rather untypical member (Henrich et al., 2005). Its proform is expressed as a type II membrane-bound protein. As with most enzymes in the proprotein convertase family, SIP undergoes auto-processing of its proform and becomes a mature enzyme that resides in the cis-Golgi. After initial signal peptidase-catalyzed cleavage at site A (Gly22-Asp23), SIP is further processed sequentially at sites B (Lys137-Tyr138) and C (Leu186-Arg187) (Espenshade et al., 1999). After proteolytic processing, the resultantly catalytically competent SIP cleaves SREBPs following their lumen loop sequence of -Arg-Ser-Val-Leu- (Duncan et al., 1997). A soluble form of SIP with its transmembrane and cytosol domain deleted was shown to possess SIP catalytic activities, which could be inhibited by phenylmethanesulfonyl fluoride (Cheng et al., 1999).

We recently described the identification of a reversible and competitive small-molecule inhibitor of SIP activity, the aminopyrrolidineamide PF-429242 (Hay et al., 2007). In this report, we describe the effects of PF-429242 on the processing of SREBP, on the expression of SREBP target genes, and on sterol and fatty acid synthesis in cultured cells and in experimental animals.

Materials and Methods

SIP Expression and Purification. Residue numbering for human SIP follows Swiss-Prot accession Q14703. SIP protein was expressed and purified using a Chinese hamster ovary (CHO) cell expression system. The expression vector for soluble site 1 protease (sS1P) was generated by cloning a human site 1 protease cDNA encoding amino acids 17 to 997, encompassing the prodomain, catalytic domain, and cysteine-rich domain (Espenshade et al., 1999) into the pSecTag/FRT/V5-His vector (Invitrogen, Carlsbad, CA). The resulting plasmid was transfected into Flp-In CHO cells (Invitrogen) following the manufacturer’s instructions. Individual clones of stable cells overexpressing sS1P were isolated and maintained in DMEM growth medium (Invitrogen) containing 10% FBS (Invitrogen; no. 16140-071), 2 mM l-glutamine (Invitrogen), 1% Pen/Strep (Invitrogen), and 500 μg/ml hygromycin B (Invitrogen). For large-scale protein expression, the Flp-In CHO/sS1P cells were adapted to suspension growth by step-wise reduction of the FBS concentration in IS-CHO (Irvine Scientific, Santa Ana, CA) serum-free medium containing 500 μg/ml Hygromycin B. Wave Bioreactors (Wave Biotech, System 20 or System 2050EH) were inoculated at ~3.5 × 10^5 cells/ml and operated at 25 racks/min and 0.2 l/min aeration with 5% CO2 at 37°C. Conditioned medium was harvested at 7 days by refrigerated continuous flow centrifugation (Heraeus Conti-fuge; Thermo Electron Corporation, Waltham, MA) at 3500 g. Leupeptin (Sigma-Aldrich, St. Louis, MO) and 1 M Tris HCl (BO850-18, pH 8.0; Moltex, Boone, NC) were added at 1 mg/ml and 25 ml/l, respectively. Harvested conditioned medium was passed through a 0.2-μm filter and stored at 4°C until purification.

Conditioned medium was concentrated 5-fold with a GE Kivcek cassette concentrator (molecular weight cut-off, 10,000; GE Healthcare, Chalfont St. Giles, UK) using a peristaltic pump at 100 rpm (Watson-Marlow Bredel Inc., Wilmington, MA; model 603S). Concentrated medium was then dialyzed with 5 volumes of 25 mM Tris HCl and 1 mM CaCl2, pH 8.0, and mixed with Ni2 ^+ -Sepharose resin (GE Healthcare) equilibrated with 25 mM Tris HCl, pH 8.0. After overnight binding at 4°C, the resin was packed into a Pharmacia XK26 column. The column was mounted on an AKTA Explorer chromatography system (GE Healthcare) and washed with 25 mM Tris HCl, pH 8.0, followed by buffer A (25 mM Tris HCl, pH 8.0, 1 mM CaCl2, 1 M NaCl, 10% glycerol (Mallinckrodt Baker, Inc., Phillipsburg, NJ)). Further sequential washes were then carried out with increasing concentrations of imidazole (10, 20, and 30 mM; Calbiochem ULTROL grade; EMD Biosciences/Calbiochem, San Diego, CA) in buffer B (25 mM Tris HCl, 1 mM CaCl2, 10% glycerol, pH 8.0) before the column was finally eluted with buffer B containing 200 mM imidazole. The eluate was then concentrated 7-fold in a Viva-science 20-ml concentrator (polyethersulfone; molecular weight cut-off, 5000; Vivascience, Stonehouse, UK), mixed with an equal volume of glycerol, and stored at −20°C. Aliquots of all fractions were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting with anti-V5 antibody (Invitrogen) to identify fractions containing sSIP.

Enzymatic Assay for SIP. The primary assay was based on the fluorogenic substrate, Ac-VFRLSLK-MCA (Peptides International Inc., Louisville, KY), as described previously (Cheng et al., 1999; Hay et al., 2007). Alternative fluorogenic substrates for SIP were based on previously described simple peptide substrates (Cheng et al., 1998; Touré et al., 2000). Each peptide was modified with a dibeyl group at the N terminus and with tetramethylrhodamine
(TMR) near the C terminus. The fluorescence of the TMR was statically quenched by contact with the dabcyl group when the substrates were intact, but enzyme-catalyzed cleavage eliminated quenching. The substrates were as follows: 1) Dabcyl-Ser-Gly-Ser-Gly-Arg-Val-Leu-Ser-Phe-Glu-Ser-Gly-Ser-Lys(TMR)-Arg-OH, from site 1 of SREBP-2, and 2) Dabcyl-Arg-His-Ser-Arg-Ser-Arg-Leu-Arg-Alg-Arg-Leu-Gly-Gly-Lys(TMR)-OH, from site B of 51P. Substrates were produced as custom orders by AnaSpec, Inc. (San Jose, CA). Assays with these substrates were conducted at 37°C in a Perseptive Biosystems CytoFluor 4000 96-well fluo-

rescence plate reader (Applied Biosystems, Foster City, CA) using 4 μM substrate in 0.025 M Tris acetate, 1 mM CaCl2, pH 8.0 (at 22°C), with the 530-nm excitation filter and 620-nm emission filter in line.

SREBP-2 Western Blot. To test for the inhibition of endogenous SREBP processing by a S1P inhibitor, Western blotting for the membrane and nuclear forms of SREBP-2 was performed as previously described with minor modifications (Sakai et al., 1996; DeBose-Boyd et al., 1999). CHO cells (2 × 10⁶) were grown in a 10-cm plate in DMEM/F12 (Invitrogen) with 5% fetal calf serum and penicillin-streptomycin (Invitrogen). After overnight culture in an incubator at 37°C with 5% CO₂, cells were switched to DMEM/F12 with 5% (v/v) delipidated FBS (Cocalico Biologicals, Inc., Reamstown, PA) containing 10 μg/ml cholesterol and 1 μg/ml 25-hydroxycholesterol or different concentrations of PF-429242 for 24 h. One hour before cells were harvested for protein extraction, α-N-acetyl-Leu-Leu-Nle-CHO (Calbiochem) was added to the medium at a final concentration of 25 μg/ml.

After harvest, cells from each plate were spun down in phosphate-buffered saline (PBS), then resuspended in 400 μl of ice-cold buffer C (10 mM HEPES/KOH, pH 7.6, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose) with freshly added 1 mM dithiothreitolo and protease inhibitors (5 μg/ml pepstatin A, 10 μg/ml leupeptin, 0.5 mM Pefabloc, 2.8 μg/ml aprotinin, 25 μg/ml α-N-acetyl-Leu-Leu-Nle-CHO). Remaining procedures were performed on ice. To shear DNA, the cells were passed 30 times through a 23-gauge needle mounted on a 1-ml syringe. The lysate was centrifuged at 1100g for 7 min at 4°C, and the resulting supernatant was cen-

trifuged again at 100,000 g for 45 min at 4°C in an ultracentrifuge. The pellet containing membrane-bound forms of SREBP was resus-

pended in 35 μl of SDS-lysis buffer (10 mM Tris HCl, 100 mM NaCl, 1% SDS, 1 mM EDTA, 1 mM EGTA, pH 6.8). To recover nuclear SREBP, the pellet from the 1100g spin was suspended in 0.1 ml of ice-cold buffer D (20 mM HEPES/KOH, 420 mM NaCl, 1.5 mM MgCl₂, 2.5% glycerol, 1 mM EDTA, 1 mM EGTA, pH 7.6) with freshly added protease inhibitors as described above. Pellets were rocked at 4°C for 1 h to 2 h, after which the samples were centrifuged at 100,000g for 45 min at 4°C. The resulting supernatant contained nuclear SREBP.

Forty-five micrograms of nuclear extract or 25 μg of membrane fraction was loaded on Criterion XT Tris-Acetate 3 to 8% gels (no. 345-0129; Bio-Rad, Hercules, CA) at 20 V for 45 min. The resulting blots were incubated in PBS/10% milk/0.05% Tween overnight at 4°C. The next day, blots were incubated with 5 μg/ml IgG-7D4, a monoclonal antibody di-

crected against the N-terminal region of hamster SREBP-2 (American Type Culture Collection, Manassas, VA; hybridoma cell line CRL-2198) (Yang et al., 1995) in PBS/10% milk/0.05% Tween on a shaker at room temperature for 2 h, followed by four washes of 10 min each with PBS/Tween 20. The blots then were incubated for 1 h with donkey anti-mouse horseradish peroxidase (Jackson Immuno-

Research Laboratories Inc., West Grove, PA) diluted at 1:50,000 in PBS/milk/Tween 20, and then washed four times for 10 min each with PBS/Tween 20. The blots were developed with Lumigen PLUS Western Blotting substrate (Roche Applied Science, Indianapolis, IN).

SRE Luciferase Reporter Assay. SRE-regulated luciferase con-

structs were generated by inserting the sterol response element SRE-38 into commercial luciferase expression vectors. Several such vectors were tested, including pLUC-MCS (SRE-TATA; Stratagene, La Jolla, CA), pTLuc (SRE-TK; Clontech, Mountain View, CA), and pGL2-enhancer (SRE-SV40; Promega, Madison, WI). The SRE-38 sequence was designed on the basis of published sequence information (Dawson et al., 1988). A BamHI restriction site sequence was added at the end of the oligonucleotides to facilitate cloning. Two complementary oligonucleotides (SRE-38 sense, gatcTTGAAA-

TCACCCCCAGGGGATTCCTCCTCCCVTTCTG; SRE-38 antisense, gatcAGCAGGGGGAGGATCCACAGTGTTGGGATTTTCAAG) were annealed, and the double-stranded SRE-38 was inserted into each plasmid at a BgIII site. SRE-38 insertions were confirmed by sequencing analysis.

For the assay of SRE-luciferase, HEK293T cells were placed in a 96-well plate at 40,000 cells per well on the day before transfection. Cells were transfected using Lipofectamine 2000 with 40 ng of SRE-2 and 2 ng of an HSV-TK-regulated Renilla luciferase control construct (pHEL-TK; Promega), according to the manufacturer’s instructions. One hour after transfection, cell culture media were replaced with 10% delipidated serum (LPDS)/DMEM containing either compounds in dimethyl sulfoxide or dimethyl sulfoxide alone. After overnight exposure to compound, both luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). The ratio between firefly luciferase activity and Renilla luciferase activity obtained reflected SRE-specific compound effects on transcription.

Quantitation of SREBP Target Gene Expression. The effects of compounds on the expression of the SREBP target genes were evalu-

ated in HepG2 cells. HepG2 cells were plated in 24-well plates at 300,000 cells/well and incubated overnight in 10% LPDS/DMEM at 37°C, 5% CO₂. The next day, cells were treated in triplicates with PF-429242 in 10% LPDS/DMEM for 24 h. Total RNA was isolated from each well with an RNaseasy Mini Kit (QIAGEN, Valencia, CA) following the manufacturer’s instructions. On-column DNase treatment was performed with RNase-free DNase (QIAGEN).

To test the in vivo efficacy of PF-429242 in regulating SREBP target genes, male CD1 mice (n = 6) were dosed i.p. with 10 or 30 mg/kg PF-429242 or saline once every 6 over a 24-h period. Mice were euthanized 6 h after the final dose, and liver tissue was collected, frozen rapidly in liquid N₂, and stored at –80°C. For RNA isolation, 50 to 100 mg of frozen liver tissue from each sample was homoge-

nized in 1 ml of TRIzol reagent (Invitrogen). Total RNA was ex-

tracted following the manufacturer’s instructions, and the resulting total RNA from each sample underwent DNA-free treatment (DNA-

free kit; Ambion, Austin, TX).

Quantitative PCR analysis was performed for RNAs from individ-

ual cell culture wells (n = 3) and individual mouse livers (n = 6) as described previously. One microgram of total RNA was used in 50 μl of reverse transcription reactions using Applied Biosystems Taqman Reverse Transcription Reagent (Applied Biosystems, Foster City, CA). For each sample, the same reverse transcriptase product was used for all subsequent quantitative PCR reactions. Real-time quanti-

tative PCR primer sets were based on published sequences (Yang et al., 2001; Liang et al., 2002). Quantitative PCR reactions were per-

formed using ABI SYBR Green PCR Master Mix (Applied Biosys-


tem with an ABI 7700 instrument. The expression levels of HMG-

CoA reductase, fatty acid synthase, LDL receptor, and ATP-binding cassette transporter A1 (ABCA1) were normalized with cyclophilin and presented as average plus S.D. for individual RNA samples.

Quantitation of Cholesterol and Fatty Acid Synthesis. Chol-

esterol and fatty acid synthesis were evaluated in HepG2 cells by measuring incorporation of [2-¹⁴C]acetate into cellular lipids as de-

scribed previously (Harwood et al., 2003), but with the following modifications to allow simultaneous assessment of both cholesterol and fatty acid synthesis. HepG2 cells were plated in 24-well plates at 1.2 × 10⁶ cells/well and incubated at 37°C, 5% CO₂. Cells reaching 70
to 80% confluence were switched to 10% LPDS/DMEM. The next
day, cells were treated with PF-429242 in 0.5 ml of 10% LPDS/
DMEM at the indicated concentrations. Twenty-four hours after
treatment, 25 µl of media containing 4 Ci of [2-14C]acetate [CFA14
(GE Healthcare), 56 mCi/mmol] was added to each well, and the
plates were incubated at 37°C for 6 h. After incubation, the cells were
dissolved in 0.5 ml of 0.2 N KOH, and an aliquot was taken for
protein determination. The remaining dissolved cells were combined
with the media for extraction. [1,2-3H]cholesterol [50,000 dpm/tube,
TRK530 (GE Healthcare), 38 Ci/mmol], and 150 µg of cholesterol was
added as count- and thin-layer chromatography (TLC)-visualizable
internal standards, respectively. Samples were saponified by the
addition of 0.30 ml of 50% KOH and 2.0 ml of ethanol, followed first
by incubation for 2 h at 75°C and then by overnight incubation at
room temperature. The saponified mixtures were extracted three
times with 4.5 ml of hexane. The pooled organic fractions were dried
under N2, resuspended in 40 µl of chloroform-hexane (1:1), and
spotted onto plastic-backed PE-Sil G TLC plates for development in
hexane-diethyl ether-acetic acid [90:30:1 (v/v)]. The region indicated
by the cholesterol standard was cut out, placed in 7 ml of Aquasol-2
liquid scintillation fluid, and counted on a Beckman LS6500 liquid
scintillation counter (Beckman Coulter, Fullerton, CA).

After collection and assessment of the hexane fraction containing
cholesterol, the remaining aqueous phase (containing fatty acid so-
dium salts) was acidic to pH < 2 by the addition of 0.5 ml of 12 M
HCl. One hundred micrograms of linoleic acid was added to each
sample as a standard for TLC visualization. The resulting mixtures
were then transferred to glass conical tubes and extracted three
times with 4.5 ml of hexane. The pooled organic fractions were dried
under N2, resuspended in 50 µl of chloroform-hexane (1:1), and
applied to plastic-backed PE-Sil G TLC plates for development in
hexane-diethyl ether-acetic acid [70:30:2 (v/v)]. The region indicated
by the linoleic acid standard was cut out, placed in 7 ml of Aquasol-2
liquid scintillation fluid, and counted on a Beckman LS6500 liquid
scintillation counter. Cholesterol and fatty acid synthesis are ex-
pressed as disintegrations per minute [2-14C]acetate incorporated into
either nonsaponifiable or saponifiable lipids per milligram of
acellular protein during the 6-h incubation at 37°C.

To detect changes in rates of sterol and fatty acid synthesis in
response to PF-429242 treatment in vivo, we measured the incorpo-
ration of [2-14C]acetate into sterols and fatty acids as described
previously (Harwood et al., 1997). Male CD1 mice (n = 10) were
dosed i.p. with PF-429242 at 10 and 30 mg/kg or saline once every 6 h
over a 24-h period. Five hours after the final dose, mice were given a
0.2-ml i.p. injection of [2-14C]acetate. One hour after the administra-
tion of radiolabel, mice were euthanized by asphyxiation with CO2,
and fatty acid synthesis are expressed as disintegrations per minute
[2-14C]acetate incorporated into either nonsaponifiable or saponifiable lipids per milligram of
acellular protein during the 6-h incubation at 37°C.

Studies Using Experimental Animals. All procedures using
experimental animals were approved by the institutional Animal
Care and Use Procedures Review Board. CD1 mice were given food
(RMH32000) and water ad libitum and treated i.p. with either saline
(vehicle) or saline containing PF-429242 as outlined above.

Results

A soluble recombinant form of site 1 protease, sSIP, in
which residues 17 to 997 of the human protein were linked with C-terminal V5 and hexahistidine tags, was purified by
metal affinity chromatography from conditioned medium of
CHO cells overexpressing sSIP. The transmembrane and
cytosolic domains of S1P (residues 998-1052) were absent from
this product (Sakai et al., 1998), which caused it to be a
secreted protein by replacing the native signal sequence with
that of IgK. N-terminal sequence analysis of purified sSIP
gave a result of Arg-Ala-Ile-Pro-Arg, corresponding to the
“C-form” of S1P (Esensen and al., 1999). Propeptides
(14- and 6-kDa forms) were also detected in purified sSIP,
although the percentage of purified sSIP that remained
associated with propeptide was not determined. The yield of
sSIP averaged 1 mg/l conditioned medium, but a large frac-
tion of this protein was aggregated or otherwise inactive.
Despite this, the presence of activity demonstrating all the
properties expected for sSIP allowed screening to proceed.

High-throughput screening for inhibitors of S1P led to
identification of three hits with micromolar potency. Com-
binatorial synthesis of analogs around one of these hits gen-
érated compound A (Hay et al., 2007), a racemic aminopyr-
rolidineamide S1P inhibitor with an IC50 of 393 nM (Fig. 1A).
Upon resolution, it was found that the less potent enantiomer
(compound B; IC50 = 971 nM) possessed the S stereochem-
istry, whereas the more potent enantiomer (PF-429242) pos-
sessed the R stereochemistry. PF-429242 was shown to be a
reversible, competitive S1P inhibitor, which has an IC50 of
175 nM in the MCA assay for sSIP (Fig. 1A). Testing against
a panel of serine proteases indicated that PF-429242 was a
highly selective inhibitor of S1P (Hay et al., 2007).

The secondary enzymatic assay was performed using al-
ternative fluorogenic substrates based on two previously re-
ported peptide substrates of S1P, corresponding to SREBP-2
site 1 and S1P autocleavage site B (Cheng et al., 1999).
Unlike the substrate for the high-throughput screen, the
secondary substrates contained amino acid residues on both
sides of the scissile bond. Cleavage by S1P was shown to
occur at the Leu-Ser bond in the first substrate and at the
Leu-Arg bond in the second. Compounds tested exhibited the
same relative potency against both the primary and second-
ary substrates (data not shown).

S1P inhibitors should inhibit processing of SREBP and
reduce or prevent formation of the nuclear form of SREBP.
Because there is no evidence that S1P acts differentially
upon SREBP-1 and SREBP-2, the fate of SREBP-2 in cells
treated with PF-429242 was used as an indicator of the
compound’s ability to affect SREBP processing. Western blot-
ing was used to analyze the respective levels of the nuclear
and membrane-bound forms of SREBP-2 (Fig. 1B). In the
absence of sterol, the nuclear form of SREBP-2 was predom-
nant, and the membrane form was much less abundant,
indicating that proteolytic processing of SREBP was favored
(Fig. 1B, lane 1). In the presence of sterol, SREBP-2 re-
mained in the ER in its membrane-bound form, and the
nuclear form of SREBP-2 was present at a much lower level
(Fig. 1B, lane 2). A 10 µM concentration of PF-429242

blocked the processing of endogenous SREBP-2 in CHO cells in the absence of sterol, as indicated by a much lower level of the nuclear form of SREBP-2 (Fig. 1B, lane 3). Similar inhibition of SREBP processing by PF-429242 was also observed in HepG2 cells (data not shown).

The nuclear forms of SREBP regulate their target genes by binding to SREs and stimulating transcription. Inhibition of SREBP processing should therefore cause the expression of SRE-regulated genes to be down-regulated. To test the ability of PF-429242 to down-regulate SREBP transcriptional activity, SRE-luciferase reporter constructs were generated. Of three SRE-luciferase constructs tested in transfected HEK293 cells, SRE-TATA luciferase (based on the pLuc-MCS vector) was selected for the assay because it gave the largest percentage of inhibition when sterol was added to the culture medium. DNA sequencing confirmed that the SRE-TATA luciferase construct had two copies of the SRE 38 sequence connected head-to-tail and inserted into pLuc-MCS (Fig. 2A). In HEK293 cells transfected with the SRE-TATA luciferase construct, SREs are regulated by endogenous SREBPs; therefore, the assay was done in the presence of LPDS to induce endogenous SREBP activity. Increased concentrations of PF-429242 gave dose-dependent inhibition of luciferase activity (Fig. 2B), suggesting that the compound was inhibiting the processing of endogenous SREBP to their nuclear forms. The luciferase activity was inhibited by up to 93% at 30 μM PF-429242, with an IC50 of 1.8 μM. The inhibitory profiles of compounds C and D, two related analogs of PF-429242, are also shown in Fig. 2B, and the structures of these compounds are shown in Fig. 2C.

The ability of PF-429242 to affect the expression of SREBP target genes in cultured cells was evaluated by treating HepG2 cells with 1, 3, 10, and 30 μM PF-429242 for 24 h in DMEM/LPDS. The expression of endogenous SREBP target genes was tested by real-time quantitative PCR, using HMG-
CoA synthase and fatty acid synthase to represent genes involved in sterol and fatty acid synthesis. Both genes were down-regulated in a dose-dependent fashion by PF-429242, with maximal inhibition of 95% for HMG-CoA synthase and 80% for fatty acid synthase at 3 \( \mu \text{M} \) PF-429242 (Fig. 3A). The expression of LDL receptor, another SREBP target gene, was also down-regulated (Fig. 3B), albeit less effectively than either HMG-CoA synthase or fatty acid synthase (e.g., 30% reduction in LDL receptor gene expression at a concentration of 1 \( \mu \text{M} \) PF-429242 versus 60% inhibition of HMG-CoA synthase and fatty acid synthase gene expression at the same inhibitor concentration; Fig. 3, A and B). Inhibition of gene expression in HepG2 cells was observed under both 10% LPDS and 10% FBS-containing culture conditions (data not shown). The expression of a non-SREBP target gene, ABCA1, was not affected by PF-429242 (Fig. 3C), indicating that the reductions in expression of the SREBP target genes were not due to cytotoxicity of the compound, an observation confirmed by the absence of lactate dehydrogenase release with compound treatment.

To evaluate the ability of PF-429242 to inhibit cholesterol and fatty acid synthesis in cultured cells, HepG2 cells were treated with 0.03, 0.1, 0.3, 1, 3, and 10 \( \mu \text{M} \) PF-429242 for 24 h. Cells were then incubated with [2,\(^{14}\)C]acetate for 6 h, and the incorporation of [2,\(^{14}\)C]acetate into cholesterol and fatty acids was determined. The rate of cholesterol synthesis was inhibited by PF-429242 in a dose-dependent manner, with an IC\(_{50}\) of 0.53 \( \mu \text{M} \) (Fig. 3D). However, under these experimental conditions, there was no significant inhibition of fatty acid synthesis.

The short-term in vivo efficacy of PF-429242 was tested in mice by measuring its effect on the expression of SREBP target genes and the rate of lipid synthesis in the liver. Because of its high clearance rate (75 ml/min/kg) and poor oral bioavailability (~5%) (Hay et al., 2007), mice were dosed i.p. with either 10 or 30 mg/kg PF-429242 at 6-h intervals for 24 h. This dose was chosen to allow continuous exposure levels at or exceeding compound IC\(_{50}\) for S1P over the time period tested. After this regimen, the level of HMG-CoA synthase RNA in mouse liver was down-regulated by 72% (*, \( p < 0.01 \)) and 83% (**, \( p < 0.01 \)), and fatty acid synthase expression was reduced by 41% (not statistically significant) and 77% (**, \( p < 0.01 \)), in the 10 and 30 mg/kg dosing groups, respectively (Fig. 4A). LDL receptor gene expression was also reduced by treatment but, as observed in HepG2 cell-based assays, to a much lesser extent (20% reduction; not statistically significant) in the 10 mg/kg-treated group and 30%...
reduction (**, p < 0.01) in the 30 mg/kg-treated group than the expression of either lipogenic enzyme (Fig. 4A).

Consistent with the reductions in HMG-CoA synthase and fatty acid synthase gene expression, the rates of cholesterol and fatty acid synthesis were also decreased in vivo by short-term treatment with PF-429242. Incorporation of [2-14C]acetate into cholesterol was decreased by 40% (p = 0.062) and 75% (***, p < 0.001) at 10 and 30 mg/kg doses, respectively, compared with vehicle-treated mice (Fig. 4B). Incorporation of [2-14C]acetate into fatty acid was reduced by 34% (not statistically significant) and 78% (***, p < 0.001), respectively (Fig. 4C).

Discussion

Western blotting of subcellular fractions from cells treated with PF-429242 provided the most direct evidence of S1P inhibition-mediated effects on SREBP. The relative abundance of nuclear and membrane-bound SREBP was distinctly different after PF-429242 treatment, compared with cells incubated with sterols. In the presence of excess sterol, the SCAP/SREBP complex remains in the ER. SREBP is not exposed to S1P and, therefore, is not processed. Under the culture conditions used, high levels of sterols resulted in the expected decrease of nuclear SREBP and an increase in membrane-bound SREBP, whereas low sterol conditions resulted in increased nuclear and decreased membrane SREBP (Fig. 1B). In contrast, treatment of cells with PF-429242 led to a marked change in the relative abundance of nuclear and membrane-bound forms of SREBP, compared with sterol-treated cells. This observation is consistent with the down-regulation of SREBP gene expression after S1P inhibition, based on observations previously reported in S1P knockout mice (Yang et al., 2001). In these animals, both SREBP-1 and SREBP-2 precursors and nuclear forms of SREBP are greatly reduced, and this reduction is accompanied by a reduction in both SREBP-1 and SREBP-2 mRNA levels. Because expression of the SREBPs is also regulated by the nuclear forms of the SREBPs themselves, through SREs in their promoters, the reduction of precursor SREBP levels in these animals occurs as a consequence of down-regulation of SREBP expression (Yang et al., 2001). In our experiments, SREBP-1 mRNA levels in HepG2 cells treated with 10 μM PF-429242 were also reduced by 61%, consistent with a secondary down-regulation of SREBP gene expression in response to S1P inhibition and similar to that observed in the S1P knockout mice (Yang et al., 2001).

Another major player in the regulation of SREBP processing is SCAP. Reduction in nuclear and membrane SREBPs is also observed in SCAP knockout mice (Matsuda et al., 2001). Based on the results from our cell and animal efficacy studies, we cannot rule out the possibility that our compounds might also have inhibited SCAP action. However, the close correlation between the potency of inhibitors in the S1P enzymatic assay and in the SRE-luciferase reporter expression assay across the series of S1P inhibitors suggests that SREBP processing inhibition as a result of S1P enzyme inhibition is the most probable explanation.

Although in vitro proteolytic cleavage assays had initially yielded an IC50 of 175 nM, PF-429242 inhibited the expression of SRE-luciferase in transfected HEK293 cells, with an IC50 of 1.85 μM. LDL receptor message levels were reduced approximately 50% at this same concentration. In contrast, when expression levels of the endogenous SREBP targets HMG-CoA synthase and fatty acid synthase were quantitated by real-time PCR in HepG2 cells, PF-429242 had a somewhat higher potency, with an EC50 < 1 μM for both genes. Maximal reduction in RNA expression was also clearly gene-specific, with HMG-CoA synthase inhibited essentially completely (>95%), and fatty acid synthase inhibited only approximately 80% at 30 μM concentration. We have tested multiple small-molecule S1P inhibitors and compared their ability to inhibit HMG-CoA synthase and fatty acid synthase by real-time reverse transcriptase-PCR. All the compounds tested followed the same order of potency for inhibition of both target genes. Given that differential reduction in cholesterol and fatty acid biosynthetic gene activity has also been observed in the published SREBP, S1P, and SCAP knockout mouse genetic models (Shimano et al., 1997; Matsuda et al., 2001; Yang et al., 2001), these results potentially reflect the differential interaction of SREBP family with the complex promoters of these genes and raise the possibility of “dialing in” relatively more cholesterol and fatty acid biosynthetic inhibition than LDL receptor reduction with this mechanism. However, our results do suggest that all compounds tested inhibited the processing of SREBP-1 and SREBP-2 to similar extents, with no indication that S1P preferentially cleaves one SREBP rather than another.

Because SREBP regulate multiple genes in cholesterol and fatty acid synthesis pathways (Horton et al., 2002), the inhibition of cholesterol and fatty acid synthesis should reflect the overall results of inhibiting many steps in the synthesis pathways. Potential lowering of LDL receptor is considered a major caveat of any mechanism aimed at disrupting SREBP, including S1P inhibition. Overall, acute treatment with PF-429242 yielded results highly reminiscent of the previously published S1P knockout mouse, including effects on hepatic cholesterol, fatty acid biosynthesis, and lowered LDL receptor levels (Yang et al., 2001). Despite this significant LDL receptor lowering, S1P knockout mice demonstrated reduced plasma cholesterol and triglyceride levels. It is unfortunate that we were unable to test plasma cholesterol and triglyceride changes after prolonged treatment with compound due to the extremely poor pharmacokinetic properties of our compound. Although definitive results await the identification of a more bioavailable and in vivo stable chemical tool, it is encouraging that cell-based data support the possibility of differential regulation of target genes, with cholesterol and fatty acid biosynthetic target genes relatively more sensitive to reduced nuclear SREBP levels than the LDL receptor.

Despite its pharmacokinetic limitations, PF-429292 has enabled key cellular proof of mechanism and initial in vivo proof of concept studies for a new class of therapeutic agents. Taken together, these findings confirm the potential of S1P inhibitors to exhibit a profile that combines the benefits of a cholesterol synthesis inhibitor (e.g., HMG-CoA reductase inhibitor) with the benefits of a fatty acid metabolism modulator (e.g., acetyl-CoA carboxylase inhibitor). New medicines from this class would be potentially useful not only for patients with dyslipidemia but also for those with a variety of cardiometabolic risk factors associated with insulin resistance, diabetes, obesity and the metabolic syndrome. It is clear that a compound with improved pharmacokinetic properties that could be administered chronically to experimental
animals is required to further confirm that the inhibition of SIP activity would indeed possess these combined benefits.

References


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