Cancer Investigation, 26:118–127, 2008
ISSN: 0735-7907 print / 1532-4192 online
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DOI: 10.1080/07357900701522612

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ORIGINAL ARTICLE
Cellular and Molecular Biology

The Synthetic Triterpenoid CDDO-Im Inhibits Fatty Acid Synthase Expression and Has Antiproliferative and Proapoptotic Effects in Human Liposarcoma Cells

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ABSTRACT

Liposarcomas constitute a rare group of tumors of mesenchymal origin that are often poorly responsive to therapy. This study characterizes a novel human liposarcoma cell line (LiSa-2) and defines the mechanism of its response to a synthetic triterpenoid. Fatty acid synthase (FAS) is a key enzyme of de-novo fatty acid synthesis and is highly expressed in both human liposarcoma tissue specimens and LiSa-2 cells. Treatment of the LiSa-2 cell line with the synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic imidazolide (CDDO-Im) markedly inhibited FAS mRNA expression, FAS protein production and FAS gene promoter activity. As expected, fatty acid synthesis was down regulated, but there was no effect on cellular fatty acid uptake or glycerol-3-phosphate synthesis suggesting a selective inhibition of endogenous fatty acid synthesis. Importantly, CDDO-Im produced a dose-dependent apoptotic effect in the LiSa-2 cell line, and simultaneous treatment with CDDO-Im and the fatty acid synthase inhibitor Cerulenin produced a synergistic cytotoxic effect. Thus, CDDO-Im and Cerulenin act at different loci to inhibit long chain fatty acid synthesis in liposarcoma cells. This study's demonstration of CDDO-Im inhibition of FAS and Spot 14 (S14) expression is the first report of triterpenoid compounds affecting the fatty acid synthesis pathway. The observed dependence of liposarcomas on lipogenesis to support their growth and survival provides a novel approach to the treatment of liposarcomas with agents that target fatty acid production.

Keywords: Liposarcoma, Fatty acid synthase, Spot-14, Lipogenic, Triterpenoid, CDDO
We thank the following individuals for their contributions to this study: Harker Rhodes (Dartmouth Medical School) for completion of immunohistochemical staining procedures, Michael Sporn (Dartmouth Medical School) for supplying synthetic triterpenoids, Johannes Swinnen (Catholic University of Leuven, Belgium) for the FAS promoter vector, and Martin Wabitsch (University of Ulm, Germany) for the LiSa-2 cell line. This study was supported in part by the Norris Cotton Cancer Center Prouty Grant (to Burton Eisenberg).
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INTRODUCTION

Liposarcoma (LS) is a relatively rare tumor with an incidence estimated at 5000 cases yearly in the United States (1). Large tumors, particularly in the retroperitoneum, have a high recurrence rate and respond poorly to current therapies (2). The morphological and molecular resemblance of LS to mature adipocytes is compatible with the theory that liposarcomas arise from an adipogenic precursor cell. In fact, global gene expression array analysis of a panel of LS revealed that the majority of up regulated genes in LS are related to fatty acid metabolism (3). However, the lack of suitable *in vitro* models has limited the elucidation of LS pathobiology and experimental therapeutic discovery. The immortalized LiSa-2 cell line was cloned from a human pleomorphic LS and expresses several adipocyte-specific genes associated with lipid metabolism (4). LiSa-2 cells

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differentiate in response to adipogenic stimuli including insulin, thyroid hormone, and hydrocortisone, as is the case for normal preadipocytes (4, 5). LiSa-2 cells therefore provide an excellent *in vitro* model for further evaluation of LS pathobiology and identification of the molecular changes induced by pharmacological intervention.

The apparent reliance of LS, a mesenchymal derived tumor, on adipogenic genes may provide a unique therapeutic opportunity, as this malignancy is likely to conform to the defining characteristics of a "lipogenic" tumor. Lipogenic tumors not only exhibit high fatty acid synthase (FAS) expression, but also demonstrate a dependence on fatty acid synthesis for proliferation and survival (6). Lipogenic characteristics have been described in several different tumor types including breast, lung, colon, prostate and ovary (7, 8). FAS is a multifunctional enzyme, which plays a key role in the synthesis of palmitate from malonyl-CoA. Spot 14 (S14) is a nuclear protein associated with fatty acid synthesis that is highly expressed in lipogenic tumors (6, 9). In our initial evaluation of the LiSa-2 cell line, we noted high levels of FAS and S14 mRNAs, compatible with the lipogenic phenotype, and this prompted in-depth analysis of these genes.

Synthetic triterpenoids are a novel class of drugs designed from the naturally occurring triterpenoids oleanolic and ursolic acid. These compounds have a variety of actions in various cell lines, including anti-proliferative, anti-inflammatory and differentiating effects (10–13). Their exact mode of action has not been entirely elucidated and may be cell type specific. Some of these effects, particularly those of differentiation, are mediated through the PPAR-γ receptor (peroxisome proliferator-activated receptor-γ) (14–16). Since PPAR-γ has relevance to the maintenance of the malignant phenotype, synthetic triterpenoids may be useful in cancer therapy, and presently are in phase I clinical testing (11, 13). Although previous attempts to promote differentiation of human LS met with only minimal success, we hypothesized that the triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic imidazolide (CDDO-Im) would produce more potent effects (17, 18). We now report that CDDO-Im indeed exerts marked actions on adipocyte-specific gene expression, lipid metabolism, apoptosis, proliferation, and sensitivity to pharmacological inhibition of fatty acid synthesis in liposarcoma cells.

MATERIALS AND METHODS

Reagents

CDDO-Im was manufactured under the NIH RAID Program. Stock solutions were prepared in DMSO, and final concentrations of vehicle in cell culture media were $< 0.05\%$. Sodium acetate 1-14C (1.0 mCi/mL), Palmitic-[carboxy-14C] Acid (0.1 mCi/mL) and Cerulenin were obtained from Sigma (St. Louis, Missouri, USA)

Cell lines and media

LiSa-2 cells were grown in DMEM/Ham's F12 50:50 media supplemented with 10% FBS (Mediatech Inc, Herndon, Virginia, USA), 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2 mM L-glutamine. Adipogenic media contained 1 nM insulin, 20 pM triiodothyronine, and 1 μM hydrocortisone. Cells were cultured at 37°C at 4.5% CO_2, and media was changed every 3 days.

Immunohistochemistry

LS surgical specimens cut from paraffin blocks were stained with rabbit anti-human FAS (1:1000 dilution, Novus Biologicals, Littleton, Colorado, USA) or mouse anti-human S14 antibodies (1:1,000 dilution of hybridoma "KVB7," an IG2a as prepared by Martel et al (9)). Visualization was achieved with horseradish peroxidase, and counterstaining was performed with hematoxylin. Connective tissue and adipose tissue in the slides served as internal negative and positive controls, respectively. The 3 separate human LS that were stained included well differentiated LS, dedifferentiated pleomorphic LS, and dedifferentiated pleomorphic LS with myxoid degeneration.

Quantitative real-time reverse transcription-PCR

RNA was extracted from LiSa-2 cells using Qiagen's RNeasy Kit (Valencia, California, USA). RNA was shown to be intact by gel electrophoresis and to be free of contaminating DNA by the failure of PCR using primers corresponding to the cyclophilin gene to yield a product. Random primers and reverse transcriptase were from New England Biolabs (Ipswich, Massachusetts, USA). Quantitative real-time PCR was carried out using the MyiQ Single Color Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA) with SYBR green fluorescence (Applied Biosystems, Warrington, UK). Expression of specific mRNAs was expressed as a ratio to that of cyclophilin mRNA to correct for the efficiency of the amplification. cDNA from human preadipocytes and adipocytes was obtained from Zen-bio (Research Triangle Park, North Carolina) and was used as controls for stages of differentiation. Primers were created as described previously (9).

Western blot

Promega Lysis Buffer (Madison, Wisconsin, USA) was used to harvest cell protein and the Bio-Rad assay (Hercules, California, USA) was used to determine protein concentrations. Blotting was performed with rabbit anti-human FAS or mouse anti-human S14 antibodies as described in immunohistochemistry section, and protein A-alkaline phosphatase conjugate (Sigma, St Louis, Missouri, USA), using NBT/BCIP alkaline phosphatase system (Pierce, Rockford, Illinois, USA) for visualization (9).

Gene transcription

Cells were transiently transfected with a 178-bp human FAS gene promoter fragment driving luciferase (Promega, Madison, Wisconsin, USA). Cells were transfected for 6 h with FuGene transfection agent (Roche, Indianapolis, Indiana, USA),

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trypsinized, and dispersed in 6 well plates to assure constant transfection efficiency within experiments. After 4 days of treatment with CDDO-Im or DMSO-containing media, luciferase activity was determined in a luminometer. Non-transfected cells were used as a negative control.

Fatty acid synthesis

Cells were cultured for 10 days in CDDO-Im or DMSO media. C14 acetate incorporation into total lipids was measured as described previously (9, 19).

Glycerophosphate dehydrogenase activity

LiSa-2 cells were cultured for 15 days with CDDO-Im 100 nM or DMSO media, and glycerophosphate dehydrogenase enzyme activity was determined as described previously (20).

Palmitic acid uptake

Cells were cultured for 10 days with CDDO-Im 100 nM or DMSO media. Cells were then incubated for 1 hour in media containing C14-palmitic acid (0.3 μCi/mL, Amersham, Biosciences, Piscataway, New Jersey, USA). Cells were trypsinized, washed in PBS and the uptake of radioactivity was determined in a liquid scintillation counter.

Cell proliferation

Cells seeded at 10,000 cells/cm^2 in 48-well plates grew for five days, with media replaced after 3 days. DNA content/well was determined using the Hoechst assay as described previously (21).

Apoptosis

Cells were cultured for 5 days in CDDO-Im (50-100 nM) or DMSO, at which time Cerulenin (1–10 μg/mL) or vehicle was added. A TUNEL assay using the FlowTACS Apoptosis Detection System (R&D Systems, Minneapolis, Minnesota, USA) was performed 48 hours after Cerulenin addition.

In silico microarray analysis

Data from a cDNA microarray analysis of a panel of sarcomas, including LS, was evaluated specifically for FAS expression using the database at: http://watson.nhgri.nih.gov/sarcoma/ (3). Determination of expression of FAS in LS was determined using relative gene expression of LS compared to the median expression for all 181 sarcoma specimens.

Statistics

Experiments were performed at least twice with 4–10 samples for each experimental group. Error bars in figures represent standard error of the mean. ANOVA was used to determine the statistical significance of differences between means.

RESULTS

Adipocyte-specific gene expression in the LiSa-2 cells

We evaluated a panel of genes related to fatty acid metabolism, including FAS, S14, fatty acid binding protein, lipoprotein lipase, sterol responsive element binding protein 1c, PPAR-γ and S14-related peptide using reverse transcriptase PCR. We analyzed LiSa-2 cells cultured in control and adipogenic media and compared them to human preadipocytes and mature adipocytes to better define the LiSa-2 lipogenic phenotype. All the lipogenic genes analyzed were absent in preadipocytes, but present in LiSa-2 and adipocytes (Table 1). FAS was noted to be induced with adipogenic stimuli, consistent with previous findings suggesting LiSa-2 can be differentiated with adipogenic media (4). Overall, this revealed that adipogenic gene expression in the LiSa-2 cell line is much closer to that of a normal adipocyte than to the preadipocyte. We chose to focus on the response of the FAS and S14 genes to CDDO-Im because they are known to be key components of the lipogenic tumor phenotype (6, 9).

Immunohistochemistry (IHC) of 3 human LS specimens for expression of FAS and S14 proteins was performed to determine similarity of paraffin embedded randomly selected human LS specimens to the LiSa-2 cell line. IHC of the LS specimens showed cytoplasmic staining for FAS and nuclear staining for S14 in all 3 different patient specimens, which represented a variety of morphologic subtypes (Figure 1a, b). Normal adipose tissue in the specimens served as an internal positive control and showed similar staining patterns for FAS and S14. Thus, both LiSa-2 cells and liposarcoma tissues exhibit brisk expression of these key genes.

CDDO-Im effects on FAS

Quantitative real-time PCR analysis demonstrated a 70% decrease in FAS mRNA with triterpenoid treatment compared to vehicle control after 15 days in culture (Figure 2a). Analysis of S14 mRNA expression in these samples likewise showed an 85% decrease after CDDO-Im treatment (Figure 2b). In both experiments differences in cyclophilin mRNA expression was not significant ($p = 0.41$) between treatment groups indicating no change in generalized gene transcription with triterpenoid treatment. In contrast to the expected increase in lipogenic gene expression that accompanies adipocyte differentiation, CDDO-Im treatment decreased the expression of FAS and S14 (5). Thus, the induction of differentiation may not be the primary mechanism of triterpenoid action in these cells.

Western blot analysis confirmed that FAS enzyme content was reduced in the triterpenoid-treated cells compared to the control group, while overall protein synthesis was unchanged as determined by Bio-rad total protein assay (Figure 2c). Thus, the inhibition at the transcriptional level corresponded to FAS protein down regulation. Effects of CDDO-Im on S14 protein could not be confirmed with standard western blotting techniques due to the relatively low abundance of this nuclear protein.

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Table 1. RT-PCR gene profile screening of lipogenesis-related genes.

	CYC	FAS	S14	S14rp	PPAR gamma	LPL	SREBP-1c	FABP
Preadipocyte								
LiSa-2								
Adipocyte								

RT-PCR was performed with a panel of lipogenesis-related gene primers against LiSa-2 cDNA from cells cultured in serum media for 15 days. Preadipocyte and adipocyte cDNA were used as controls for stages of differentiation. Cyclophilin (CYC) was used as positive control. CYC = cyclophilin, FAS = fatty acid synthase, S14 = Spot-14, S14rp = Spot-14 related peptide, PPAR-gamma = peroxisome proliferator-activated receptor γ, LPL = lipoprotein lipase, SREBP1c = sterol response element-binding protein c, FABP = fatty acid binding protein.

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To assess the impact of CDDO-Im on the activity of the FAS gene, we transiently transfected LiSa-2 cells with a reporter plasmid containing the proximal 178 bp of the human FAS gene promoter, which contains the sterol response element-binding protein 1c (SREBP1c) binding site (Figure 2d) (22). Cells treated with CDDO-Im exhibited a 60% decrease in luciferase activity compared to the control group while total protein production between samples was unchanged as determined by Bio-rad total protein assay (Figure 2e). This assay demonstrated that increasing levels of CDDO-Im lead to decreasing levels of promoter activity, indicating that transcription is the locus of triterpenoid action on FAS expression.

Lipid metabolism

In order to assess the impact of altered expression of adipocyte-specific genes after CDDO-Im administration, we determined rates of fatty acid synthesis, cellular fatty acid uptake, and the activity of a key enzyme of triglyceride formation, glycerophosphate dehydrogenase. Lower concentrations of triterpenoid (50-100 nM) were used in these metabolic assays to prevent the cell proliferative and apoptotic effects of higher dose triterpenoids (1–10 μM) from distorting results.

Metabolic activity of FAS was assessed by the incorporation of radiolabeled acetate, a substrate in fatty acid synthesis, into

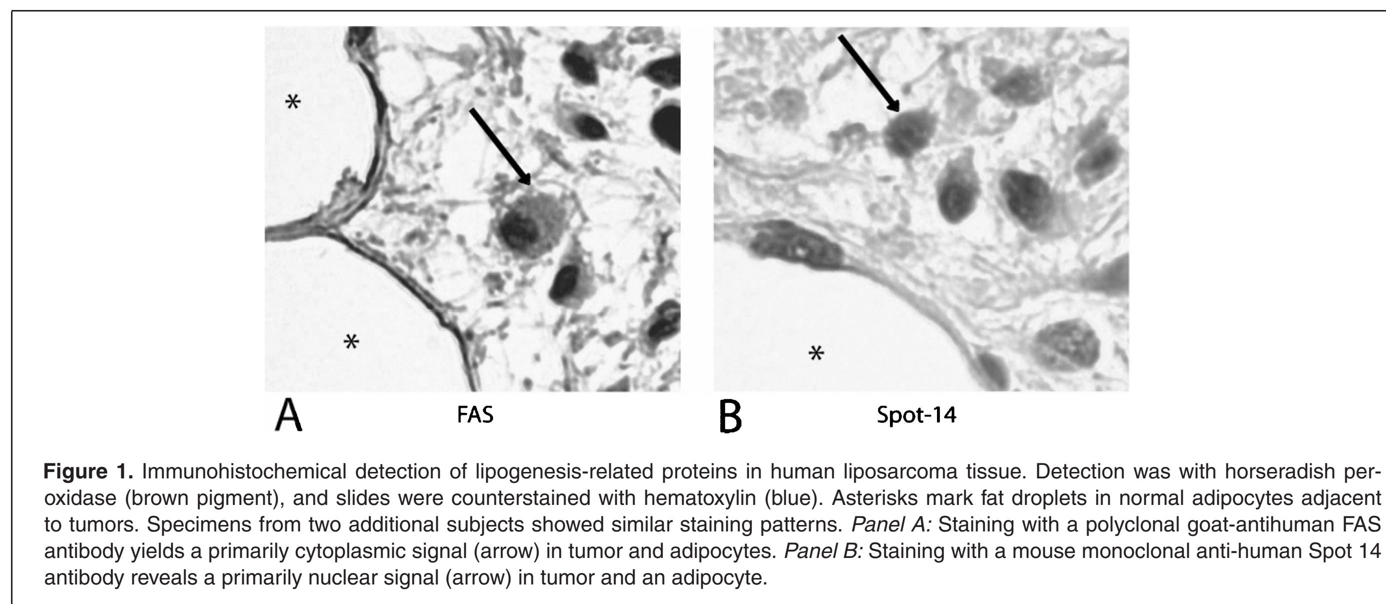

Figure 1. Immunohistochemical detection of lipogenesis-related proteins in human liposarcoma tissue. Detection was with horseradish peroxidase (brown pigment), and slides were counterstained with hematoxylin (blue). Asterisks mark fat droplets in normal adipocytes adjacent to tumors. Specimens from two additional subjects showed similar staining patterns. *Panel A:* Staining with a polyclonal goat-antihuman FAS antibody yields a primarily cytoplasmic signal (arrow) in tumor and adipocytes. *Panel B:* Staining with a mouse monoclonal anti-human Spot 14 antibody reveals a primarily nuclear signal (arrow) in tumor and an adipocyte.

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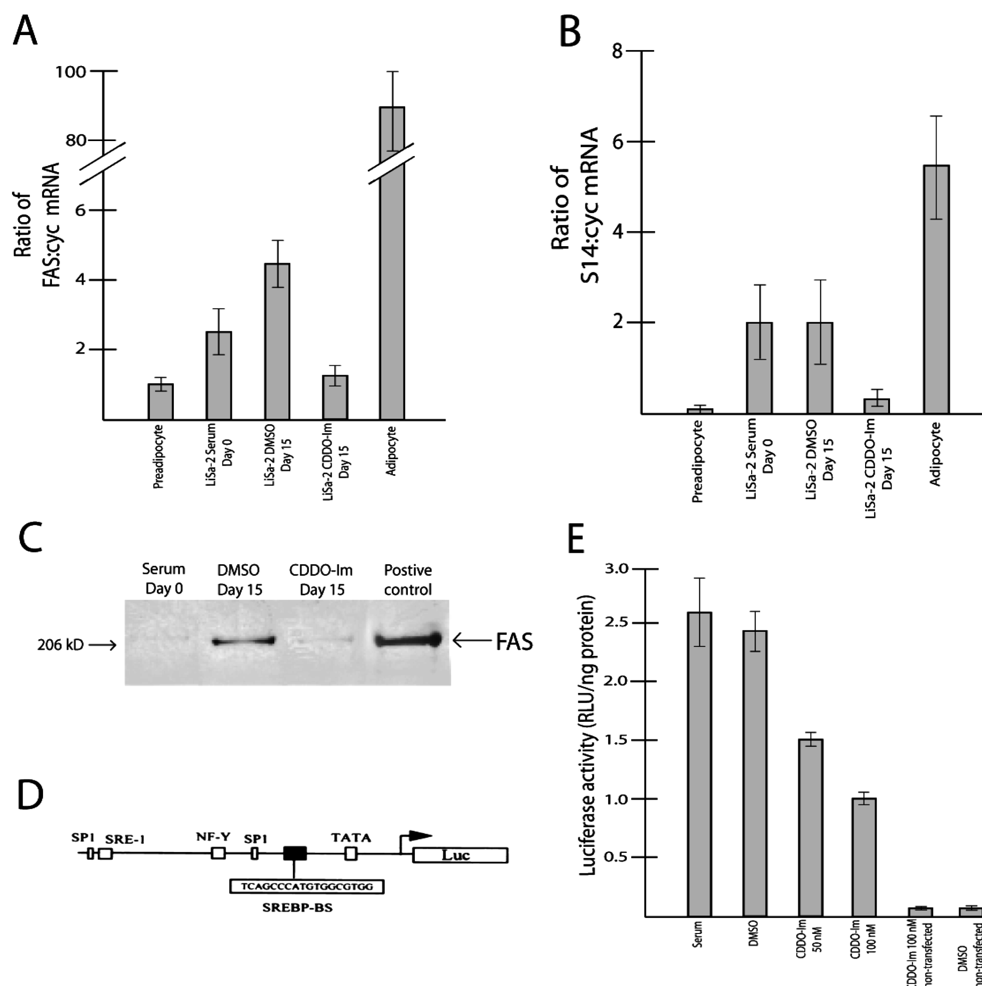

Figure 2. CDDO-Im down-regulates FAS expression in LiSa-2 cells. *Panel A:* Quantitative real-time PCR demonstrates a 70% decrease in FAS mRNA after 15 day exposure to 100 nM CDDO-Im compared to control plates. Data (mean ± SEM, n=6-10/group) are expressed as the ratio of the FAS signal to that of cyclophilin. *Panel B:* Quantitative real-time PCR demonstrates an 85% decrease in S14 mRNA after 15 day exposure to 100 nM CDDO-Im compared to control plates. Data (mean ± SEM, n = 6/group) are expressed as the ratio of the S14 signal to that of cyclophilin. *Panel C:* Western blot (20 μg protein/lane) demonstrates reduced FAS protein in cells treated with CDDO-Im, as opposed to diluent, for 15 days. Serum day 0 indicates subconfluent LiSa-2 cells in serum-containing medium. T47D human breast cancer cells were used as a (+) control. The FAS band migrates at ∼ 206 kD. *Panel D:* Diagram of the 178 bp fragment of the proximal human FAS gene promoter used to drive luciferase expression in transfection experiments. SREBP-BS denotes the binding site for SREBP-1c; the position of binding sites for transcription factors NF-Y and SP1 are indicated, as are a non-essential sterol response element sequence (SRE) and the TATA box. *Panel E:* Luciferase activity (mean ± SEM, n= 3/group) measured 4 days after transfection is shown. FAS gene promoter activity is reduced by ∼ 60% in cells exposed to 100 nM CDDO-Im. Non-transfected LiSa-2 cells acted as negative controls.

de novo synthesized fatty acids (19). After treatment of LiSa-2 cells with CDDO-Im 100 nM or DMSO control for 10 days, we observed a 4-fold decrease in C14 acetate incorporation into total lipids in the triterpenoid treated group and no change in total protein (Figure 3a). This confirmed that the triterpenoid-mediated down regulation of FAS and S14 gene expression is reflected functionally at the level of fatty acid synthesis.

Glycerophosphate dehydrogenase (GPDH) enzyme activity is a measure of differentiation of human preadipocytes, with increasing levels of activity corresponding to achievement of the mature adipocyte phenotype (20). GPDH activity showed a 12-fold induction after 15 days of culture in confluent LiSa-2 cells in DMSO media compared to cells in serum media at sub-confluency (Figure 3b). There was a slight decrease in GPDH enzyme activity in CDDO-Im 100 nM treated cells at 15 days, but there was also substantial up regulation compared to cells in serum media at sub-confluency (Figure 3b). The results indicate the 100 nM dose of CDDO-Im does not induce further differentiation in the LiSa-2 cell line and that the capacity to differentiate with achievement of confluency is preserved in its presence.

Exogenous uptake is an alternative mechanism for cells to obtain fatty acids, and this can be measured by uptake of radio-labeled palmitic acid carried by albumin (23). We demonstrated

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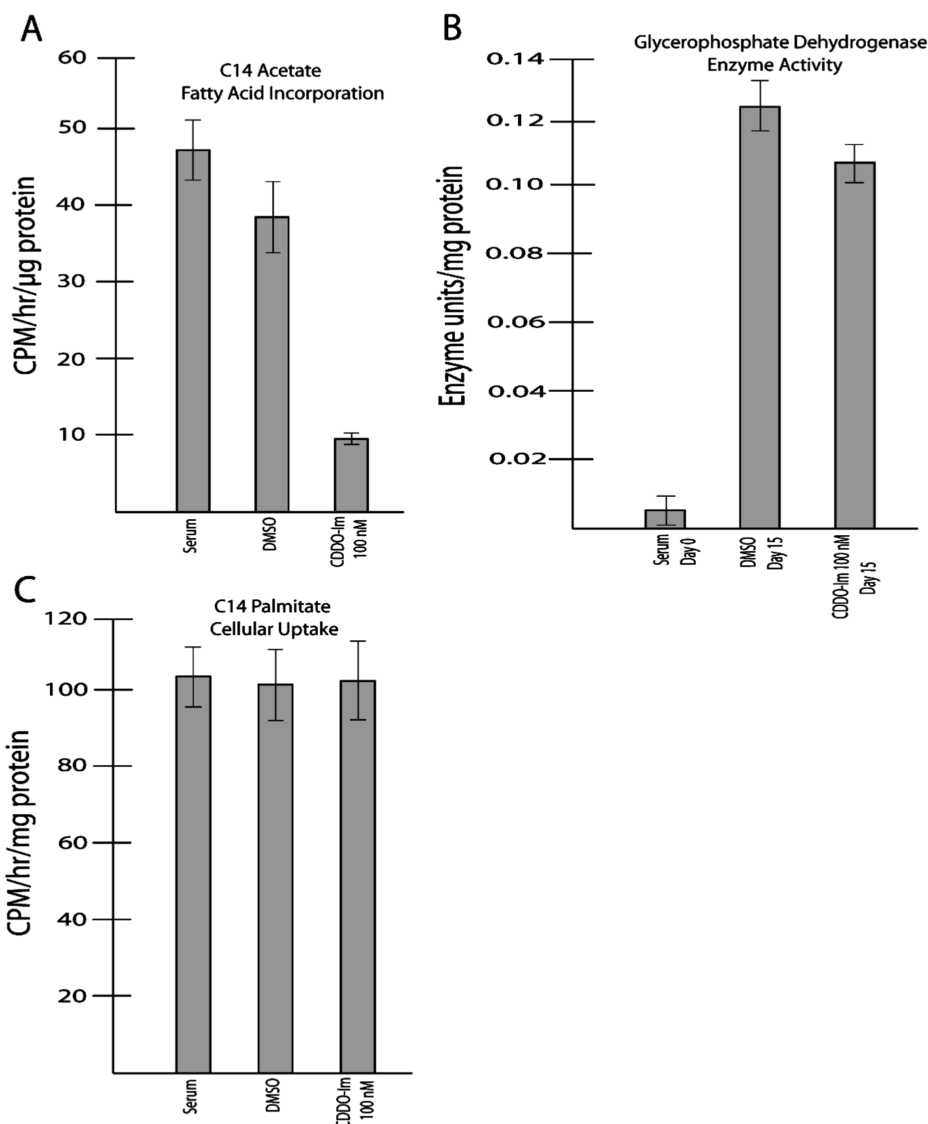

Figure 3. Metabolic effects of CDDO-Im in LiSa-2 cells. *Panel A:* CDDO-Im inhibits incorporation of [14]-C-acetate into lipids. Cells were labeled for 3 hours, at which time total lipids were extracted, and incorporation was measured in a liquid scintillation counter. Data are mean cpm ($\times 10^2$)± SEM (n = 4/group). *Panel B:* CDDO-Im does not exert a significant effect on the activity of the major enzyme of triglyceride synthesis. GPDH enzyme activity (mean ± SEM, 8 wells/group) was measured before and after 15 day exposure to vehicle or 100 nM CDDO-Im. *Panel C:* The uptake of exogenous fatty acids is unaffected by exposure to CDDO-Im. Cells were incubated in the presence of the indicated culture media spiked with [14]-C-palmitic acid for 1 hour, and cell-associated radioactivity was determined. Data are mean cpm (x 10^4)± SEM (n = 4/group).

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no difference in the levels of palmitic acid uptake between LiSa-2 cells treated with CDDO-Im or DMSO control (Figure 3c), indicating that no compensatory increase in exogenous fatty acid uptake is elicited by the reduced *de novo* fatty acid synthesis.

Effects of CDDO-Im and Cerulenin on LiSa-2 proliferation and apoptosis

CDDO-Im effects on LiSa-2 cell accumulation were determined by the Hoechst DNA assay. A dose-response curve was evident with CDDO-Im treatment at concentrations greater than 100 nM, with complete cell kill occurring at >10 μM (Figure 4a). This is consistent with previous studies demonstrating apoptosis of a variety tumor cells with higher levels of triterpenoid (11, 24). Our novel finding of FAS down regulation with CDDO-Im led us to apply Cerulenin, an inhibitor of FAS enzyme activity, to determine if synergistic cytotoxicity could be achieved through inhibition of FAS at both the transcriptional and protein level (25–27). A similar dose-response was seen with Cerulenin alone at doses 1-10 μg/mL with complete killing occurring at concentrations above 5 μg/mL (Figure 4b). Co-treatment with CDDO-Im 100 nM increased sensitivity of the LiSa-2 cells to Cerulenin, allowing for a 50% reduction in the Cerulenin

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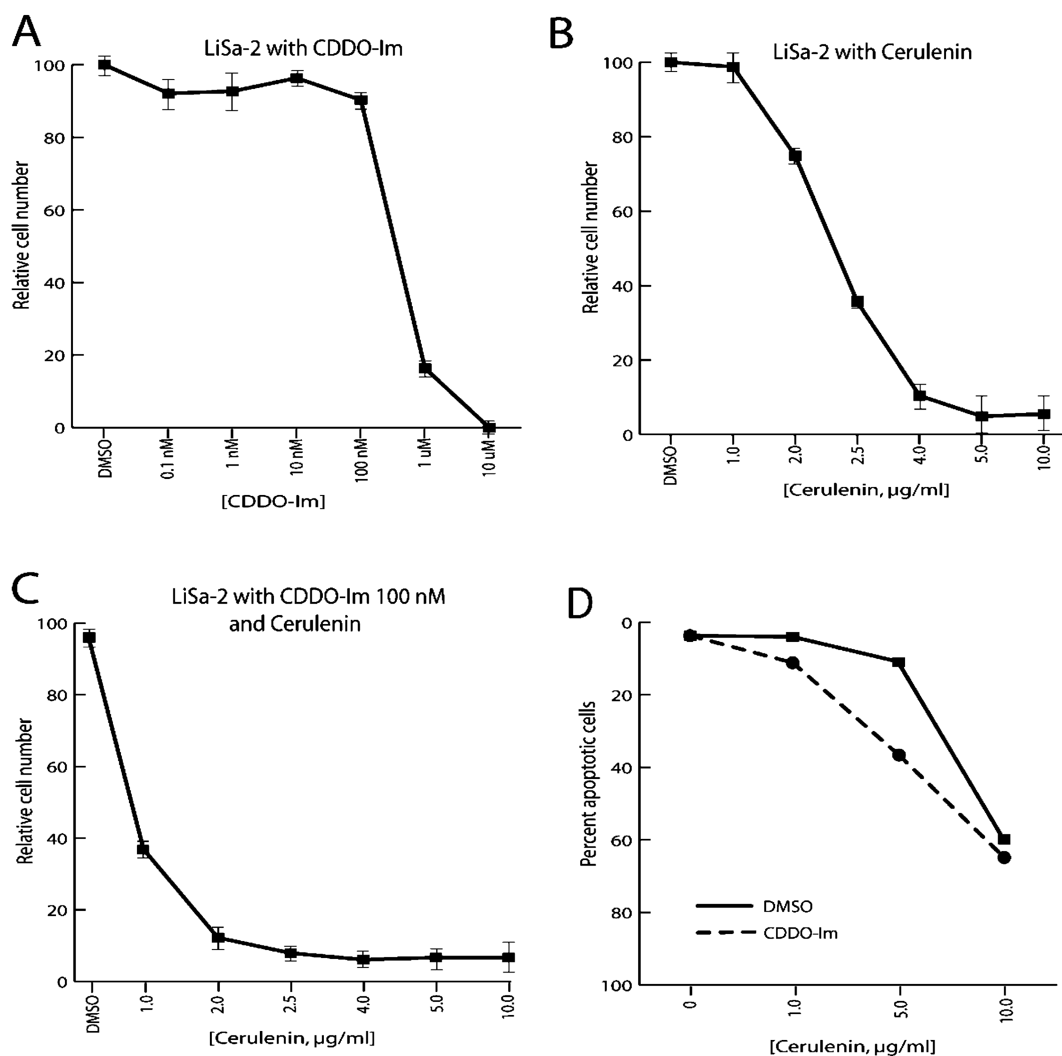

Figure 4. CDDO-Im-treated LiSa-2 cells exhibit dose-dependent apoptosis and increased sensitivity to the cytotoxic effect of Cerulenin. *Panel A:* Net cell accumulation after 5 day exposure to CDDO-Im, determined by DNA content/well is shown (mean ± SEM, n = 4 wells/data point). *Panel B:* Cerulenin inhibits LiSa-2 cell growth. Cells were incubated in the indicated concentrations of Cerulenin x 5 days, and assayed as in panel A. *Panel C:* Cells exposed to Cerulenin exhibit reduced growth at a sublethal concentration of CDDO-Im. Cells were exposed to 100 nM CDDO-Im combined with the indicated concentrations of Cerulenin for 5 days, and analyzed for DNA content/well. *Panel D:* CDDO-Im causes enhanced sensitivity to Cerulenin-induced apoptosis. Cells were grown x 5 days in 100 nM CDDO-Im in the presence of the indicated concentrations of Cerulenin or diluent were analyzed in a TUNEL assay. Addition of a submaximally-lethal concentration of Cerulenin (5 μg/mL) to cells exposed to 100 nM CDDO-Im caused a 5-fold increase in apoptosis. Note that the interaction is obscured at a maximally cytotoxic level of Cerulenin (10 μg/mL).

dose required to achieve 100% cytotoxicity, thus demonstrating synergy between CDDO-Im and Cerulenin (Figure 4c).

We performed a TUNEL assay to determine if reduced cell number was due to induction of apoptosis or inhibition of growth. LiSa-2 cells were cultured for 5 days in either CDDO-Im or DMSO media with Cerulenin added at various concentrations for the final 48 hours. The TUNEL assay demonstrated a dose-dependent apoptotic effect of Cerulenin from 1-10 μg/mL, thus confirming that the decrease in cell number seen in the Hoechst assay was caused by increased apoptosis (Figure 4d). Increased sensitivity with co-treatment with CDDO-Im was demonstrated by a 4-fold increase in apoptosis at the 5 μg/mL Cerulenin dose when compared to Cerulenin alone. CDDO-Im at 50 and 100 nM did not exert an apoptotic effect above that of the control, as was expected at these sub-apoptotic concentrations (Figure 4a, d). The results of the proliferation and apoptosis assays, therefore, confirmed that the triterpenoid-induced down regulation of fatty acid synthesis ultimately results in decreased LiSa-2 proliferation and a lowering of the threshold of the response to the FAS inhibitor Cerulenin.

DISCUSSION

We evaluated the effects of CDDO-Im in an immortalized LS cell line. Triterpenoid treatment had dose-related apoptotic

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effects consistent with previous studies in various tumor cell lines; however, the differentiating effects seen at lower doses were not dramatic in this cell line as they were in osteosarcoma, leukemia and 3T3-L1 cells (10, 11, 14, 28). Although differentiation was evident in CDDO-Im treated LiSa-2 cells as assessed by the GPDH enzymatic assay, this was not significantly higher compared to the control cells, which demonstrated increased GPDH activity with achievement of confluency alone (Figure 3b). The ability of the LiSa-2 cells to increase GPDH enzyme activity with confluency, similar to the preadipocyte 3T3 cells, indicates that the response to this stimulus for differentiation is preserved in LiSa-2 cells whether or not CDDO-Im is present (29). The gene expression pattern related to fatty acid synthesis observed in LiSa-2 cells indicates that they are quite dissimilar to the preadipocyte and are developmentally arrested in a near-adipocyte state (Table 1). Therefore, these cells have already completed a number of steps in the adipocyte differentiation sequence.

While the synthetic triterpenoid did not foster further differentiation of LiSa-2 cells, the effects of increased apoptosis and decreased cell proliferation were evident and were mediated, at least in part, by the newly-described down regulation of FAS and S14. Our data indicate that CDDO-Im inhibition of FAS expression is mediated through a succinct fragment of the FAS gene promoter, and that this decreases FAS mRNA and protein (Figure 2a, c, e). The metabolic impact of FAS down regulation is evident in the decreased *de novo* synthesis of fatty acids (Figure 3a). The specificity of triterpenoid action on *de novo* lipogenesis, as opposed to other lipid-related pathways, is confirmed by the unchanged uptake of exogenous fatty acids and GPDH activity (Figure 3b, c). This analysis provided a comprehensive view of the pathways of fatty acid metabolism (30). Our demonstration of CDDO-Im down regulation of FAS and S14 is the first report of triterpenoid compounds affecting the fatty acid synthesis pathway in any tumor cell line.

Reduced growth of LiSa-2 cells during CDDO-Im exposure appeared to be mediated by the down regulation of genes concerned with fatty acid formation because Cerulenin-mediated FAS inhibition simulated the actions of the triterpenoid in this regard. Thus, LS exhibit a key aspect of the lipogenic tumor phenotype in that their growth depends on fatty acid synthesis (6). This concept is consistent with published cDNA microarray data from a panel of 181 human sarcomas (3). In the 27 LS specimens analyzed in this microarray, the fatty acid metabolism gene category was the most highly expressed in the LS compared to all sarcomas (3). The LS specimens showed an average 0.5 log over-expression of FAS compared to the median for all other sarcomas (Figure 5). LS was the only adult sarcoma to over express FAS, while low expression was seen in malignant fibrous histiocytomas and leiomyosarcomas (Figure 5). Although the S14 cDNA was not dotted on this microarray, its expression has been linked closely with the expression of FAS in lipogenic malignancies (6, 9). We also noted the presence of S14 and FAS protein in human LS via IHC staining, indicating that these genes are important to the LS phenotype *in vivo* (Figure 1). The importance of fatty acid metabolism in LS makes FAS a particularly

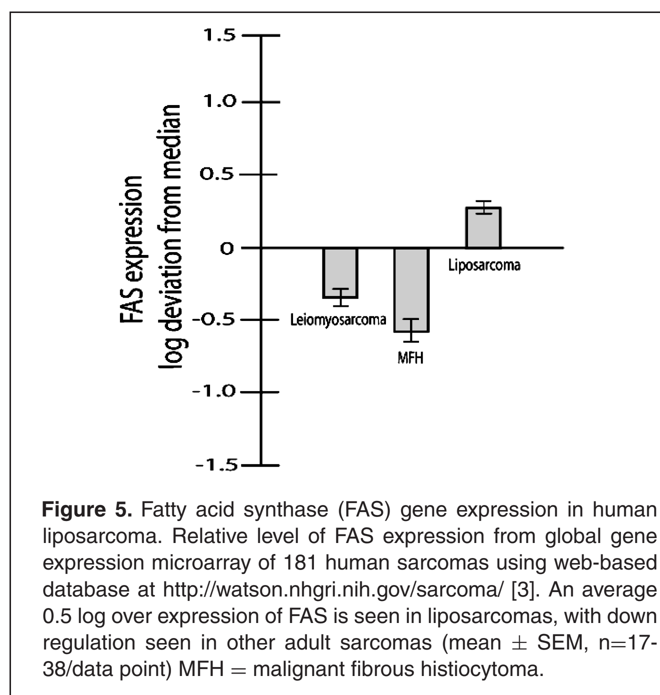

Figure 5. Fatty acid synthase (FAS) gene expression in human liposarcoma. Relative level of FAS expression from global gene expression microarray of 181 human sarcomas using web-based database at http://watson.nhgri.nih.gov/sarcoma/ [3]. An average 0.5 log over expression of FAS is seen in liposarcomas, with down regulation seen in other adult sarcomas (mean ± SEM, n=17-38/data point) MFH = malignant fibrous histiocytoma.

attractive candidate target in this malignancy, which is generally resistant to medical therapy.

The consequences of FAS inhibition by CDDO-Im are mediated by decreased proliferation and increased apoptosis of the LiSa-2 cells (Figure 4a). Similar dose-responsive apoptosis with higher doses of triterpenoids has been demonstrated in several tumor types (11, 24). The dose-dependency of triterpenoid action indicates that down-regulation of FAS can occur at sublethal doses. At higher doses, CDDO-Im will initiate apoptosis at a critical level of FAS down regulation (Figure 4a). Thus, lethal FAS inhibition can be achieved through either reduction of gene expression mediated by CDDO-Im or at the enzymatic level by Cerulenin. Cerulenin inhibits fatty acid biosynthesis through its specific post-translational inhibition of FAS (31). The inhibition of FAS gene expression by the triterpenoid is associated with reduced proliferation, while enzyme inhibition by Cerulenin induces apoptosis, yielding a synergistic cytotoxic effect (Figure 4c, d). This synergism may allow for lower doses of the drugs, thereby reducing toxicity. The inhibitory effect of CDDO-Im on cancer cell lipid metabolism may be clinically exploitable.

This study confirms that the LiSa-2 cell line is an excellent *in vitro* model for human LS and establishes the lipogenic nature of this tumor type manifest by high expression of lipogenesis-related genes, such as FAS and S14, and establishes its dependence on de-novo fatty acid synthesis for proliferation and survival. While the full array of triterpenoid effects on gene expression and cellular metabolism have yet to be defined, the demonstration of inhibition of fatty acid synthesis by CDDO-Im may have therapeutic relevance beyond LS, as recent studies indicate that a lipogenic phenotype is frequent in several common human tumor types (25, 27, 32). Pharmacologic inhibition of endogenous fatty acid synthesis would be expected to have few systemic ill-effects, due to the low activity of this

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pathway in non-malignant cells (33). Therapeutic targeting of fatty acid synthesis using compounds such as CDDO-Im may provide a novel and sorely-needed medical approach to treatment for LS. These findings also provide the basis for studies of the effects of synthetic triterpenoids in *in vivo* models of LS.

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