A Fatty Acid Synthase Blockade Induces Tumor Cell Cycle Arrest
by Down-regulating Skp2

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2The abbreviations used are: DMSO, dimethyl sulfoxide; FAS, fatty acid synthase; FP-PEG-TAMRA, fluorophosphonate-poly-ethyleneglycol-tetramethyl rhodamine; IB, immunoblotting; IP, immune-precipitation; PI, propidium iodide; siRNA, small interfering RNA.
SUMMARY

In eukaryotes, fatty acid synthase (FAS) is the enzyme responsible for synthesis of palmitate, the precursor of long-chain nonessential fatty acids. FAS is up-regulated in a wide range of cancers and has been suggested as a relevant drug target. Here, two independent approaches are taken toward knocking down FAS and then probing its connection to tumor cell proliferation. In one approach, Orlistat, a drug approved for treating obesity, is used as a potent inhibitor of the thioesterase function of FAS. In a separate strategy, the expression of FAS is suppressed by targeted knock-down with small interfering RNA. In both circumstances the ablation of FAS activity causes a dramatic down-regulation of Skp2, a component of the E3 ubiquitin ligase that controls the turnover of p27^Kip1. These effects ultimately tie into the retinoblastoma protein pathway and lead to a cell-cycle arrest at the G1/S boundary. Altogether, the findings of the study reveal unappreciated links between fatty acid synthase and ubiquitin-dependent proteolysis of cell-cycle regulatory proteins.
INTRODUCTION

Breast cancer is the second-leading cause of cancer death and morbidity for women in the United States (1,2). Although advances in early detection and treatment have led to a decline in mortality, the survival rate for patients with advanced-stage breast cancer is still low (1). Consequently, there is still a great need to identify and validate new molecular targets for antitumor therapy. This study focuses on mammary carcinoma, where fatty acid synthase (FAS) has attracted considerable attention as a potential drug target. Much of the interest in FAS stems from the fact that the enzyme is up-regulated in about 50% of breast cancers and is an indicator of poor prognosis (3-7).

Fatty acid synthase (FAS) is the enzyme responsible for cellular synthesis of palmitate, the precursor of long-chain nonessential fatty acids (8-11). FAS, which contains seven separate enzymatic pockets, is situated as a head-to-tail dimer with the ketoacyl synthase and malonyl/acetyl transferase domains of one monomer working together with the dehydratase, enoyl reductase, ketoacyl reductase, acyl carrier protein, and thioesterase domains on the adjacent monomer (8-11). These enzymatic domains act sequentially to condense acetyl-CoA with malonyl-CoA to form a four-carbon intermediate. Six additional turns of the enzyme’s cycle convert this intermediate to palmitate, which is then liberated from FAS by the action of the thioesterase domain (12).

Because FAS functions as a head-to-tail dimer, targeted inhibition of one of the enzymatic domains of FAS can ablate the activity of one or both FAS subunits (10,11). Cerulenin, a natural product, is an antagonist of the ketoacyl synthase domain (the condensing enzyme) of FAS and functions by covalently modifying the active site cysteine, resulting in dead-end inhibition (13). c75, a synthetic analog of cerulenin, also targets the condensing
enzyme and inhibits fatty acid synthesis (14). The inhibition of FAS by either cerulenin or c75 can suppress tumor cell proliferation and in some cases can induce tumor cell apoptosis (14-20). These observations support the contention that FAS is a relevant drug target in oncology. However, both cerulenin and c75 are now known to have other molecular targets (21-23), so searches for additional antagonists of FAS with better selectivity and distinct mechanisms of action are certainly warranted.

We recently reported that Orlistat, a drug approved for treating obesity, is a rather potent and selective inhibitor of FAS in prostate carcinoma cells (24). The drug elicits its effects by inhibiting the thioesterase domain of FAS, which is responsible for releasing palmitate from the enzyme’s acyl carrier protein. By virtue of this activity, Orlistat was able to slow the growth of xenograft tumors of PC-3 prostate carcinoma cells in mice (24). The objective of the present study was twofold. First, we sought to use an independent strategy to confirm that the antiproliferative effects of Orlistat result from inhibition of FAS. Second, we sought to elucidate the mechanism by which a FAS blockade interferes with tumor cell proliferation. Both Orlistat and siRNA targeting FAS cause a dramatic down-regulation of Skp2, a component of an E3-ligase ubiquitin ligase that tags p27^Kip1 for degradation by the proteasome. These findings mechanistically connect two biochemical pathways being explored as drug targets in cancer, fatty acid biosynthesis, and ubiquitin-dependent proteolysis.
MATERIALS AND METHODS

Cell lines. The MDA-MB-435 cell line was obtained from Janet Price at the University of Texas Southwestern. MDA-MB-231, MCF-7 were purchased from American Type Culture Collection (Manassas, VA). Tumor cells were maintained in Minimal Eagle’s Media (MEM) Earl’s Salts (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (Irvine Scientific), 2 mM L-glutamine (Invitrogen Life Technologies, Inc., Carlsbad, CA), MEM vitamins (Invitrogen Life Technologies, Inc.), nonessential amino acids (Irvine Scientific) and antibiotics (Omega Scientific, Inc., Tarzana, CA).

Profiling serine hydrolase activity in mammary epithelial cells. Cultured cells were washed with ice-cold phosphate buffered saline (PBS), harvested with a cell scraper, and collected by centrifugation. Cell pellets were resuspended in 50 mM Tris-HCl (pH 8.0) and lysed by sonication as previously described (25,26). The soluble and insoluble cell fractions were separated by ultracentrifugation at 64,000 rpm for 1 h at 4°C. Protein concentrations were determined using the BCA assay kit from Pierce (Rockford, IL). The resulting extracts were diluted in lysis buffer to yield a 1 mg/ml final protein concentration.

The fluorophosphonate probes—FP-PEG-TAMRA, FP-PEG-BODIPY, and FP-PEG-biotin—were synthesized and generously provided by Activx Biosciences (La Jolla, CA). Serine hydrolase activity was examined by incubating the soluble cell fractions (40 µl) with FP-PEG-TAMRA (2 µM) for 1 h at room temperature. Nonspecific labeling was monitored by denaturing samples for 10 min at 100°C prior to labeling with FP-PEG-TAMRA. The reaction was terminated by the addition of 2x Laemli sample buffer, boiled for 5 min, and resolved by 10% SDS-PAGE. Fluorescent labeled hydrolases were visualized at 605 nm using a Hitachi flatbed scanner and quantitated using Image Analysis (MiraiBio, Alameda, CA).
Purification and identification of serine hydrolases by avidin-biotin affinity chromatography and MALDI-TOF mass spectrometry. Serine hydrolases were identified using the FP-PEG-biotin probe (26). Briefly, cell lysates were preabsorbed with avidin-agarose to reduce nonspecific binding of proteins during purification. Lysates were labeled with FP-PEG-biotin (5 µM) at room temperature for 1 h, after which proteins were separated from unincorporated FP-PEG-biotin by gel filtration on Nap 25 columns. Following the addition of 0.5% SDS, the eluate was boiled for 10 min to denature proteins. Samples were diluted with 50 mM Tris (pH 7.5) and 150 mM NaCl and absorbed with avidin-agarose for 1 h at room temperature. Avidin-agarose beads were pelleted by centrifugation and washed eight times with 50 mM Tris (pH 7.5), 150 mM NaCl, and 1% Tween 20. Labeled proteins were eluted with 2X sample buffer, resolved by 10% SDS-PAGE, and detected by silver staining. Specific bands were extracted and subjected to in-gel trypsin digestion and peptide mass fingerprinting with MALDI-TOF using methods previously described (27,28).

Inhibition of serine hydrolase activity with Orlistat. Orlistat was extracted from Xenical® capsules (Roche, Indianapolis, IN) by solubilizing each pill in 1 ml ethanol. Insoluble product was removed by centrifugation (14,000 rpm for 5 min). The supernatant yielded a solution of Orlistat (250 mM), which was aliquoted and stored at −80°C. Soluble cell extracts (40 µl) were incubated with Orlistat (0-1 µM) for 20 min prior to FP-PEG-TAMRA addition. Lysates were labeled with FP-PEG-TAMRA, and reactions were terminated and processed as described above. The final concentration of DMSO or ethanol in each reaction was 10%.

Gene silencing using siRNA. FAS siRNA sequences corresponding to 5′ CAA CTA CGG CTT TGC CAA T (nucleotides 6213-6231), 5′ GCA ACT CAC GCT CCG GAA A (nucleotides 6657-6675), 5′ GCC CTG AGC TGG ACT ACT T (nucleotides 6146-6164) and 5′
GGT ATG CGA CGG GAA AGT A (nucleotides 7515-7533), were custom designed and pooled together by Dharmacon (Lafayette, CO). MDA-MB-435 cells were plated at 3.125 × 10⁴/cm² in 6 cm plates for 24 h prior to transfection with 100 nM FAS, Skp2 (Dharmacon Smartpool # M-003324-01) or scrambled control (Dharmacon #D-001206-13) siRNA in Opti-MEM medium (Invitrogen) using lipofectamine 2000 reagent (Invitrogen) according to manufacturer instructions. Cells were placed into normal culture medium 6 h post-transfection and grown for an additional 42 h.

**Fatty acid biosynthesis.** The incorporation of [¹⁴C]-malonyl CoA into cellular fatty acids was measured according to published methods (15). Briefly, MDA-MB-435 cells were harvested with a cell scraper and centrifuged at 2,000 rpm for 5 min, and then frozen at -80°C. Cell pellets were hypotonically lysed in 20 mM Tris (pH 7.5), 1 mM DTT and 1 mM EDTA and insoluble material was removed by centrifugation (14,000 rpm) for 15 min at 4°C. Lysates (380 ug) were exposed to Orlistat (0.1-10 µM) or vehicle at 25°C for 1 h. Lysates, in 80 ul volumes, were added to 520 µl of solution containing 581 µM NADPH, 193 µM acetyl CoA, 116 mM KCl (pH 6.6). Reactions were mixed with 0.4 µCi [2-¹⁴C]-malonyl CoA (Amersham Biosciences) for 25 min at 37°C. Cold malonyl CoA (208 uM) was added to reaction mixtures which were incubated for an additional 15 min at 37°C. Reactions were terminated by the addition of chloroform:methanol (1:1). The chloroform extracts were dried under N₂ and extracted with water-saturated butanol. The butanol extract was evaporated under N₂, and labeled fatty acids were quantified by scintillation counting. The identity of the labeled fatty acid was verified by comparison to a palmitate standard on thin layer chromatography. Briefly, lipid extracts from Orlistat and vehicle-treated lysates of MDA-MB-435 cells were resuspended in 40 µl chloroform, spotted on Silica Gel (EM Science) and chromatographed in hexane/diethyl
ether/acetic acid (45:5:1). Tritiated palmitate (Perkin Elmer) and cold palmitate (Sigma) were used as standards. Chromatographed lipids were detected by exposing the plate in iodine vapor and on Biomax film (Kodak).

**Cell proliferation assays.** Cells were plated at $6.25 \times 10^4/cm^2$ in 96-well plates for 24 h. Cells were washed with PBS and incubated in serum-free RPMI 1640 medium for 24 h prior to Orlistat (0–100 µM) addition. Cell proliferation was measured 72 h later using the Cell Proliferation BrdU ELISA kit (Roche) according to manufacturers directions.

**Cell synchronization and cell-cycle analysis.** Tumor cells were plated at $6.25 \times 10^4/cm^2$ in 6-well plates for 24 h, washed with PBS, and serum-starved for an additional 24 h. M phase synchronization was achieved by treating cells with 100 nM nocodazole (Sigma-Aldrich) for 16 h. Synchronized cells were treated with Orlistat (0-50 µM) immediately following release from the block and harvested at various times over 24 h. Cells were collected by trypsinization, rinsed in cold PBS, fixed in 70% ethanol, and stored at −20°C. Cellular DNA was stained by the addition of PBS containing 200 units/ml RNase (Roche) and 18 µg/ml propidium iodide (Molecular Probes, Eugene, OR). Fluorescence was monitored on 15,000 cells per sample using a BD FACSort tabletop cytometer (Becton Dickinson, San Jose, CA). Data were analyzed using Modfit LT software (Verity Software House, Topsham, ME).

**Western blot analysis.** Cells were synchronized with nocodazole, treated with Orlistat (0-50 µM), harvested by trypsinization, and rinsed with PBS. Pellets were lysed in 2x SDS sample buffer, passed 20 times through an 18-gauge needle, boiled for 5 min, and stored at −80°C. Insoluble material was removed by centrifugation (14,000 rpm) for 10 min at 4°C, and protein was separated by SDS-PAGE. Following electrophoresis, protein was transferred onto nitrocellulose and probed overnight at 4°C with anti-FAS (BD Transduction Laboratories).
anti-p27 (Santa Cruz Biotechnology #sc-1641, Santa Cruz, CA), anti-β-tubulin (Santa Cruz Biotechnology #sc-5274), anti-Rb (Santa Cruz Biotechnology #sc-102), anti-phospho-Rb (Ser780; Cell Signaling Technology #9307, Beverly, MA), anti-phospho-Rb (Ser795; Cell Signaling Technology #9301), anti-phospho-Rb (Ser907/811; Cell Signaling Technology #9308), and anti-p45 Skp2 (Zymed Laboratories #32-3400, South San Francisco, CA). Immunoreactivity was detected using anti-mouse or anti-rabbit IgG conjugated peroxidase and visualized by enhanced chemiluminescence.
RESULTS

Serine Hydrolase Profile of Normal and Neoplastic Mammary Epithelial Cells.

Activity-based protein profiling was used to visualize the profile of active serine hydrolases present in mammary carcinoma cells. We used an activity-based probe of serine hydrolases consisting of a fluorophosphonate moiety linked to tetramethyl rhodamine (FP-PEG-TAMRA). The fluorophosphonate warhead tags the active site serine of serine hydrolases, forming an adduct that is stable to SDS gel electrophoresis (29). Activity profiles of three human breast cancer cell lines, MCF7, MDA-MB-231, and MDA-MB-435, were compared (Fig. 1). In each case, more than 20 active serine hydrolases were detected as fluorescent bands on the SDS gel. Preheating the sample to denature the enzymes in the lysate eliminated reaction with the FP-PEG-TAMRA probe. The identity of many of these enzymes was determined using mass spectrometry. They included, among others, dipeptidyl peptidases 7 and 9, prolyloligopeptidase, lysophospholipase-1, and FAS.

Orlistat suppresses tumor cell proliferation by interfering with G1/S progression.

Our previous work showed that Orlistat inhibits FAS in prostate carcinoma cells and that such inhibition slows their growth in vivo (24). Given the presence of FAS in mammary carcinoma cells (Fig. 1), we conducted studies to determine if Orlistat would inhibit their proliferation. Studies were conducted to determine whether Orlistat interferes with the proliferation of mammary carcinoma cells. Cells were incubated with Orlistat for 72 h, and DNA synthesis was measured by incorporation of BrdU. Orlistat inhibited proliferation of the MCF7, MDA-MB-231, and MDA-MB-435 cell lines (Fig. 2A). Slight differences were observed in the response of each cell line to Orlistat. While proliferation of the MDA-MB-231
cells was completely inhibited by Orlistat, proliferation of the MDA-MB-435 cells was knocked down by 70–80%, and proliferation of the MCF7 cells was suppressed by about 50%.

To determine the effect of Orlistat on cell-cycle progression, we used synchronized cultures of MDA-MB-435 cells. Cells were synchronized in the M phase using a nocodazole block. Following release of the block, cells were exposed to a saturating concentration of Orlistat and analyzed for the distribution of cells in the G1 and S phases every 4 h by flow cytometry. Orlistat dramatically slowed the entry of the cells into S phase compared to untreated cells (Fig. 2B). The amplitude of the G1 peak in Orlistat-treated cells declines slowly after a period of several hours. This decline in the G1 population results partially from apoptosis (unpublished observation) and also partially because the blockade is leaky. Similar results were obtained when cells were synchronized at the G1/S border using thymidine, released from the block, and allowed to progress through the next cell cycle (data not shown).

**Orlistat and anti-FAS siRNA block fatty acid biosynthesis.**

We sought an independent means of inhibiting FAS to solidify the role of this enzyme in regulating tumor cell proliferation. Therefore, we compared Orlistat and siRNA targeting FAS for the ability to knock down the enzyme’s activity in MDA-MB-435 mammary carcinoma cells (Fig. 3). As expected, Orlistat was without effect on the level of FAS (Fig. 3A, left panel) but did ablate the activity of the thioesterase domain as indicated with the activity-based probe FP-PEG-TAMRA (Fig. 3A, right panel). The siRNA targeting FAS reduced the level of FAS protein (Fig. 3B, left panel), and thereby reduced the labeling of the enzyme with the activity-based probe (Fig. 3B, right panel). Both antagonists also blocked the synthesis of palmitate, the
end product of FAS, as indicated by reductions to the incorporation of $[^{14}C]$-malonyl CoA into fatty acids (Fig. 3C and D).

**A FAS blockade alters the key regulatory steps in the retinoblastoma protein pathway.**

We examined the effects of a FAS blockade on key regulatory steps in the retinoblastoma protein (Rb) protein pathway, a primary regulator of the G$_1$/S transition (30). This analysis included measures of 1) the phosphorylation status of Rb, a parameter that governs the interaction of this protein with E2F-1 and subsequent entry into S phase (30); 2) p27$^{kip1}$ which negatively regulates cyclin-dependent kinase activity (31); and 3) Skp2, a protein component of the E3 ubiquitin ligase that regulates degradation of p27$^{kip1}$ (32). The effect of a concentration range of Orlistat on each of these parameters was measured by Western blotting (Fig. 4A). Orlistat reduced phosphorylation of the Rb protein, up-regulated p27$^{kip1}$, and down-regulated Skp2. These effects were evident at levels of the drug consistent with the cellular IC$_{50}$ of Orlistat for inhibition of the FAS thioesterase (~ 1-3 uM). To independently verify that the effects of Orlistat could be attributed to its ability to block FAS, similar experiments were conducted with siRNA targeting FAS (Fig. 4B). Like Orlistat, the siRNA targeting FAS decreased the phosphorylation of Rb, increased the level of p27$^{kip1}$, and reduced the level of Skp2. An siRNA targeting Skp2 had identical effects on its downstream target, p27$^{kip1}$, and on the phosphorylation status of the Rb protein. Together these findings provide strong support for the idea that a FAS blockade acts on the Rb pathway via regulation of Skp2, and also indicate that a FAS blockade is likely to have effects similar to a Skp2 blockade.
DISCUSSION

Results from this study solidify the idea that FAS is a relevant drug target in oncology and further support the notion that FAS is the relevant target for Orlistat in tumor cells. The inhibitory effects of Orlistat on FAS are rather unexpected because the drug has been studied for more than 15 years with no mention of the effects on fatty acid synthesis. The effects of the drug on FAS are likely to have been overlooked because Orlistat is administered orally but is not significantly absorbed into the bloodstream. The drug acts in preventing absorption of dietary fat by inhibiting pancreatic lipase (another serine hydrolase) in the digestive tract (33,34). The results of the present study indicate that Orlistat and other \( \beta \)-lactones should be considered as a promising class of thioesterase antagonists that could be exploited for antitumor therapy.

The evidence supporting the conclusion that Orlistat’s antiproliferative effects are mediated by inhibition of FAS is as follows: First, our activity-based protein profiling experiments indicate that FAS is the only serine hydrolase target for Orlistat in the breast cancer cell lines. We have noted three other bands with \( M_r \sim 90-150 \text{ kDa} \) that are also inhibited by Orlistat, but these appear to be breakdown products of FAS because they can be immune-precipitated with anti-FAS antibody and because they are knocked down with siRNA targeting FAS (see Fig. 3B). Second, Orlistat blocks the incorporation of \([^{14}\text{C}]-\text{malonyl} \text{ CoA}\) into palmitate, a biosynthetic reaction mediated by FAS. Third, the effects of Orlistat on tumor cell proliferation and regulation of Rb, p27\text{ Kip}^1 and Skp2 all occur at concentrations of the drug that approximate its cellular IC\text{50} for FAS (between 1 and 3 \( \mu \text{M} \)). Fourth, our prior work shows that Orlistat directly inhibits the activity of the recombinant thioesterase of FAS (24). Fifth, the effects of Orlistat on tumor cell proliferation and on regulation of Rb and p27\text{ Kip}^1 are mimicked by siRNA targeting FAS.
The present study also provides a new insight into the mechanisms underlying the connection between FAS and tumor cell proliferation. Orlistat arrests the cell cycle at the G₁/S transition. This effect was noted in all breast cancer cells we tested, along with tumor cells derived from the prostate (not shown). These findings suggest that the block in G₁/S progression is a common mechanism mediating Orlistat’s antiproliferative effects. Substantial decreases in DNA synthesis have likewise been noted in response to cerulenin and c75, other antagonists of FAS (19).

The G₁/S cell-cycle arrest elicited by a FAS blockade is mediated through the Rb pathway. Cells treated with either Orlistat or siRNA targeting FAS show decreased phosphorylation of Rb, and increased levels of the complex between Rb and E2F-1 (data not shown). The increased association of these two proteins prevents the transcriptional activity of E2F-1 essential for entry into S phase. We have traced the effects of a FAS blockade upstream of Rb, and found such a blockade to alter the levels of p27^Kip1 and Skp2. p27^Kip1 acts as a negative regulator of the cyclin-dependent kinases that ultimately phosphorylate Rb and therefore acts as a negative regulator of G₁/S transition (35). Inhibition of FAS led to increases in p27^Kip1 protein levels without affecting its transcription (data not shown), indicating a stabilization of p27^Kip1. Interestingly, inhibition of FAS also substantially reduces the levels of Skp2, an F-box protein essential for proteasome degradation of p27^Kip1. Like siRNA targeting FAS, an siRNA targeting Skp2 also increased p27^Kip1 levels and blocked phosphorylation of Rb. Consequently, we conclude that inhibition of FAS acts upstream of the proteasome to control p27^Kip1 levels and ultimately block cell-cycle progression. The mechanistic connections between the FAS blockade by Orlistat and reductions in Skp2 are the subject of current investigation.
The observations in this report are different from prior work in which cerulenin and c75 were employed as FAS inhibitors. These compounds block both G\textsubscript{1}/S and G\textsubscript{2}/M progression (16,19,36). We found no evidence that FAS blockade with Orlistat affected the G\textsubscript{2}/M transition point. There are two potential explanations for this difference in effect. One possibility is that inhibition of the different enzymatic pockets of FAS elicits distinct downstream effects. Although we cannot definitively exclude this possibility, it is difficult to envision how antagonists of distinct enzymatic pockets, each leading to inhibition of product formation, could elicit different effects. Another possibility is that cerulenin and c75 bind to additional molecular targets that account for the effects on G\textsubscript{2}/M. In this regard, we have begun to analyze clonal variants of MDA-MB-435 cells resistant to Orlistat. We have found these cells to retain sensitivity to cerulenin (data not shown), a finding that lends support to the idea that these two compounds have different mechanisms of action.

It is now clear that several avenues for antitumor therapy converge at the G\textsubscript{1}/S transition. Recent work indicates that Skp2 is one of the important G\textsubscript{1}/S regulatory points because it is necessary for ubiquitin-dependent degradation of p27\textsuperscript{Kip1} (32). Consequently, Skp2 is a positive regulator of cell-cycle progression. In fact, Skp2 has even been suggested as a potential drug target (37), an idea that is significantly strengthened by the recent observation that Skp2 levels are associated with reduced survival in prostate cancer (38). The present study shows that a FAS blockade ultimately decreases Skp2 levels. Therefore, FAS represents an upstream leverage point for targeting Skp2. Identifying and understanding the mechanism and molecular players that make this connection is an important future direction.
FIGURE LEGENDS

Figure 1. Serine hydrolase profile of mammary carcinoma cells.

Cell lysates were generated from three breast cancer cell lines (MDA-MB-435, MDA-MB-231, and MCF-7). Lysates were labeled with FP-PEG-TAMRA to tag active serine hydrolases (lanes marked -). Nonspecific labeling with the probe was measured in samples that were boiled prior to the addition of FP-PEG-TAMRA (lanes marked +).

Figure 2. Orlistat inhibits tumor cell proliferation by blocking G1/S progression.

(A) The effect of Orlistat on cell proliferation was monitored by measuring BrdU incorporation into cellular DNA. MDA-MB-435, MDA-MB-231 and MCF7 cells were exposed to Orlistat (0–100 µM) for 72 h and monitored for DNA synthesis during the final 24 h of treatment. Cell growth is normalized to untreated controls and expressed as a percentage of proliferation. Values are means ± SEM of 4 replicates per treatment.

(B) MDA-MB-435 cells were synchronized in the M phase using nocodazole. Following nocodazole removal and refeeding with fresh media at time 0, cells were exposed to 50 µM Orlistat (upper panel) or vehicle control (lower) and analyzed at various time points during a 24-h period. The distribution of cells in different phases of the cell cycle was monitored as a function of time using flow cytometry. The panels show representative flow cytometry profiles obtained from two experiments.

Figure 3. Orlistat and siRNA targeting FAS block fatty acid synthesis.

(A) The effect of Orlistat on serine hydrolase activity was measured by incubating MDA-MB-435 cell lysates with the drug for 20 min. The level of FAS protein was quantified by Western
blot (left panel), and the profile of active serine hydrolases was probed with the activity-based probe FP-PEG-TAMRA (right panel).

(B) The effect of siRNA targeting FAS was gauged by transfecting MDA-MB-435 cells with FAS siRNA, scrambled siRNA or only the lipofectamine reagent. The level of FAS protein was measured by Western blot (left panel), and the level of active FAS thioesterase was gauged with the activity-based probe FP-PEG-TAMRA (right panel).

(C) The effect of Orlistat on the synthesis of palmitate was measured in lysates of MDA-MB-435 cells incubated with 0.1-10 µM Orlistat for 1 h. Subsequently, the lysate was incubated with [14C]-malonyl CoA, and labeled fatty acids were extracted and quantified by scintillation counting. Values are means ± SEM of 3 replicates per treatment.

(D) The effect of siRNA targeting FAS on palmitate synthesis was measured 48 h after transfection using the approaches described for (B).

Figure 4. Inhibition of FAS leads to down regulation of Skp2.

(A) The effect of Orlistat on Rb phosphorylation and on the levels of p27 and Skp2 was measured in nocodazole synchronized MDA-MB-435 cells. Cells were exposed to a range of Orlistat for 20 h, lysed and subjected to analysis by Western blot.

(B) The effect of siRNA targeting FAS or Skp2 on Rb phosphorylation, and the levels of p27 was measured in MDA-MB-435 cells. Following a 48 h incubation after transfection with siRNA, cells were lysed and samples analyzed by Western blot.
REFERENCES


Acknowledgements

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Figure 1

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Markers:
- **FAS**
- **Dipeptide peptidases**
- **N-acyl peptide hydrolase**
- **Prolyl oligopeptidase**
- **Peroxisomal long-chain acyl-CoA thioesterase**
- **Serine proteases and epoxide hydrolases**
- **Lysophospholipase-1**
Figure 2

A

Orlistat [µM]

BrdU Incorporation (% Control)

B

Orlistat

Counts

Incubation time (h)

0 4 8 12 16 20 24

G1 → G2/M → S

Control

Counts

Incubation time (h)

0 4 8 12 16 20 24

G1 → G2/M → S
Figure 3

A  Orlistat

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B  siRNA

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C

![Graph C](image13)

D

![Graph D](image14)
Figure 4

A  Phospho-Rb  
Ser 807/811
  p27\textsuperscript{Kip1}  
  Skp2  
  β-tubulin

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B  Phospho-Rb  
Ser 807/811
  p27\textsuperscript{Kip1}  
  Skp2  
  β-tubulin

Mock  Scrambled  F-AS  Skp2
Supplemental Figure 1. Orlistat blocks G1/S progression by altering Rb, p27\textsuperscript{Kip1} and skp2 protein expression patterns. MDA-MB-435 cells were synchronized in the M phase using nocodazole. Following nocodazole removal and refeeding with fresh media at time 0, cells were exposed to 50 µM Orlistat or vehicle control and analyzed at various times over a 24 h period. The panels show representative data from two experiments. A, Total and phosphorylated Rb was measured in synchronized MDA-MB-435 cells. Orlistat suppressed Rb phosphorylation without altering total Rb protein expression. B, Effects of Orlistat on the Rb/E2F-1 complex were measured in nocodazole synchronized MDA-MB-435 cells. Cell lysates sampled at each time point were immunoprecipitated with E2F-1 antibody, and immunoblotted with antibody specific for Rb and E2F-1. Orlistat enhanced the association of Rb with E2F-1 and also decreased total E2F-1 protein levels. C, Orlistat (right panel) caused a delay in the increase in p27\textsuperscript{Kip1} and also prevented degradation of this protein over a period of 16–24 h. D,E, The status of the complex between cyclin E/cdk2/p27\textsuperscript{Kip1} was examined by immunoprecipitating lysates with anti-p27\textsuperscript{Kip1} (D) or anti-cyclin E (E) antibody, separating extracts by SDS-PAGE and immunoblotted with antibody specific for p27\textsuperscript{Kip1}, cyclin E and cdk2. Orlistat increased the levels of cyclin E and cdk2 bound to p27\textsuperscript{Kip1}. F, Orlistat prevented the accumulation of skp2 protein (16–24 h).
Supplemental Figure 1

A

Phospho-Rb

Rb

116 kDa

110 kDa

Phospho-Rb

Ser 780

Ser 795

Ser 807/811

Control

Orlistat

8 12 16 20

8 12 16 20

B

Rb/E2F-1 Complex: IP E2F-1

IB Rb

IB E2F-1

Control

Orlistat

0 4 8 12 16 20

4 8 12 16 20 24

C

p27Kip1

Control

Orlistat

0 4 8 12 16 20 24
**D**

Cyclin E/cdk2/p27 Complex: IP p27

IB Cyclin E
IB cdk2
IB p27

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**E**

Cyclin E/cdk2/p27 Complex: IP cyclin E

IB cyclin E
IB cdk2
IB p27

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**F**

skp2

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A fatty acid synthase blockade Induces tumor cell cycle arrest by down-regulating Skp2
Lynn M. Knowles, Fumiko Axelrod, Cecille D. Browne and Jeffrey W. Smith
J. Biol. Chem. published online May 11, 2004

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