Nelfinavir induces liposarcoma apoptosis and cell cycle arrest by upregulating sterol regulatory element binding protein-1

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'HIV protease-induced lipodystrophy syndrome' is associated with the use of HIV protease inhibitors for treatment of HIV infection. In-vitro studies suggest that alteration of sterol regulatory element binding protein-1 levels underlie its pathogenesis. We postulated that HIV protease inhibitors may represent a novel class of antiliposarcoma agents. SW872, FU-DDLS-1 and LiSa-2 liposarcoma, and HT1080 and 293 nonliposarcoma cell lines were treated with HIV protease inhibitors (nelfinavir, ritonavir, saquinavir, indinavir and amprenavir), and clonogenic assays were performed. Nelfinavir exhibited the most potent inhibition of clonogenicity, and further assays for proliferation, cell cycle and apoptosis were performed with nelfinavir. Immunoblots were performed for sterol regulatory element binding protein-1, proapoptotic and cell cycle-related protein expression after nelfinavir treatment. Finally, a sterol regulatory element binding protein-1-inducible SW872 cell line was developed to examine the phenotype resulting from upregulated sterol regulatory element binding protein-1. Nelfinavir selectively inhibited clonogenicity and proliferation, and induced G_1 cell cycle block and induced apoptosis in a dose-dependent manner in SW872 and LiSa-2 cells, whereas it had minimal or no effect on these parameters in FU-DDLS-1 or nonliposarcoma cells. Nelfinavir induced significant sterol regulatory element binding protein-1 expression in a dose-dependent and time-dependent fashion in sensitive SW872 and LiSa-2 cells, modestly in HT1080 cells, but not in nelfinavir-insensitive FU-DDLS-1 and 293 cells without inducing adipocytic differentiation. Forced expression of sterol regulatory element binding protein-1 in inducible-SW872 cells led to the induction of proapoptotic and antiproliferative proteins, and consequent reduction of cellular proliferation. Our data indicate that nelfinavir represents a novel class of antiliposarcoma agent that acts by selectively upregulating sterol regulatory element binding protein-1 expression in liposarcomas. *Anti-Cancer Drugs* 17:891–903 © 2006 Lippincott Williams & Wilkins.

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Introduction

Liposarcomas are the second most common adult soft-tissue sarcoma [1]. They range from low-grade to highly malignant variants [2]. Liposarcomas are relatively insensitive to cytotoxic chemotherapy. Their common adipocyte origin provides an opportunity to investigate targeted approaches for all liposarcomas.

HIV protease inhibitors (PIs) prevent cleavage of gag and gag–pol precursors in HIV-infected cells, and thereby block the infectivity of nascent virions [3]. The PIs (saquinavir, ritonavir, indinavir, nelfinavir and amprenavir) are structurally related, but differ based upon the HIV aminoacid sequences recognized and cleaved [3]. Their use has fundamentally improved the HIV epidemic [4].

HIV PI use is linked to 'HIV protease-induced lipodystrophy syndrome', a clinical syndrome consisting of peripheral lipoatrophy and central fat accumulation associated with insulin resistance and hyperlipidemia [5]. Its pathophysiology has focused on dysregulation of the adipogenic transcription factors, peroxisome proliferator-activated receptor-γ (PPAR-γ) and sterol regulatory element binding protein-1 (SREBP-1). PPAR-γ is a nuclear receptor which forms a heterodimeric complex with the retinoid X receptor-γ and is a central regulator of adipocyte differentiation [6]. SREBP-1 is a member of the basic helix–loop–helix leucine zipper transcription factor family [7]. As the master transcriptional regulator of fatty acid and cholesterol synthesis, it promotes lipogenic gene expression, including PPAR-γ [7,8]. SREBP-1 and PPAR-γ cooperatively promote adipogenesis, and HIV PI-induced perturbations in their expression are suspected to result in the lipodystrophy phenotype.

Three SREBP isoforms exist, SREBP-2, SREBP-1a and SREBP-1c; the latter two are alternative splice products of a single SREBP-1 gene [9–12]. SREBP-1c is the primary isoform in white adipose tissue [7,13]. SREBPs

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are produced as inactive, membrane-bound precursors in the endoplasmic reticulum bound at their COOH-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 endoplasmic reticulum [16]. Insigs block the SCAP-mediated proteolytic processing of SREBP required for its activation [16]. During periods of cholesterol depletion, SCAP and Insig fail to interact, and the SREBP–SCAP complex is transported to the Golgi apparatus where it is processed in two sequential cleavage steps by Site-1 protease and Site-2 protease to release the transcriptionally active NH_2-terminus of SREBP [16–18]. SREBP is degraded by the ubiquitin/proteasome-dependent pathway [19]. Additionally, SREBPs are phosphorylated by the extracellular signal-regulated kinase, Jun N-terminal kinase and p38 signaling pathways [20–22]. These posttranslational changes provide tight control of SREBP-1 function.

HIV PIs can both suppress or induce preadipocyte differentiation [23–27]. Their effect on SREBP-1 expression is more consistent. Ritonavir induces preadipocyte differentiation, associated with a 3-fold increase in mature SREBP-1 expression [27]. In ritonavir-treated HIV patients, SREBP-1 protein levels were increased 2.6-fold in adipose tissue compared with seronegative controls despite reduced SREBP-1 mRNA levels [28]. These studies suggest that HIV PIs enhance SREBP-1 expression despite reduced transcription.

Despite the essential requirement for SREBP-1 in adipogenesis, it is surprising that adipose tissue mass and expression of adipogenic genes are unaffected in SREBP-1 null mice [29]. As SREBP-2 expression is increased in these mice, it may replace SREBP-1 function. In contrast, in SREBP-1c transgenic mice, adipose tissue mass is reduced to less than half of wild-type mice [30]. Additionally, insulin resistance, diabetes mellitus, fatty liver and hyperlipidemia develop [30]. These features are nearly identical to congenital generalized lipodystrophy, an autosomal recessive disorder in humans [31], and virtually indistinguishable from 'HIV protease-induced lipodystrophy syndrome'. This demonstrates a substantial link between SREBP-1 overexpression and congenital and acquired lipodystrophy syndromes. On the basis of these clinical and laboratory observations, we hypothesized that HIV PIs would inhibit liposarcoma growth by upregulating SREBP-1 expression.

Materials and methods

Drugs

HIV PIs amprenavir, nelfinavir, ritonavir, saquinavir and indinavir sulfate were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Rockville, Maryland, USA). Ten micromolar stock solutions were made of each HIV PI in dimethyl sulfoxide, except for indinavir (distilled water).

Cell lines

SW872 high-grade liposarcoma, 293 embryonic kidney and HT1080 fibrosarcoma cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). The FU-DDLS-1 dedifferentiated liposarcoma cell line was a kind gift from Drs Jun Nishio and Hiroshi Iwasaki (Department of Pathology, Fukuoka University School of Medicine, Fukuoka, Japan). The LiSa-2 poorly differentiated pleomorphic liposarcoma cell line was a kind gift from Drs Silke Brüederlein and Peter Möller (Institute of Pathology, University of Ulm, Ulm, Germany).

Plasmid DNA

pTet-On and TK-Hygromycin plasmids were purchased from Clontech (Palo Alto, California, USA). The nuclear active (na)-SREBP-1 expression vector (PUHD10-3-naSREBP-1) was kindly provided by Dr Haiyan Wang (University Medical Centre, Geneva, Switzerland) [32] and the pUHC13-3 luciferase reporter plasmid was provided by Dr Jiing-Kuan Yee (Beckman Research Institute, Duarte, California, USA) [33].

Clonogenic assays

SW872 cells were treated for 4 h with 2.5, 10 and 20 μmol/l of the HIV PIs, nelfinavir, ritonavir, indinavir amprenavir and saquinavir, or with vehicle alone. Following treatment, cells were washed in phosphate-buffered saline and collected. Cells (10^3) were plated in triplicate into 60-mm tissue culture plates. Colonies were grown for 7–10 days, stained and scored ($\geq$40 cells/colony). Clonogenic assays with nelfinavir only were also performed with HT1080 and 293 cells.

Proliferation assays

Cells were plated in 96-well microtiter plates (20 000 cells per well) and incubated overnight. Nelfinavir was added at the indicated concentrations (0, 2.5, 10 and 20 μmol/l) the next day. At the end of the incubation period (20 h at 37°C), cellular proliferation was determined by a tetrazolium dye assay (Promega, Madison, Wisconsin, USA). Values for the experimental conditions were normalized to a control value of 1 for each experiment. Assays were reported as the average of at least three separate experiments ± standard error of the mean (SEM).

Cell cycle assays

Cells (3×10^6) were treated with 10 μmol/l nelfinavir for 2, 8 and 24 h. At the end of the treatment period, the cells were trypsinized, collected and resuspended in 1 ml of modified Krishnan Buffer (0.1% sodium citrate, 0.3% Nonidet P-40 [NP-40], 0.05 mg/ml propidium iodide and 0.02 mg/ml DNase-free RNase A) [34], vortexed for 10 s

and incubated at 4°C for 30 min. The cells were pelleted, resuspended in fresh modified Krishnan Buffer and passed through a 27-gauge needle. The resulting single-cell suspension was analyzed on a four-laser MoFlo MLS Flow Cytometer (DakoCytomation, Fort Collins, Colorado, USA).

Apoptosis assays

Cells (3×10^6) were treated with 0, 2.5, 10 and 20 μmol/l nelfinavir for 24 h, trypsinized, and collected. The cells were washed once with media containing serum, once with phosphate-buffered saline, and resuspended in 400 μl of 1 × assay buffer containing 1 μg/ml annexin V fluorescein isothiocyanate and 1 μg/ml propidium iodide (Santa Cruz Biotechnology, Santa Cruz, California USA). Samples were analyzed using a single laser emitting light at 488 nm for fluorescein isothiocyanate on the MoFlo MLS Flow Cytometer.

Western blot analysis

Cells (2×10^7) cells were treated with 0, 2.5, 10 and 20 μmol/l nelfinavir for the indicated time, trypsinized, and collected for protein extraction. Western blot analysis was performed for the expression of: SREBP-1 (H-160), PPAR-γ (E-8), p21 (C-19), Fas (C-20), Bax (N-20), proliferating cell nuclear antigen (PCNA) (FL-261), β-actin (I-19), p53 (FL-393) and glyceraldehyde-3-phosphate dehydrogenase (V-18) (Santa Cruz Biotechnology) as previously described [32].

Oil red O staining

Five days after incubation with nelfinavir or dimethyl sulfoxide vehicle only, the cells were fixed in 10% formalin in isotonic buffer for 2 h, washed with 60% isopropanol, and stained with 0.6% (w/v) oil red O solution (60% isopropanol, 40% water) for 10 min. Oil red O was removed and immediately washed 4 times with water to remove unbound dye. Pictures were then taken of the stained cells in their wells.

Quantitative real-time reverse transcription-polymerase chain reaction

The sequence of SREBP-1c is identical to that of SREBP-1a except for a shortened NH_2-terminal acidic domain (24 amino acids in SREBP-1c versus 42 amino acids in SREBP-1a) [9,10]. Forward and reverse primers 3′ of this sequence were selected to detect an amplicon that reflected expression of both isoforms. The sequences of primers and probes are given Table 1.

The probe concentration was 0.3 μmol/l for each gene. The primer concentration was 0.4 μmol/l for SREBP-1 detection and 0.8 μmol/l for β-actin detection. Polymerase chain reaction amplification was performed as previously reported [35].

Table 1

Gene	Primer sequence
β-actin	
Sense	5′-TGAGCGCGGCTACAGCTT-3′
Antisense	5′-CCTTAATGTCACACACGATT-3′
Probe	TET-5′ACCACCACGGCCGAGCGG-3′TAMRA
SREBP-1	
Sense	5′-CGACATCGAAGACATGCTTCA-3′
Antisense	5′-AGGCTTCAAGAGAGGAGCTCAA-3′
Probe	5′-/56FAM/CTGGCCTATTTGACCCACCCTATGCTG/36-TAMTph/-3′

Establishment of SW872 cells permitting inducible expression of nuclear active sterol regulatory element binding protein-1c

Stable pTet-On-expressing SW872 cells were created by electroporation method. Neomycin-resistant pTet-On SW872 clones were transfected with the luciferase reporter plasmid pUHC13-3 by the calcium phosphate method [33] to test for doxycycline-dependent transactivation of luciferase activity. One clonal line that exhibited very high doxycycline-inducible luciferase activity was chosen for cotransfection with the naSREBP-1 and hygromycin-resistance (TK-Hygromycin) plasmids by the electroporation method. Twenty hygromycin-resistant colonies were screened for optimal doxycycline-inducible expression of SREBP-1 by Western blot analysis.

Statistics

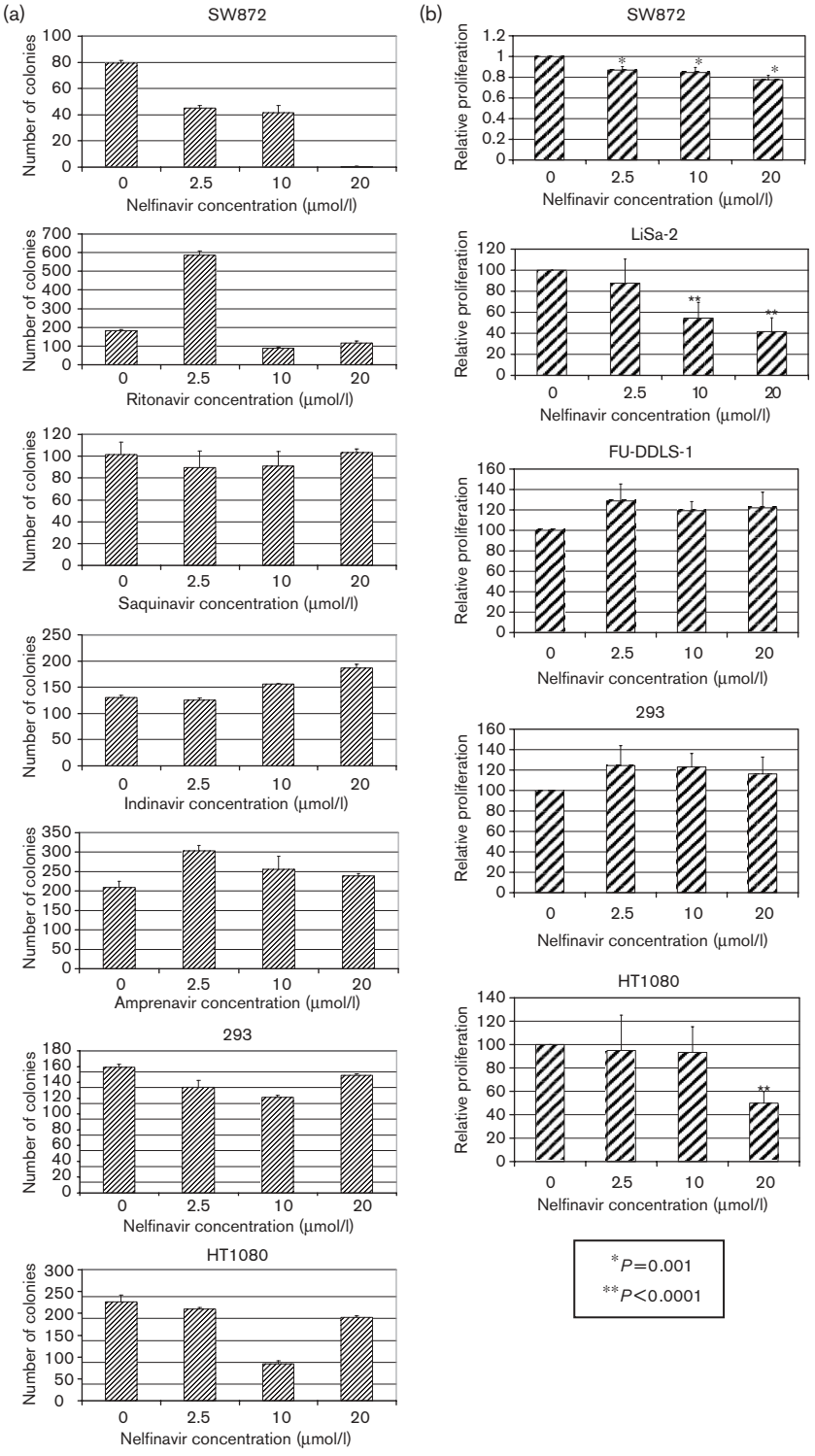
A two-way analysis of variance was performed for each cell line to determine significance of nelfinavir dose on cellular proliferation. Main effects were nelfinavir dose level and experiment. Global statistical significance of nelfinavir on expression level was determined using an *F*-statistic. A Duncan multiple range test ($P = 0.05$) was used to determine statistical significance between dose levels.

Results

Nelfinavir inhibits liposarcoma clonogenicity and proliferation

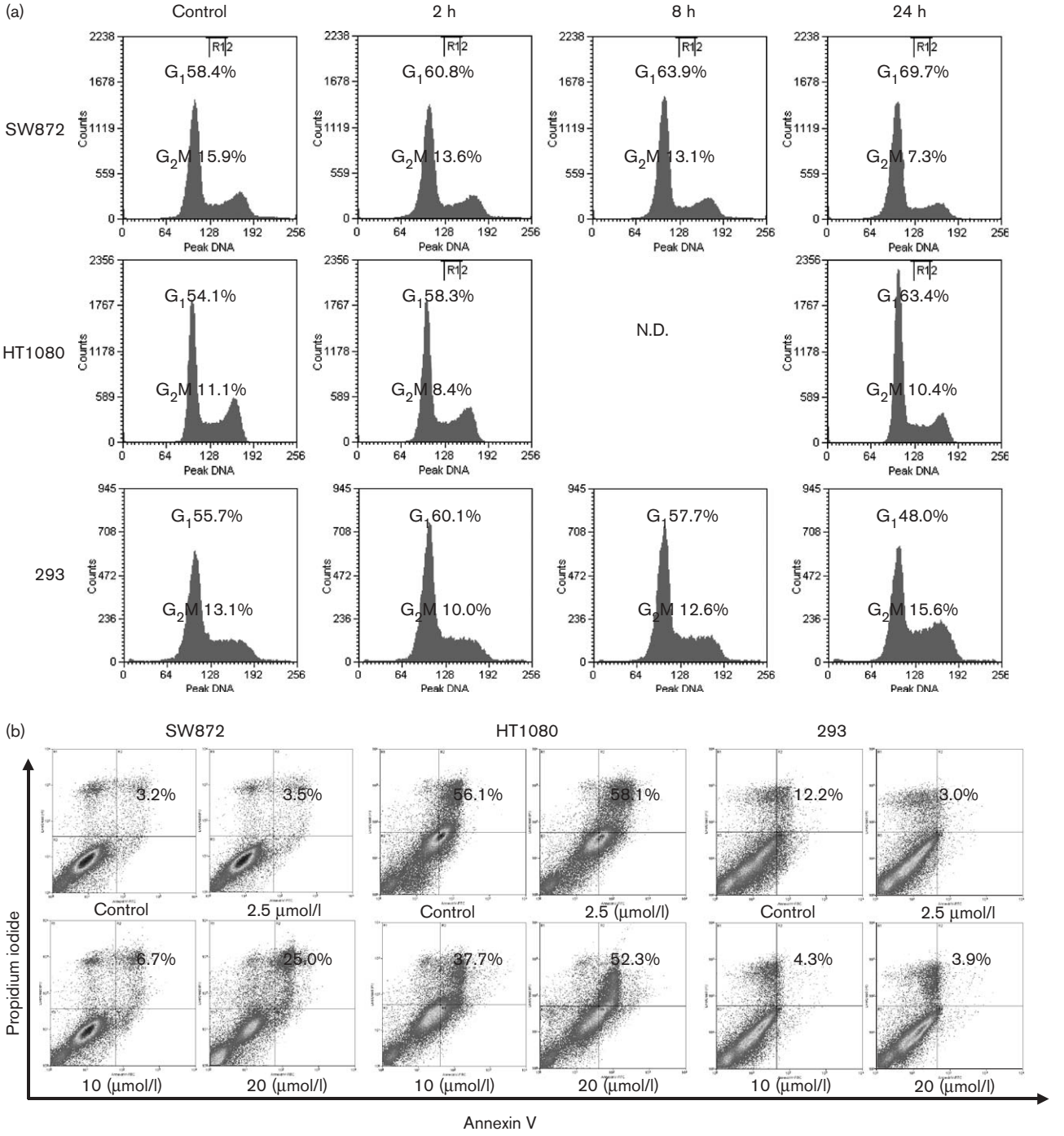
To determine whether HIV PIs would affect the capacity for liposarcoma cells to form colonies, nelfinavir, ritonavir, saquinavir, indinavir and amprenavir were assayed for inhibition of SW872 clonogenicity. At the approved oral dose, the C_{max} for each HIV PI is nelfinavir 6.97 μmol/l, ritonavir 15.53 μmol/l, saquinavir 3.67 μmol/l and indinavir 12.6 μmol/l [3]. The concentrations employed for these experiments were intended to bracket the biologically relevant concentrations of the HIV PIs. Nelfinavir exhibited the most potent inhibition of clonogenicity (Fig. 1a) and all further assays were performed with nelfinavir only. A tetrazolium dye-based assay was then used to investigate whether nelfinavir would inhibit liposarcoma proliferation. Figure 1(b) demonstrates that

Fig. 1



(a) Nelfinavir inhibits SW872 liposarcoma clonogenicity more potently than other HIV protease inhibitor (PIs) without inhibiting 293 embryonic kidney nor HT1080 fibrosarcoma clonogenicity. SW872, 293 and HT1080 cells were treated for 4 h with the indicated concentration of HIV PI, and 1000-treated cells were plated into 60-mm tissue culture plates in triplicate. Data are presented as the mean number of colonies ± standard error of the mean (SEM). (b) Nelfinavir inhibits proliferation of SW872 and LiSa-2 without affecting the proliferation of FU-DDLS-1 liposarcoma cells. Nelfinavir does not inhibit 293 proliferation, but at the highest concentration (20 μmol/l), it does inhibit HT1080 proliferation. The indicated cell lines were plated in 96-well microtiter plates and treated with the indicated concentrations of nelfinavir for 20 h, and cellular proliferation was determined by a tetrazolium dye assay. Light absorbance was measured at 590 nm. Data are reported as the mean of at least three separate experiments relative to control ± SEM. *P=0.001; **P<0.001.

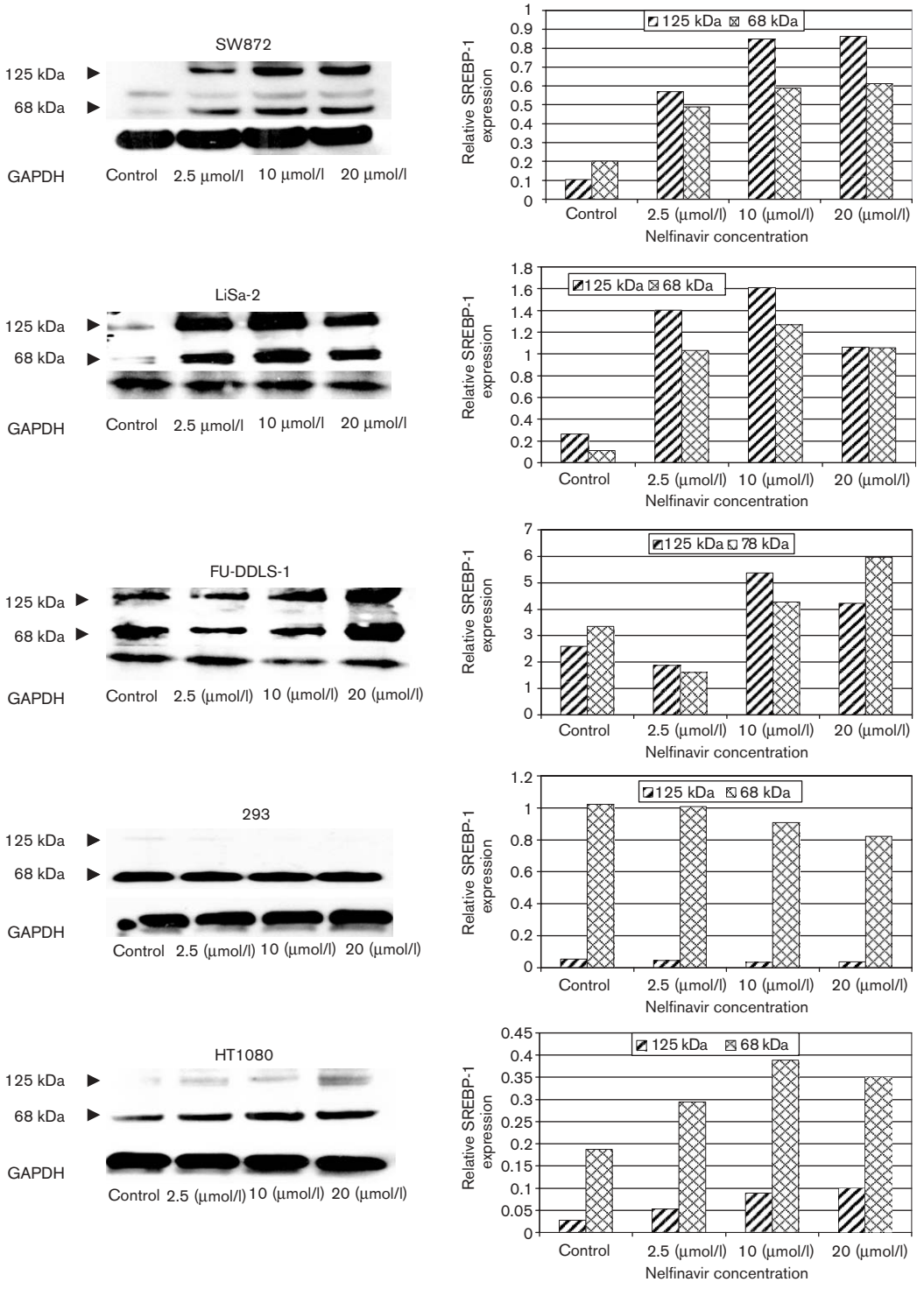
Fig. 2



(a) Nelfinavir induces a time-dependent G_1 cell cycle arrest in SW872 and HT1080 cells, but not in 293 cells. The indicated cell lines were treated with 10 μmol/l nelfinavir for the indicated time period and subjected to fluorescence-activated cell sorting analysis with propidium iodide. (b) Nelfinavir induces dose-dependent apoptosis in SW872 cells, but not in HT1080 nor 293 cells. Cells were treated with 2.5, 10 and 20 μmol/l nelfinavir, or vehicle (dimethyl sulfoxide) for 24 h before FACS analysis for annexin V expression.

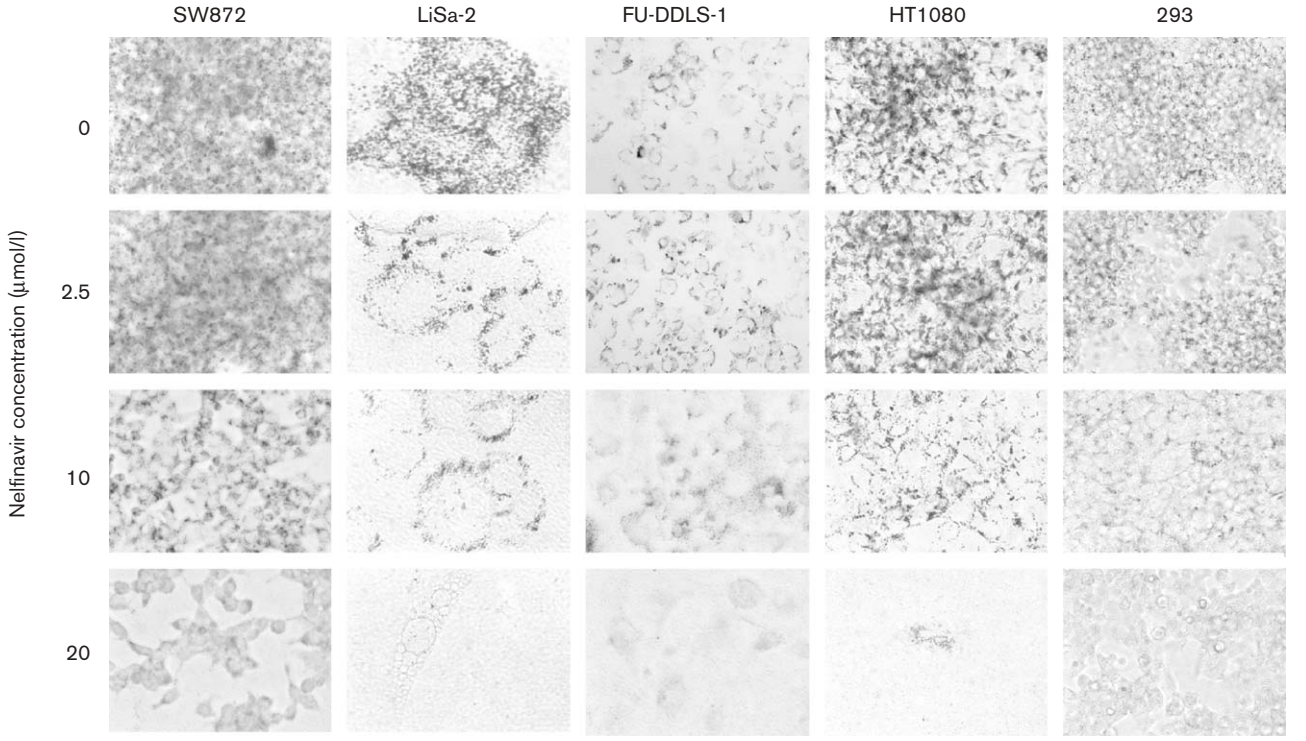
nelfinavir significantly inhibited SW872 ($P = 0.001$ for all nelfinavir concentrations) and LiSa-2 liposarcoma proliferation ($P < 0.0001$ for 10 and 20 μmol/l nelfinavir) in a dose-dependent fashion, whereas it had no effect upon FU-DDLS-1 liposarcoma ($P = 0.16$) nor 293 proliferation ($P = 0.28$). At the highest concentration of nelfinavir (20 μmol/l), HT1080 proliferation was significantly inhibited ($P < 0.0001$).

Fig. 3



Nelfinavir leads to the upregulation of precursor (125 kDa) and mature (68 kDa) forms of sterol regulatory element binding protein (SREBP)-1 in SW872 and LiSa-2, but not in FU-DDLS-1 liposarcoma nor 293 cells. Nelfinavir modestly induces expression of SREBP-1 in HT1080 cells. Cells were treated with the indicated concentrations of nelfinavir for 24 h before protein collection for Western analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were examined as a loading control.

Fig. 4



Nelfinavir does not induce differentiation in liposarcoma or nonliposarcoma cells. Cells were treated with 2.5, 10 and 20 μmol/l nelfinavir or vehicle (dimethyl sulfoxide) for 5 days before oil red O staining.

Nelfinavir induces a G_1 cell cycle block

To determine whether nelfinavir would affect cell cycling, cell cycle analysis was performed by fluorescence-activated cell sorting analysis for each cell line after treatment with 10 μmol/l nelfinavir for the designated time. Consistent with the proliferation results, nelfinavir induced a time-dependent accumulation of cells in G_1 phase in SW872 (58.4–69.7%) and HT1080 cells (54.1–63.4%) consistent with a G_1 cell cycle block, whereas it had no effect on the kinetics of 293 cells (Fig. 2a). These results demonstrate that a nelfinavir-induced G_1 cell cycle block contributes to the inhibition of clonogenicity and proliferation observed for the sensitive liposarcoma cell lines, and inhibition of proliferation at the highest concentration in HT1080 cells.

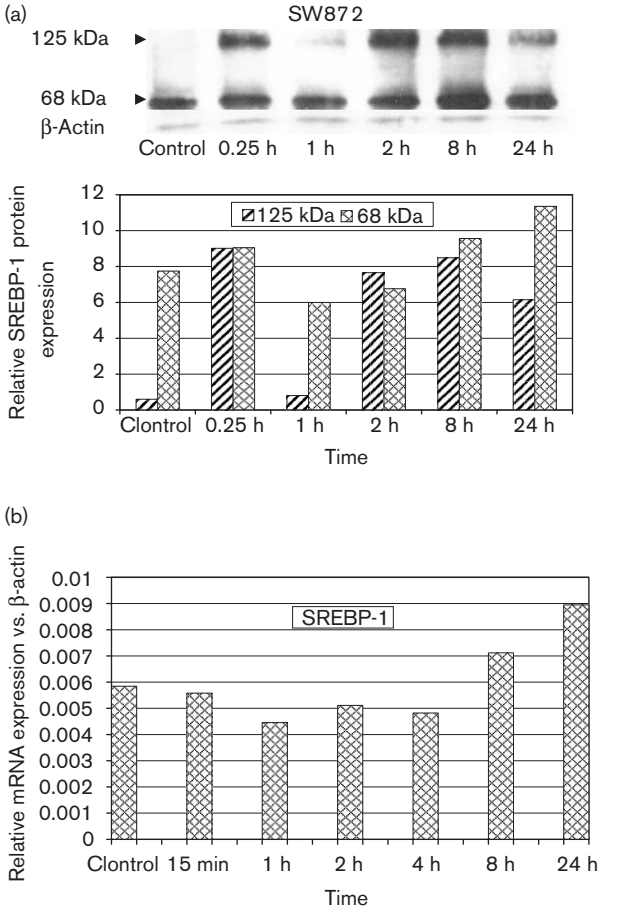
Nelfinavir selectively induces liposarcoma apoptosis

To determine whether nelfinavir would induce apoptosis in liposarcoma cells, fluorescence-activated cell sorting analysis for annexin V expression was performed after 24 h incubation with nelfinavir. As shown in Fig. 2(b), nelfinavir induced dose-dependent apoptosis specifically in SW872 cells. The fraction of apoptotic cells increased from 3.2% at baseline to 25.0% in the nelfinavir-treated SW872 cells. In contrast, there was no increase in the fraction of apoptotic cells in the 293 nor HT1080 cell lines. The high rate of spontaneous apoptosis in HT1080 cells (56.1%) is likely related to the marked proliferative rate of these cells in contrast to the lower proliferative SW872 and 293 cells. Consequently, it is possible this high proliferative rate may partially obfuscate the actual incidence of nelfinavir-induced apoptosis in these cells. Regardless, these results demonstrate that nelfinavir selectively induces apoptosis in SW872 liposarcoma cells.

Nelfinavir leads to sterol regulatory element binding protein-1 upregulation in the absence of proliferator-activated receptor-γ upregulation in sensitive liposarcoma cells

Expression of SREBP-1 was determined by Western blot analysis in the liposarcoma and nonliposarcoma cell lines after 24 h nelfinavir treatment. There was a dose-dependent accumulation of both the unprocessed, 125-kDa, and the processed, transcriptionally active 68-kDa forms of SREBP-1 in the nelfinavir-sensitive SW872 and LiSa-2 cells, whereas there was minimal change in SREBP-1 expression in the nelfinavir-insensitive FU-DDLS-1 cells (Fig. 3). Interestingly, the processed form of SREBP-1 was detectable in the untreated

Fig. 5



(a) Nelfinavir leads to rapid upregulation of precursor (125 kDa) and mature (68 kDa) sterol regulatory element binding protein (SREBP)-1 protein expression in SW872 cells. Cells were treated with 10 μmol/l nelfinavir for the indicated time period before protein collection for Western analysis. β-Actin levels were examined as a loading control. (b) Nelfinavir modestly induces delayed SREBP-1 gene expression relative to β-actin gene expression in SW872 cells. Cells were treated with 10 μmol/l nelfinavir for the indicated time period before RNA collection for quantitative reverse transcription-polymerase chain reaction of SREBP-1 and β-actin gene expression.

nonadipogenic cell lines; however, nelfinavir did not alter its expression in 293 cells, whereas it did moderately increase in HT1080 cells.

Demetri *et al.* [36] reported induction of terminal adipocytic differentiation in a clinical trial of the PPAR-γ ligand troglitazone in patients with liposarcoma. Importantly, PPAR-γ is a target gene of and regulated by SREBP-1 [7,8]. Consequently, Western analysis for PPAR-γ expression was performed in SW872 cells; however, PPAR-γ expression was undetectable in both control and nelfinavir-treated cells demonstrating that the effects of nelfinavir are not mediated through PPAR-γ (data not shown). Consistent with these results was the absence of evidence of adipocyte differentiation by oil red O staining of the liposarcoma cell lines SW872, LiSa-2 and FU-DDLS-1, and the nonliposarcoma cell lines HT1080 and 293 (Fig. 4).

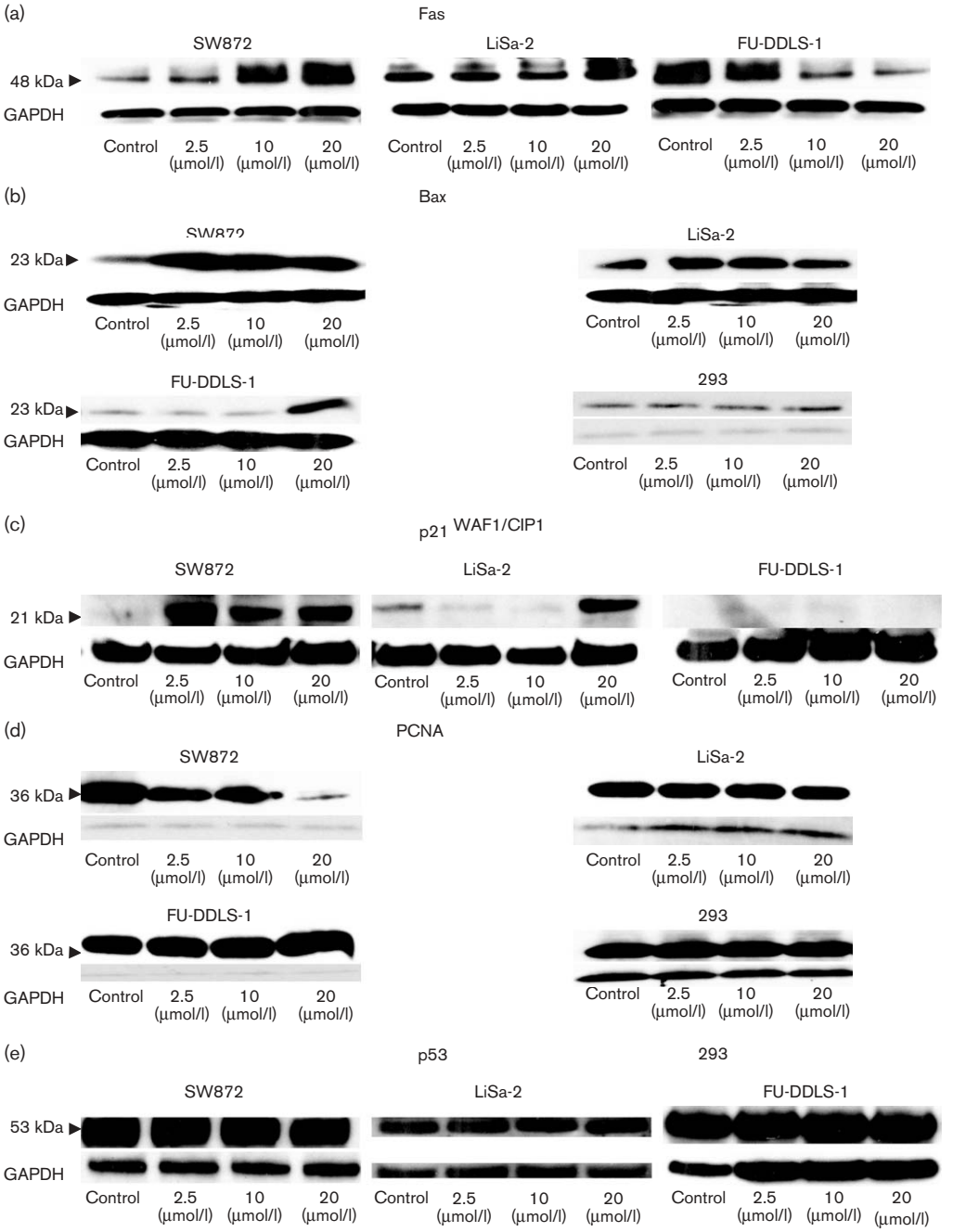
Nelfinavir rapidly upregulates sterol regulatory element binding protein-1 protein expression without inducing sterol regulatory element binding protein-1 gene expression

To examine the kinetics of SREBP-1 induction, SW872 cells were treated with 10 μmol/l nelfinavir for 0.25, 1, 2 and 24 h before Western analysis and reverse transcription-polymerase chain reaction. As shown in Fig. 5(a), nelfinavir upregulated SREBP-1 protein expression within 0.25 h, reaching maximal levels by 8 h and declining by 24 h. As demonstrated in Fig. 5(b), SREBP-1 mRNA expression was only modestly induced by nelfinavir at 8 and 24 h. These results suggest that nelfinavir mediates prompt regulation of SREBP-1 primarily via posttranscriptional means.

Nelfinavir increases expression of $p21^{WAF1/CIP1}$, Fas and Bax, and reduces expression of proliferating cell nuclear antigen in sensitive liposarcoma cells without affecting p53

To investigate the signaling pathways responsible for the G_1 cell cycle block and apoptosis observed in the cellular assays, Western analyses for Fas, Bax, $p21^{WAF1/CIP1}$, p53 and PCNA expression were performed in nelfinavir-sensitive SW872 and LiSa-2, and nelfinavir-insensitive FU-DDLS-1 liposarcoma cells. As shown in Fig. 6(a), nelfinavir induced expression of Fas in SW872 cells at all concentrations, at 20 μmol/l in LiSa-2 cells, but not at all in insensitive FU-DDLS-1 cells. As shown in Fig. 6(b), Bax expression is maximally induced between 2.5 and 10 μmol/l nelfinavir in SW872 and LiSa-2 cells, whereas only at 20 μmol/l in FU-DDLS-1 cells. In contrast to liposarcoma cells, Bax expression is unaltered in 293 cells. As shown in Fig. 6(c), $p21^{WAF1/CIP1}$ expression is upregulated in SW872 cells at all concentrations of nelfinavir, at 20 μmol/l in LiSa-2 cells, whereas it is unaltered in FU-DDLS-1 cells. To examine the consequence of enhanced $p21^{WAF1/CIP1}$ expression, PCNA expression in nelfinavir-treated cells was examined (Fig. 6d). Nelfinavir reduced expression of PCNA in SW872 and to a lesser degree in LiSa-2 cells, whereas it had no effect in 293 nor FU-DDLS-1 cells. Finally, to determine whether $p21^{WAF1/CIP1}$ expression is upregulated in a p53-dependent manner, expression of p53 was examined in the liposarcoma cell lines after treatment with nelfinavir. Figure 6(e) demonstrates that expression of p53 is unaltered in any of the liposarcoma cell lines treated with nelfinavir, demonstrating that nelfinavir-mediated $p21^{WAF1/CIP1}$ induction is independent of p53.

Fig. 6



(a) Nelfinavir induces expression of Fas in a dose-dependent fashion in sensitive SW872 and at the highest concentration in LiSa-2 cell lines, but reduces Fas expression in insensitive FU-DDLS-1 cells. Cells were treated with the indicated concentrations of nelfinavir for 24 h before protein collection for Western analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were examined as a loading control. (b) Nelfinavir upregulates the expression of Bax in sensitive SW872 and LiSa-2 cell lines at low concentrations (2.5 μmol/l), whereas in insensitive FU-DDLS-1 liposarcoma cells, Bax is upregulated only at the highest concentration of nelfinavir (20 μmol/l). Bax is not upregulated in control 293 cells. Cells were treated with the indicated concentrations of nelfinavir for 24 h before protein collection for Western analysis. GAPDH levels were examined as a loading control. (c) Nelfinavir upregulates expression of $p21^{WAF1/CIP1}$ in sensitive SW872 cells at all concentrations of nelfinavir and at the highest concentration of nelfinavir in LiSa-2 cell lines (20 μmol/l). In contrast, nelfinavir does not induce $p21^{WAF1/CIP1}$ expression in insensitive FU-DDLS-1 cells. Cells were treated with the indicated concentrations of nelfinavir for 24 h before protein collection for Western analysis. GAPDH levels were examined as a loading control. (d) Nelfinavir leads to reduced expression of proliferating cell nuclear antigen (PCNA) in sensitive SW872 and LiSa-2 cells, but not in insensitive FUDDLS-1 nor 293 cells. Cells were treated with the indicated concentrations of nelfinavir for 24 h before protein collection for Western analysis. (e) Nelfinavir does not alter p53 expression in liposarcoma cells. SW872, LiSa-2 and FU-DDLS-1 cells were treated with the indicated concentrations of nelfinavir for 24 h before protein collection for Western analysis. GAPDH levels were examined as a loading control.

Forced expression of nuclear active sterol regulatory element binding protein-1c in inducible SW872 cells increases expression of Fas and $p21^{WAF1/CIP1}$, reduces expression of proliferating cell nuclear antigen, and reduces proliferation

To determine whether forced expression of nuclear active SREBP-1 (naSREBP-1; N-terminal 1–403 amino acids) [7] in SW872 cells would recapitulate the nelfinavir-generated phenotype, inducible-naSREBP-1c SW872 cells were created. Two clones [naSREBP-1c-(12) and naSREBP-1c-(9)] showed inducible expression of the precursor and mature forms of SREBP-1 after doxycycline induction (Fig. 7a). Interestingly, even in the absence of doxycycline, the transfected naSREBP-1c (around 50 kDa) could be detected indicating uninduced leakage of the Tet-On-inducible system. Importantly, sterol regulatory element (SRE) binding sites have been identified in the promoter region of the SREBP-1c gene [37]. Consequently, SREBP-1 mRNA can increase in response to nuclear SREBPs, creating a feed-forward activation [36]. This explains the presence of the 125- and 68-kDa forms of SREBP-1 in the uninduced cells. Doxycycline-treated parental, nontransfected SW872 cells demonstrated no significant change of expression of SREBP-1, which served as an important negative control (Fig. 7b).

Western analyses were performed on naSREBP-1c-(12) and naSREBP-1c-(9) for Fas, $p21^{WAF1/CIP1}$ and PCNA expression after doxycycline induction. As shown in Fig. 7(c), doxycyline dose-dependent induction of Fas and $p21^{WAF1/CIP1}$ expression was observed in both clones, whereas PCNA expression was reduced; however, naSREBP-1c-(12) demonstrated a greater response to doxycycline induction. Consequently, cellular proliferation was analyzed in naSREBP-1c-(12). Figure 7(d) demonstrates that doxycycline induction results in a significantly reduced proliferative phenotype for all doxycycline concentrations relative to control ($P < 0.0001$). These results demonstrate that forced expression of SREBP-1 in SW872 cells in the absence of nelfinavir produces an analogous phenotype to that generated by nelfinavir.

Discussion

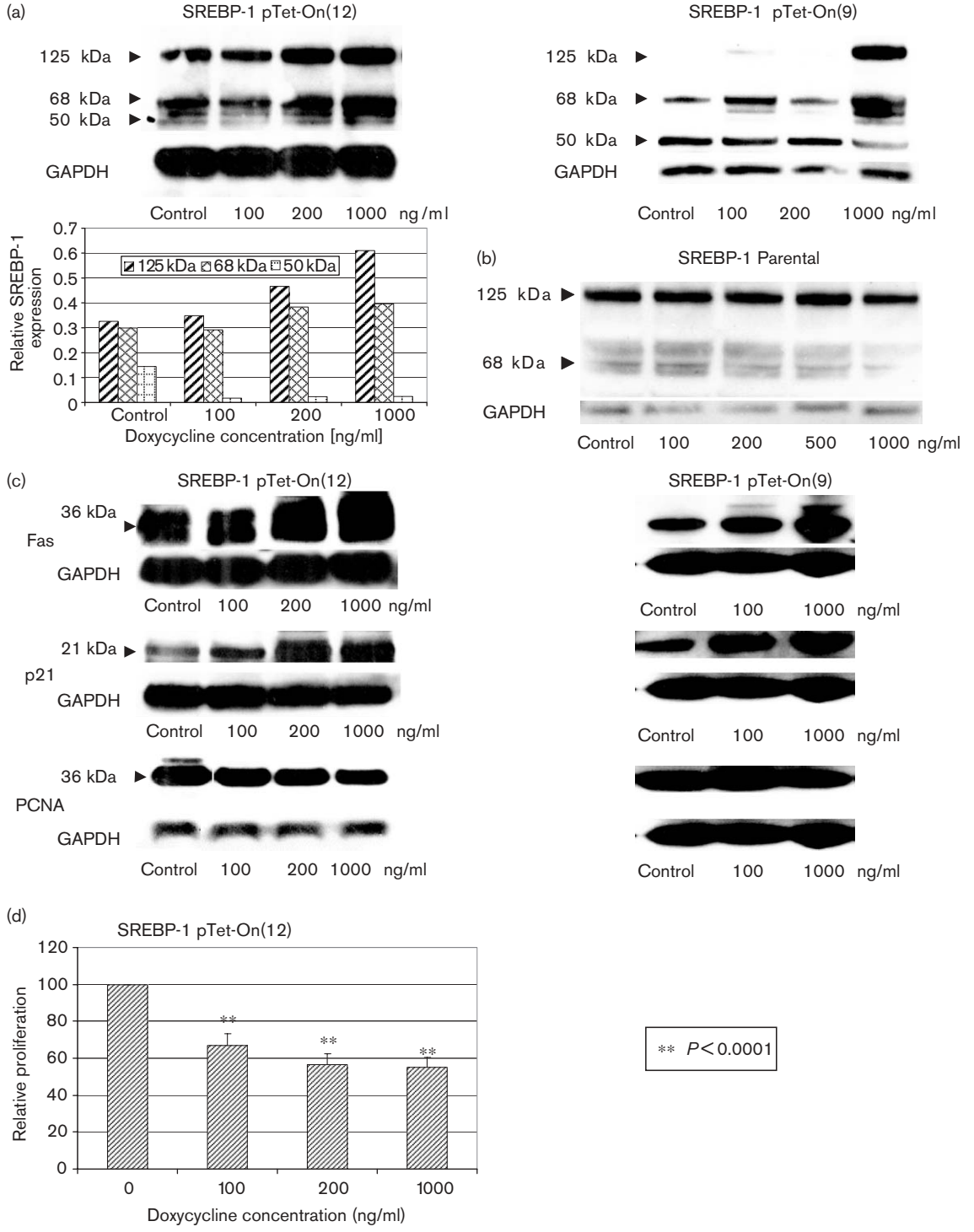
In this study, we show that nelfinavir upregulates expression of SREBP-1 in two sensitive liposarcoma cell lines (SW872 and LiSa-2), modestly in HT1080 cells, but not in insensitive FU-DDLS-1 liposarcoma nor in 293 cells. We also demonstrate that nelfinavir induces expression of proapoptotic and antiproliferative proteins, which correlates with induction of apoptosis and G_1 cell cycle block. Finally, our inducible-SREBP-1 liposarcoma cell line allows us to conclude that the induction of apoptosis and reduced proliferation we observed is a direct consequence of SREBP-1 upregulation. Our results highlight diametric signaling pathways through SREBP-1; whereas physiologic expression of SREBP-1 promotes adipogenesis, pharmacologic expression of SREBP-1 promotes liposarcoma apoptosis.

'HIV protease-induced lipodystrophy syndrome' was first reported in 1998 [38]. Preclinical and clinical studies have demonstrated a persuasive link between HIV PI use and alterations in SREBP-1 expression [23–27], which was the stimulus for the present study, in which we assessed the effect of HIV PIs on liposarcoma and nonliposarcoma cell lines. Our preliminary studies evaluating HIV PI effect on liposarcoma clonogenicity demonstrated a substantial inhibitory effect of nelfinavir compared with the other HIV PIs. This is unlikely to be a class effect; rather, it is likely limited to nelfinavir. Significantly, only nelfinavir and amprenavir are nonpeptidomimetic PIs [3]. Despite their structural and functional similarity, amprenavir had no effect upon SW872 clonogenicity. Additionally, the nelfinavir effect was relatively selective, because nonliposarcoma cell lines, 293 and HT1080 were generally unaffected. Further, cellular assays for proliferation, cell cycle arrest and apoptosis demonstrated potential mechanisms for the clonogenicity results. These initial results provided an impetus to investigate the signaling pathways initiated by nelfinavir.

We began by investigating whether SREBP-1 expression was altered. Precursor and processed forms of SREBP-1 were consistently upregulated by nelfinavir in sensitive SW872 and LiSa-2 cells, whereas SREBP-1 expression was unaffected in nelfinavir-insensitive FU-DDLS-1 and 293 cells. Mature SREBP-1 expression was only modestly induced in HT1080 cells. The kinetics of SREBP-1 protein upregulation were rapid (Fig. 4a), but did not correlate with SREBP-1 gene expression (Fig. 4b). This raises the hypothesis that nelfinavir primarily affects posttranslational modification of SREBP-1. We are actively investigating this possibility. Importantly, although nelfinavir upregulated SREBP-1 expression, it did not affect PPAR-γ expression in SW872 cells, a direct target of SREBP-1 during adipogenesis [7,8]. This result suggests an alternative SREBP-1 signaling pathway unrelated to adipogenesis, and consistent with the antiproliferative and proapoptotic properties observed with nelfinavir.

As inducible expression of na-SREBP-1c has been shown to elicit rat insulinoma growth arrest and apoptosis via upregulation of Fas, Bax and $p21^{WAF1/CIP1}$, SREBP-1c has been proposed to act as a proapoptotic gene in pancreatic β-islet cells [32]. Consequently, these parameters were investigated here in nelfinavir-treated cells. Our results confirm that nelfinavir increased expression of $p21^{WAF1/CIP1}$, Fas and Bax, and reduced expression of PCNA in sensitive SW872 and LiSa-2 cells (Fig. 5a–d). The induction of $p21^{WAF1/CIP1}$ was independent of p53,

Fig. 7



(a) Doxycycline-dependent forced expression of nuclear active sterol regulatory element binding protein (naSREBP)-1c in SW872 cells leads to dose-dependent increased expression of mature and processed SREBP-1. Note Tet-On system leakage by detection of around 50-kDa na-SREBP-1c in uninduced cells. Two Tet-On-inducible na-SREBP-1c clones [naSREBP-1c-(12), and naSREBP-1c-(9)] were treated with the indicated concentrations of doxycycline (ng/ml) for 24 h before protein collection for Western analysis. (b) Doxycycline does not induce expression of parental SW872 cells. SW872 cells were treated with the indicated concentrations of doxycycline (ng/ml) for 24 h before protein collection for Western analysis. (c) Doxycycline-dependent forced expression of na-SREBP-1c in SW872 cells leads to dose-dependent increased expression of Fas and $p21^{WAF1/CIP1}$, and reduced expression of proliferating cell nuclear antigen (PCNA). Tet-On-inducible na-SREBP-1c clones, naSREBP-1c-(12) and naSREBP-1c-(9), were treated with the indicated concentrations of doxycycline (ng/ml) for 24 h before protein collection for Western analysis. (d) Doxycycline-dependent forced expression of na-SREBP-1c in SW872 cells leads to a dose-dependent reduction in proliferation. Tet-On-inducible na-SREBP-1c-(12) cells were treated with the indicated concentrations of doxycycline (ng/ml) for 24 h before assaying for proliferation by a tetrazolium dye assay. Light absorbance was measured at 590 nm. Data are reported as the mean of at least three separate experiments relative to control ± standard error of the mean. $^{**}P < 0.0001$.

as p53 expression was unaltered by nelfinavir treatment (Fig. 5e). Significantly, FU-DDLS-1 cells served as an important control. Despite its liposarcoma origin, nelfinavir failed to upregulate SREBP-1 and reduce proliferation in FU-DDLS-1 cells. Accordingly, it also failed to increase expression of $p21^{WAF1/CIP1}$, Fas and Bax, and reduce expression of PCNA in this cell line. Finally, our Tet-On-inducible system demonstrated that conditional expression of na-SREBP-1c in SW872 cells induced Fas and Bax expression, as well as $p21^{WAF1/CIP1}$, and consequently reduced PCNA expression and cellular proliferation (Fig. 6a–c). These findings are identical to the phenotype of nelfinavir-treated cells.

In summary, this study demonstrates that nelfinavir-induced SREBP-1 upregulation leads to an alternative signaling pathway generating an antiproliferative and proapoptotic phenotype relatively selectively in sensitive liposarcoma cells. These results suggest that nelfinavir represents a novel class of antiliposarcoma agents that act by upregulating SREBP-1 expression in liposarcomas. Exploiting this unique property may have therapeutic implications.

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