Nelfinavir induces liposarcoma apoptosis through inhibition of regulated intramembrane proteolysis of SREBP-1 and ATF6

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TRANSLATIONAL RELEVANCE

This manuscript “Nelfinavir induces liposarcoma apoptosis through inhibition of regulated intramembrane proteolysis of SREBP-1 and ATF6,” describes a novel anti-cancer pathway elucidated for the HIV protease inhibitor, nelfinavir. The mechanism involves inhibition of regulated intramembrane proteolysis (RIP) though inhibition of site 2 protease (S2P) activity. This results in accumulation of precursor sterol regulatory element binding protein-1 (SREBP-1) and activating transcription factor 6 (ATF6). The resulting accumulation of unfolded SREBP-1 and ATF6 results in overwhelming ER stress and an impaired unfolded protein response (UPR). The liposarcoma cells respond by undergoing apoptosis. This novel approach to cancer therapeutics is heretofore unreported. A clinical trial for nelfinavir in liposarcoma supported by an FDA Orphan Products Development grant (R01 FD003006) is currently ongoing, which has demonstrated early activity (NCT00233948: A Phase I/II Study of Nelfinavir in Liposarcoma; Chow WA et al. Anti-HIV drugs for cancer therapeutics: back to the future? Lancet Oncol 2009;10:61-71).
ABSTRACT

Purpose: We previously reported nelfinavir induces $G_1$ cell cycle block and apoptosis selectively in liposarcoma cell lines due to increased SREBP-1 expression in the absence of increased transcription. We postulate that nelfinavir interferes with regulated intramembrane proteolysis of SREBP-1 and ATF6.

Experimental Design: Time-lapse, confocal microscopy studies demonstrate that nelfinavir inhibits the nuclear translocation of full-length SREBP-1-EGFP and ATF6-EGFP fusion proteins. siRNA-mediated knockdown of site-1 and/or site-2 protease leads to inhibition of SREBP-1 intracellular trafficking to the nucleus and reduces liposarcoma cell proliferation. Treatment of LiSa-2 liposarcoma cells with DCI, a serine protease inhibitor of S1P, did not affect SREBP-1 processing. In contrast, 1, 10-phenanthroline, an S2P-specific inhibitor, reproduces the molecular and biological phenotype observed in nelfinavir-treated cells, which implicates S2P as a target of nelfinavir. In vivo evaluation of nelfinavir in a murine liposarcoma xenograft model leads to inhibition of tumor growth without significant toxicity.

Results: Nelfinavir-induced up-regulation of SREBP-1 and ATF6 results from inhibition of site-2 protease, which together with site-1 protease, mediates regulated intramembrane proteolysis from their precursor to their transcriptionally-active forms. The resulting endoplasmic reticulum stress and concurrent inhibition of the unfolded protein response induces caspase-mediated apoptosis.
Conclusions: These results provide new insight into the mechanism of nelfinavir-mediated induction of ER stress and cell death in liposarcomas, and are the first to report targeting S2P for cancer therapy.
INTRODUCTION

The HIV protease inhibitor (PI), nelfinavir (Viracept®), has shown promising anti-cancer activity via: induction of apoptotic cell death; induction of endoplasmic reticulum (ER) stress and autophagy; inhibition of epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor (IGFR)-mediated PI-3K/Akt activation (1-4); and induction of chemoradiosensitization (5). Nelfinavir also decreases vascular endothelial growth factor (VEGF)/hypoxia-inducible factor-1α (HIF-1α) expression, tumor hypoxia, and angiogenesis (6). Additionally, nelfinavir inhibits signal transducer and activator of transcription 3 (STAT3) and extracellular signal-regulated kinase 1/2 (ERK1/2) (7). Such pleiotropic activities demonstrate that nelfinavir has multiple off target effects.

Liposarcomas arise from adipocytes or their precursors (8). Chemotherapy for recurrent or metastatic liposarcomas is generally palliative (9). We hypothesized that nelfinavir would inhibit liposarcoma growth because HIV PI use is linked to a clinical syndrome of peripheral lipoatrophy known as “HIV protease-induced lipodystrophy syndrome” (10). In this syndrome, adipocyte apoptosis is observed and associated with alteration of sterol regulatory element binding protein-1 (SREBP-1) expression (11-13).

SREBPs are synthesized as inactive, membrane-bound precursors in the endoplasmic reticulum (ER), tethered at their carboxy-terminal domain by SREBP cleavage-activating protein (SCAP) (14, 15). In the presence of cholesterol, SCAP binds insulin-induced gene-1 or gene-2 (Insig-1 or Insig-2) in the ER preventing SCAP-mediated proteolytic processing and activation of
SREBPs (16). Upon cholesterol depletion, SCAP and Insig fail to interact, the SREBP-SCAP complex is transported to the Golgi apparatus, where it is processed in two sequential proteolytic cleavage steps by Site-1 protease (S1P), a subtilisin-like serine protease, and Site-2 protease (S2P), a metalloprotease protease, to release the transcriptionally-active, amino-terminal fragment to the nucleus in an evolutionarily-conserved process, regulated intramembrane proteolysis (RIP) (16-18). SREBP-1 is a key transcription factor necessary for adipogenesis, cholesterol biosynthesis and adipocyte differentiation (19). Its target genes include genes related to cholesterol synthesis such as fatty-acid synthase (FASN) (20) and anti-proliferative and pro-apoptotic genes such as: p21WAF1/CIP1, Fas and Bax (21, 22).

Most proteins fold and mature in the ER lumen. An imbalance between the load of unfolded proteins that enter the ER (ER stress) and the capacity of the cellular machinery that handles this load (unfolded protein response, UPR) eventually triggers apoptosis (23, 24). Activating transcription factor 6 (ATF6) is a precursor ER-bound transcription factor that activates UPR genes. When unfolded proteins accumulate in the ER, ATF6 is RIP-processed by S1P and S2P to release its amino-terminal domain analogous to SREBPs (18).

Our group has shown that nelfinavir induces a G1 cell cycle block and apoptosis selectively in liposarcoma cell lines as a consequence of up-regulation of SREBP-1 expression (3). In the present report, we demonstrate that nelfinavir-induced up-regulation of SREBP-1 and ATF6 results from inhibition of S2P, leading to prevention of intracellular transport of mature SREBP-1 and ATF6 to
the nucleus. These results provide new insight into the mechanism for nelfinavir-mediated ER stress in liposarcomas.

**MATERIALS AND METHODS**

**Cell culture** SW872 dedifferentiated liposarcoma was purchased from the American Type Culture Collection (Virginia, USA) (25). LiSa-2 pleomorphic liposarcoma cell line was a kind gift from Dr. Silke Brüderlein (University of Ulm, Ulm, Germany) (26), and maintained in Iscove’s modified Dulbecco’s medium /RPMI in a 4:1 ratio supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 0.1 mg/ml gentamicin. The cells were DNA fingerprinted to confirm identity as previously described (27).

**Chemicals and reagents** Nelfinavir (NFV) was obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, MD). M8, the primary, active *in vivo* NFV metabolite was obtained from Pfizer, Inc. (Groton, CT). Stock solution of NFV and M8 were made in dimethyl sulfoxide (DMSO). 3,4-dichloroisocoumarin (DCI) and 1,10-phenanthroline were purchased from Sigma-Aldrich. SREBP-1, FASN, and GRP78 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and ATF6 antibody was purchased from Abcam, Inc. (Cambridge, MA).

**Apoptosis, clonogenicity and proliferation assays** LiSa-2 cells were treated with NFV for 24 hr for caspase 9/6 assay according to the manufacturer’s protocol (Clontech, Mountain View, CA) or annexin V detection (Santa Cruz Biotechnology, Inc.) by flow cytometry. Clonogenic assay was performed in NFV-
treated LiSa-2 cells according to the protocol previously described (3). A fluorescence-based digital image microscopy system (DIMSCAN) (Bioimaging Solutions Inc., San Diego, CA) was used to quantify the number of viable NFV- or M8-treated LiSa-2 cells as previously described (28). Briefly, 1000 cells were seeded in a 96-well plate in multiples of 10 wells and treated with NFV or M8.

**Protein T₁/₂ assay and Western blot analysis** SW872 cells were treated with 10 μM NFV or DMSO for 4 hr, followed by cycloheximide (100 μM) for the indicated time. Cell lysates were harvested to detect SREBP-1 or ATF6 by Western blot. FASN and GRP78 were detected in lysates prepared from LiSa-2 cells treated with NFV for 24hr.

**Plasmid and quantitative RT-PCR** pTK-HSV-BP1a encoding human SREBP-1a and pCGN-ATF6 encoding human ATF6 were kindly provided by Dr. Guosheng Liang (UT Southwestern Medical School, Dallas, TX) and Dr. Amy Lee (USC, Los Angeles, CA). cDNA encoding SREBP-1a and ATF6 were amplified from pTK-HSV-BP1a and pCGN-ATF6 by PCR (Supplemental data). The PCR fragments were inserted into pEGFP-C2 (Clontech, Mountain View, CA) to obtain fusion EGFP-amino terminal SREBP-1 and ATF6 plasmids (pSREBP-1-EGFP, pATF6-EGFP). Quantitative reverse transcriptional PCR (q-RT-PCR) was carried out in total RNA extracted from siRNA treated LiSa-2 cells with the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) (Supplemental data). Relative gene-expression quantification method was used to calculate the fold change of mRNA expression according to the comparative Ct method using β-actin as an endogenous control.
**S1P, S2P siRNA knockdown assay** 5 × 10⁵ / well LiSa-2 cells were cultured in six-well plates overnight prior to transfection. Human S1P and S2P gene specific siRNA and scrambled siRNA (Santa Cruz Biotechnology, Inc) were transfected into cells with Lipofectamine (Invitrogen Corp., Carlsbad, CA). After 48 hr incubation, the transfected cells were collected for q-RT-PCR of S1P and S2P or Western blot of SREBP-1 or subjected to DIMSCAN.

DIMSCAN was used to quantify the number of viable siRNA-treated LiSa-2 cells. 2000 cells were seeded in a 96-well plate in multiples of 10 wells and followed by transfection with S1P, S2P, or S1P and S2P or scrambled siRNA. 24 hr later the cells were transfected with pSREBP-1-EGFP. At 48 and 72 hr after siRNAs transfection, cells were stained and scanned.

**Confocal microscopy time-lapse imaging** On day 0, LiSa-2 cells were plated in a two-well chamber slide at 3X10⁵ cells / well. On day 2, cells were transfected with pSREBP-1-EGFP or pATF6-EGFP. On day 3, transfected cells were stained with Hoechst 33322 (Invitrogen, Carlsbad, CA) at final conc. 1 μg/ml for 5 min and transferred to the Microscopy Core Lab. Cells were maintained in the live cell chamber at 37°C in 5% CO₂ during confocal microscopy. To start the time-lapse experiment, DMSO or 10 μM NFV was added to the pretreated wells. The first image (t= 0 hr) was recorded 5 min later. N-terminal EGFP-labeled SREBP-1 or ATF6 were visualized with a Zeiss LSM510 META NLO axiovert microscope using a 488 argon laser (green) and chameleon 2P laser (blue) and the objective lens is LD-AChroplan 40x/0.6NA with correction.
collar. Digital images were captured every 2 hr for 12 hr with no significant loss of signal.

In the siRNA knockdown study, S1P, S2P or equal amounts of S1P and S2P siRNA were transfected on day 2. On day 3, cells were transfected with pSREBP-1-EGFP. On day 4, nucleus was stained for time-lapse imaging as described. The first image (t=0 h) was recorded 24 hr after pSREBP-1-EGFP transfection (48 hr after siRNA transfection) and recorded every 2 hr for 12 hr. Images were generated using Multi Time Macro in the LSM 510 software and was analyzed using LSM Image Browser and Concatination Macro software.

**In vivo evaluation of nelfinavir in a murine xenograft liposarcoma model.** A heterotopic murine liposarcoma model was established with human LiSa-2 cells in severe-combined immunodeficient (SCID) mice. Log-phase growth cells were harvested and resuspended in phosphate-buffered saline (PBS) at 1X10^7 cells/ml. 100 µl of cells were injected subcutaneously into the flanks of 6-8 week-old female SCID mice. Tumors that developed were surgically removed at week 5, cut into 2 mm^3 pieces, and placed into a surgically prepared subcutaneous pocket in the recipient mice. The recipient mice were allowed to recover 7 days before treatment with NFV (n=7) or control (n=7). The average starting weight of the mice was 20 gm and tumor volume (V) was determined by the equation: V=1/2 l x w^2.

Given our previous experience, prolonged twice daily oral gavage was not considered feasible. NFV 625 mg tablets were purchased from the City of Hope Pharmacy and crushed into a fine powder. NFV powder was admixed with
Transgenic Dough Diet (Bio-Serv, Frenchtown, NJ) to dose at 500 mg/Kg/day. Mouse feed was replaced daily with 4 gm of Transgenic Dough Diet alone (control) or admixed with NFV. Tumors were bidimensionally measured and the mice were weighed 3 times weekly.

**Nelfinavir pharmacokinetics in SCID mice.** NFV powder was resuspended in 1% carboxymethylcellulose to 100 mg/ml. Groups of three, 8-10 week-old female SCID mice underwent oral gavage with 500 mg/kg of NFV in carboxymethylcellulose then euthanized at 0, 1, 2, 4, 8, and 24 hr after oral gavage. Blood was collected and plasma was separated for analysis of NFV concentration with an UPLC-tandem mass spectrometric assay developed and validated in the Analytical Pharmacology Core Facility at the City of Hope (Supplemental data).

**Measurement of ER stress.** S1P, S2P or equal amounts of S1P and S2P siRNA were transfected on day 1. Cell lysates for Western blot detection of GRP78 were prepared from the transfected cells on day 3. Nelfinavir treated LiSa-2 cells were collected at 24 hr for Western blot detection of GRP78. Thapsigargin (0.5 µM) served as a positive control for ER stress induction.

**Statistical Analysis** Data were presented as the mean ± SD of three independent experiments. Group comparisons for continuous data were done with student's t-test for independent means or two-way ANOVA.
RESULTS

Nelfinavir induces caspase-dependent apoptosis. Caspase activation was analyzed in NFV-treated LiSa-2 and SW872 cells. As shown in Figure 1A, activity level of caspase 9/6 increases in a dose-dependent fashion and reaches a maximum of four- and five-fold over baseline activity at the highest dose (20 µM). Clonogenic assays in LiSa-2 cells (Figure 1B) confirm NFV inhibits clonogenicity in a dose-dependent manner.

M8, the primary nelfinavir metabolite, induces dose-independent cell death. NFV is metabolized in vivo to its hydroxyl-t-butylamide metabolite, M8, which possesses similar in vitro antiviral activity as its parent compound. Figure 1C reveals similar (day 2) or slightly higher survival (day 6) in M8-treated LiSa-2 cells compared to NFV at the same doses. This demonstrates M8 possesses comparable anti-tumor activity as NFV.

Nelfinavir increases SREBP-1 protein half-life. Our previous data showed NFV leads to significantly elevated expression of precursor SREBP-1 (125 KDa), in the absence of increased transcription (3). Consequently, protein stability of SREBP-1 was evaluated in the presence of NFV. DMSO-treated SW872 cells demonstrate degradation of precursor SREBP-1(125-kDa) by 1 hr and its complete absence by 4 hr (Fig. 2A) along with increased detection of processed SREBP-1 (68-kDa) up to 24 hr. In contrast, NFV leads to accumulation of precursor SREBP-1 and minimal detection of its processed form through 24 hr (Fig. 2A). The results are demonstrated quantitatively in Fig. 2A with the half-life ($T_{1/2}$) of 2 hr for control and 13 hr for NFV treated cells. Similarly,
ATF6 (Fig. 2B) was also tested in LiSa-2 cells, and demonstrates a similar dose-dependent increase of precursor ATF6 (90 KDa) by NFV, and prolonged $T_{1/2}$ from 6 to 9 hr (Supplemental Data). Importantly, NFV did not alter SREBP-1 ubiquitination nor acetylation (Supplemental Data).

**Nelfinavir inhibits intracellular trafficking of SREBP-1 and ATF6.** To explore whether the increased half-life of precursor SREBP-1 or ATF6 results from NFV-mediated inhibition of RIP, plasmids encoding the full-length SREBP-1 or ATF6 gene fused in frame at their amino terminus with EGFP were constructed and transfected into LiSa-2 cells to monitor the trafficking of their mature forms from the cytoplasm (ER-Golgi) to the nucleus. As shown in Fig. 2C and D, control cells show intense green fluorescence throughout the nucleus by 12 hr, which reflects normal RIP processing of the precursor SREBP-1-EGFP fusion protein to its transcriptionally active form. In contrast, the NFV-treated cells show retention of the fusion protein in the cytoplasm and minimal movement into the nucleus at 12 hr. Similarly, inhibition of RIP processing of precursor ATF6 was observed, as shown in Fig. 2D. These data show that NFV inhibits intracellular trafficking of SREBP-1 and ATF6 by inhibiting RIP.

**Nelfinavir inhibits Fatty Acid Synthase (FASN) expression.** FASN is a transcriptional target of SREBP-1 (29). Accordingly, FASN was evaluated in NFV-treated LiSa-2 cells to evaluate the effect of inhibition of SREBP-1 production on downstream targets. Fig. 2B demonstrates NFV dose-dependently inhibits FASN expression (confirmed by FASN q-RT-PCR gene expression,
Supplemental Data), consistent with the hypothesis that NFV interferes with SREBP-1 processing.

**siRNA-mediated inhibition of S1P or S2P blocks intracellular trafficking of SREBP-1 and reduces liposarcoma proliferation.** Both SREBP-1 and ATF6 are cleaved by S1P and S2P during RIP. To further explore the mechanism of NFV-induced accumulation of unprocessed SREBP-1 and ATF6, siRNA targeting S1P, S2P, or S1P plus S2P were transfected into LiSa-2 cells. siRNA reduced target S1P or S2P RNA level >90% (Fig. 3A). Western blot confirms an accumulation of precursor SREBP-1 (Fig. 3A) in S1P or/and S2P siRNA transfected cells. Notably, time-lapse microscopy demonstrates the absence of nuclear GFP in both S1P and S2P siRNA-transfected cells whereas control cells show diffuse nuclear fluorescence (Figs. 3B and 3C). These results indicate inhibition of S1P and S2P results in potent inhibition of SREBP-1 trafficking to the nucleus. Significantly, combined S1P and S2P knockdown leads to the greatest inhibition of SREBP-1 movement, as shown by nearly complete exclusion of fluorescence in the nucleus (Fig. 3D). To determine the consequence of S1P and S2P knockdown on cell proliferation, a fluorescence-based assay, DIMSCAN, was performed Fig. 4A shows S1P or S2P siRNA reduces cell survival to 85% of control at 72 hrs. Notably, both siRNAs reduce survival by 50%. These results demonstrate that inhibition of S1P and/or S2P-mediated RIP of SREBP-1 leads to cell death, which supports the notion that S1P and/or S2P may be potential targets of NFV.
Blocking S2P function with a small molecule inhibitor reproduces the nelfinavir-treated phenotype. To more closely examine whether the activity observed for NFV was attributable to inhibition of S1P or S2P, small molecule inhibitors were utilized. 3,4-dichloroisocoumarin (DCI) is a potent serine protease inhibitor of recombinant S1P (30). DCI does not alter the precursor SREBP-1 or ATF6 accumulation (Figs. 4B). In contrast, treatment of LiSa-2 cells with 1,10-phenanthroline, a metalloprotease-specific S2P inhibitor, leads to both dose- and time-dependent accumulation of precursor SREBP-1 and ATF6 (Fig. 4C), similar to the phenotype observed for NFV (31). Likewise, 1,10-phenanthroline leads to dose-dependent apoptosis (Fig. 4D) which highly suggests that NFV inhibits the proteolytic activity of S2P.

Nelfinavir inhibits liposarcoma growth in a murine model. To determine whether NFV possesses in vivo anticancer activity, a heterotopic SCID murine liposarcoma model was established, and the mice were treated with NFV 500 mg/Kg/day orally for 6 weeks. The results shown in Fig. 5A demonstrate that by day 24 the tumor growth curve of the NFV-treated mice begin to diverge from the control mice, and continue to so throughout the entire experimental period (P<0.05), which suggest that the in vitro observations for NFV may be equally applicable in vivo.

Clinical use of NFV in treatment of HIV infection is commonly associated with diarrhea, but rarely leads to hepatotoxicity and/or myelosuppression (32). To evaluate toxicities associated with NFV in SCID mice, after 2 weeks of continuous twice daily dosing with vehicle only (carboxymethylcellulose), NFV 1
gm/kg/day, or 2 gm/kg/day by oral gavage, the mice were euthanized and subjected to necropsy. Figure 5B demonstrates minimal or no weight loss associated with NFV with either dose level (P<0.05). Daily cage examination failed to demonstrate diarrhea or its pre-determined surrogate, 10% weight loss. Histologic evaluation of liver and bone marrow demonstrated no microscopic difference in liver architecture nor levels of myeloid nor erythroid precursors between control and NFV-treated mice (data not shown).

To determine the pharmacokinetics of oral NFV in vivo, cohorts of 3 mice underwent a single, oral gavage with 500 mg/kg of NFV, and the mice were euthanized for cardiac puncture at the indicated time points. The results demonstrate at a dose of 500 mg/kg, a peak plasma concentration of NFV that approximates the in vitro biologically active dose (~10 µM) is reached in 1 hr, and remains steady for 8 hr (Fig. 5C). These data suggest that twice daily dosing is feasible for long-term NFV dosing.

**Nelfinavir and S2P siRNA induce ER stress.** NFV induces ER stress (4). To determine whether S2P inhibition also induces ER stress, siRNA targeting S1P, S2P, or S1P plus S2P were transfected into LiSa-2 cells, and expression of the ER stress response protein GRP78 was evaluated. Thapsigargin (TG) served as a positive control for ER stress induction. Fig. 6A shows that TG, high dose NFV, and S2P or S1P plus S2P siRNA strongly induce expression of GRP78, whereas control and S1P siRNA alone does not. These results are consistent with the hypothesis that NFV inhibits S2P activity, which induces ER stress.
DISCUSSION

Nelfinavir is a PI that was originally developed for therapy against HIV. Nelfinavir may inhibit cancer growth via multiple pathways (1-6). Recently, a study in ovarian cancer suggested a novel additional mechanism that nelfinavir may target cancer stem cells (33). These reports have led support for the use of nelfinavir alone or in combination with radiation and/or chemotherapy for advanced cancers in several clinical trials including our ongoing trial in liposarcomas (5, 34).

Liposarcomas are one of the most common soft tissue sarcomas, and outcomes for recurrence remains poor. They may be particularly sensitive to nelfinavir, because it interferes with maturation of SREBP-1, an adipocytic transcription factor (19). Our data strongly suggests that nelfinavir inhibits liposarcoma proliferation, and promotes apoptosis by inhibiting RIP processing of SREBP-1 and ATF6. This results in accumulation of unfolded SREBP-1 and ATF6 precursor proteins (ER stress), and inhibition of the UPR because ER stress-induced activation of ATF6 is neutralized.

Regulation of SREBP-1 and ATF6 is similar (Figure 6B). Both transcription factors are synthesized as ER transmembrane proteins (precursor) and transported to the Golgi where RIP occurs. Luminal S1P cleavage occurs first, followed by intra-membrane S2P cleavage, which liberates the transcriptionally-active amino-terminal segments of SREBP-1 and ATF6 to migrate to the nucleus to transactivate sterol biosynthesis or UPR activation genes.
Our previous data showed precursor and processed forms of SREBP-1 were consistently upregulated by nefinavir in various liposarcoma cell lines (3). Our present results show nefinavir induces similar up-regulation of ATF6 (Fig. 2). Confocal, time-lapse microscopy (Fig. 3) demonstrates that SREBP-1 and ATF6 processing is inhibited by nefinavir with retention of precursor SREBP-1 and ATF6 in the cytoplasm. This results in SREBP-1-mediated downregulation of FASN. Accumulation of precursor SREBP-1 and ATF6 induces ER stress, activates the UPR, and induces apoptosis. Our results indicate inhibition of RIP processing of SREBP-1 and ATF6 results from NFV-mediated inhibition of S2P activity, which is supported by the demonstration that siRNA-mediated knockdown of S2P, but not S1P, induces ER stress (Fig. 6A). Interestingly, HIV PIs inhibit proteolytic activation of zinc metalloproteinase, ZMPSTE24, and matrix metalloproteinase-2 (MMP2) (35, 36). S2P is also a metalloprotease, and likely shares a similar domain with other family members recognized by a PI.

Our current model illustrated in Fig. 6B demonstrates that nefinavir-induced up-regulation of precursor SREBP-1 and ATF6 is mediated through inhibition of S2P, which leads to inhibition of RIP. Confirmatory support remains pending our current efforts to purify S2P protein for enzymatic cleavage assays. The precursor proteins are retained in the cytoplasm, their transport to the nucleus is halted, and transcriptional activation of target genes is blocked. Accumulation of unprocessed SREBP and ATF6 induces ER stress, and a deficient UPR leads to caspase-dependent apoptosis.
Although our data indicates nelfinavir inhibits S2P activity, partial S1P inhibition cannot be excluded. RIP processing of SREBP-1 and ATF6 requires sequential S1P and S2P cleavage. Prywes et al. previously noted the bulky ATF6 luminal domain blocks S2P cleavage, and the primary role of S1P is to reduce the size of the luminal domain to prepare ATF6 to be an optimal S2P substrate (37). An analogous model for SREBP-1 exists, with removal of its second transmembrane domain by S1P, which allows for movement of the substrate site within the membrane (38). This may explain why knockdown S1P by siRNA still leads to accumulation of unprocessed precursor SREBP-1 in the cytoplasm in the absence of precursor SREBP-1 accumulation in DCI-treated cells. However, knockdown of S2P by siRNA or inhibition of S2P catalytic activity by 1,10-phenanthroline leads to a phenotype characterized by nelfinavir treatment.

Our previous data demonstrated that conditional expression of SREBP-1 in SW872 liposarcoma cells reduces cellular proliferation, and induces anti-proliferative and proapoptotic genes such as p21^{WAF1/CIP1}, Fas and Bax. SREBP-1 acts as a proapoptotic gene in pancreatic β-islet cells, and caspases 2 and 7 are activated by SREBP-1 and -2 in statin-induced apoptotic gastric cancer cells (39, 40). ATF6-mediated apoptosis resulting from ER stress has been elucidated (41). Accumulation of misfolded proteins in the ER induces the UPR to promote cell survival by adjusting ER protein folding capacity. However, if homeostasis cannot be re-established, apoptosis is induced through ATF6-mediated activation of CHOP/Gadd153 (42).
A notable target gene of SREBP is FASN. One of the hallmarks of cancer is increased *de novo* fatty acid (FA) synthesis, referred to as the “lipogenic phenotype” (43, 44). FAs are essential constituents of all biological membrane lipids, and are important substrates for energy metabolism (44). FASN catalyzes the terminal steps of long chain FA synthesis (44). FASN is overexpressed in many cancers (43, 44). Inhibition of FASN by RNAi or small molecules inhibits growth and survival of tumors (44-46). We also observed nelfinavir-dependent reduction in FASN expression, which was mediated through depletion of transcriptionally-active SREBP-1. This provocative result suggests that nelfinavir may possess anticancer activity in other cancers which exhibit a “lipogenic phenotype,” an avenue that we are currently actively pursuing.

In summary, this study demonstrates that nelfinavir induces liposarcoma apoptosis through ER stress induction and deficient UPR. These biological effects arise from accumulation of precursor SREBP-1 and ATF6, as a result of S2P-regulated RIP cleavage inhibition. To our knowledge, this is the first report of a novel strategy targeting S2P and RIP for cancer therapeutics.
REFERENCES

Fig. 1 Min Guan

A

LiSa-2

SW872

Fluorescence (O.D.)

NFV μM

0 2.5 10 20

0 50 100 150 200

0 2.5 10 20

0 100 200 300 400

B

Number of Colonies

NFV μM

0 2.5 10 20

0 10 20 30 40 50 60 70

100

C

Survival (%)

Days

0 2 4 6

100 90 80 70 60 50 40 30 20 10

DMSO

NFV 2.5 μM

NFV 10 μM

NFV 20 μM

M8 2.5 μM

M8 10 μM

M8 20 μM

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Fig. 5 Min Guan
Fig. 6 Min Guan
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