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Therapeutic

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14. ABSTRACT Fatty acid synthase (FAS) is expressed at high levels in prostate cancer making it an attractive target for therapeutic intervention. Herein, we describe several important discoveries about the mechanism of action of FAS inhibitors, including orlistat. We have discovered that a primary determinant of the cell death pathway following FAS inhibition is the overall goal of this project is to understand the anti-tumor effects of FAS inhibitors. We have followed up the endoplasmic reticulum (ER) stress response. Moreover, we have identified a crosstalk between the FAS and proteasome pathway that, when perturbed, enhances ER stress signaling. In addition, preliminary data suggests that the ER stress and autophagy pathways may be acting in concert when FAS activity is reduced in tumor cells. In another line of investigation, we solved the first crystal structure of a FAS domain bound to ligand. The structure of the thioesterase domain of FAS bound to orlistat, in two states, was solved. Together, these data provide a framework, or blueprint, the design of novel FAS inhibitors, and understanding of their anti-tumor mechanisms and their future translation into the clinic.						
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2. Little, J.L., Wheeler, F., Koumenis, C., and Kridel S.J. , Inhibition of Fatty Acid Synthase Induces Endoplasmic Reticulum Stress in Tumor Cells (2007). <i>Cancer Research</i> , 67(3):1262-9.	
3. Pemble, C.W., Johnson, L.C., Kridel, S.J. , and Lowther W.T., Crystal structure of the thioesterase domain of human fatty acid synthase inhibited by Orlistat. (2007) <i>Nature Structural and Molecular Biology</i> 14(8): 704-709. (Article of the month highlight)	
4. Chen Y.Q., Edwards, I.J., Kridel, S.J. , Thornburg, T., and Berquin, I.M. Dietary fat-gene interactions in cancer. (2007) <i>Cancer and Metastasis Reviews</i> DOI 10.1007/s10555-007-9075-x (Invited Review)	
5. Kridel, S.J. , Lowther, W.T., and Pemble, C.W. Fatty acid synthase inhibitors: new directions for oncology. <i>Expert Opinion on Investigational Drugs</i> (2007) 16(11): 1817-29 (Invited Review)	

Introduction

The basic premise of this New Investigator proposal was based on two important findings. The first was the discovery that fatty acid synthase (FAS) is overexpressed in prostate cancer as well as in many other cancers [1,2]. Fatty acid synthase is the enzyme that catalyzes the synthesis of fatty acid from the precursors acetyl-CoA and malonyl-CoA [3]. The fatty acids are then utilized for subsequent phospholipid synthesis and membrane biogenesis. A body of literature has demonstrated that tumor cells are addicted to FAS derived fatty acids as inhibition of FAS activity induces cell death in tumor cells. The second was the initial discovery of the FDA-approved drug orlistat as an inhibitor of FAS [4]. Orlistat targets the thioesterase domain of FAS and induces cell death specifically in prostate tumor cells and inhibits the growth of prostate tumor xenografts in mice [4]. Based on these discoveries, three specific aims were proposed. They were to 1) determine the cellular consequences of FAS inhibition by orlistat, 2) to analyze the molecular basis for FAS inhibition by orlistat and 3) to select and characterize novel FAS inhibitor scaffolds using peptide phage display. A discussion on the final progress of these aims is reported in the body below. This funded proposal resulted in five publications (so far), presentation of ten abstracts at national meetings (including a best student poster award), four invited oral presentations and the funding of an RO1 from the NIH/NCI and an Idea Award from the PCRP.

Body

Specific aim 1. To determine the cellular consequences of FAS inhibition by orlistat. The FAS enzyme is localized and active in the cytoplasm of tumor cells. However, the fatty acid products of FAS are shuttled to the endoplasmic reticulum (ER) for downstream processes like extension to the 18-carbon fatty acid stearate, desaturation of palmitate or stearate, or incorporation of fatty acid into phospholipids. Because of this functional connection between FAS and the ER, we asked whether orlistat and other FAS inhibitors would induce ER stress in tumor cells. The ER stress response, also known as the unfolded protein response (UPR), is a protective mechanism activated to protect cells from various stresses [5]. The status of the ER is monitored by three proximal sensors; the ER resident eIF2 α kinase PERK, the kinase/endonuclease IRE1 and the ATF6 transcription factor precursor. Upon stress to the ER, protein are improperly folded which then induces a titration of the chaperone BiP (GRP78) off the proximal sensor to the unfolded proteins. Release of BiP from the proximal sensor activates the ER stress response.

As initially reported in a previous report, inhibition of FAS does indeed induce ER stress. This finding was published in *Cancer Research* in February 2007 (see **manuscript 2 [6] in Reportable outcomes and the appendix**), and will only be summarized here. We discovered that the FAS inhibitors orlistat, C75 and cerulenin induce a PERK-dependent phosphorylation of eIF2 α . Concomitant with this was the corresponding inhibition of protein synthesis. When eIF2 α is phosphorylated, several ER stress regulated genes are induced, including ATF4 and CHOP. Inhibition of FAS induces the expression of these ER stress regulated proteins as well. It is interesting to note that phosphorylation of eIF2 α is evident as soon as eight hours after treatment with FAS inhibitor, while canonical hallmarks of apoptosis like caspase activation and PARP cleavage do not become evident until approximately 24 hours after treatment. This finding indicates that ER stress precedes apoptosis in cells treated with FAS inhibitors and may actually signal a death response in the cells. Inhibition of FAS activity also activated the IRE1 pathway in tumor cells. FAS inhibition lead to an IRE1 mediated splicing of the ER stress specific transcription factor XBP1 to XBP1s. Importantly, all aspects of the ER stress response were only activated in tumor cells and not in normal cells. In addition, when protein burden on the ER was relieved by co-incubation with the general translation inhibitor cycloheximide, ER stress was reduced and death was abrogated. This indicates that ER stress may be a trigger to cell death with reduced FAS activity. Collectively these data make a number of important scientific contributions. The first is that the data provide a mechanism that may explain the anti-tumor effects of orlistat and other FAS inhibitors. Because FAS inhibitors are being developed as anti-tumor agents, these data could be important in the translation of these inhibitors into the clinic. The data also provide a potential teleological explanation for why tumors require high FAS levels; that is to support proper ER function.

The novel finding that FAS inhibitors induce ER stress prompted us to further this line of investigation. Several reports have identified a link between the FAS pathway and the proteasome pathway [7,8]. Interestingly, it has also been reported that the FDA-approved proteasome inhibitor Velcade induces ER stress in tumor cells [9]. That prompted us to determine the effects of combining FAS inhibitors with Velcade in prostate cancer cells. Surprisingly, the FAS inhibitors orlistat and C75 each induced accumulation of ubiquitin-modified proteins (Figure 1). Moreover, there was a more than additive effect on the accumulation of ubiquitin-modified proteins when FAS inhibitors were combined with Velcade. This result further suggests a functional connection, or crosstalk, between the FAS pathway and the proteasome pathway. Conversely, this notion is supported by the fact that Velcade induced fatty acid synthesis in a dose-dependent manner (Figure 2). Surprisingly, the increase in fatty acid synthesis was not due to changes in FAS levels but rather a transient increase in acetyl-CoA carboxylase (ACC) expression. ACC is the rate limiting step in the fatty acid synthesis pathway and catalyzes the carboxylation of acetyl-CoA to malonyl-CoA.

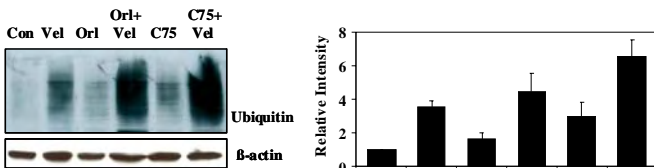


Figure 1. FAS inhibitors potentiate accumulation of ubiquitinated proteins. PC-3 cells were treated with the FAS inhibitors orlistat or C75 either alone or in combination with Velcade. Ubiquitin modified protein were detected by western blot (left) and quantified (right).

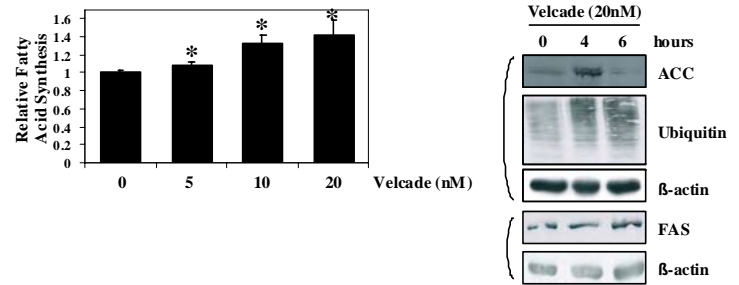


Figure 2. Velcade induces fatty acid synthesis. PC-3 cells were treated with the indicated concentrations of Velcade and fatty acid synthesis was determined by ¹⁴C-acetate incorporation (left). Protein expression levels of ACC and FAS were determined following Velcade treatment (right).

We next looked at the downstream readouts of the ER stress pathway. As mentioned above, ATF4 and CHOP are two genes whose expression is regulated by ER stress [10,11]. ATF4 is expressed as a function of eIF2 α phosphorylation. While orlistat and Velcade each induce ATF4 alone, the cells treated with both agents demonstrated a more rapid and robust expression (Figure 3). Similarly, expression of the ER stress regulated transcription factor CHOP was also robustly increased in cells treated with orlistat and Velcade (Figure 3).

CHOP has been associated with the death phase of the ER stress response [11].

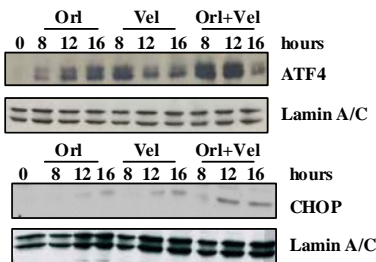


Figure 3. Increased ER signaling in FAS inhibitor and Velcade treated cells. Prostate tumor cells were treated with orlistat (25 μ M), Velcade (10 nM) or the combination for the indicated times. Lysates were collected and probed for ATF4, CHOP or Lamin by western blot.

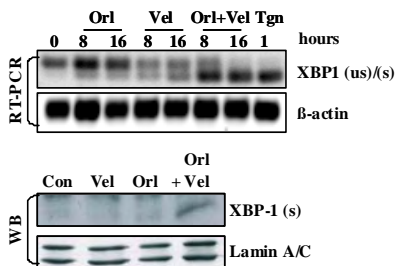


Figure 4. Increase in IRE1 signaling. Cells were treated as described above and RNA was collected by PCR analysis of XBP1 splicing (top) or lysates were collected for XBP1s protein expression.

We previously demonstrated that FAS inhibition induces the IRE1-dependent splicing of the ER stress specific XBP1 [6]. Low concentrations of FAS inhibitor when combined with low concentrations of Velcade induced the complete processing of XBP1 from the un-

spliced to the spliced form (Figure 4). Importantly, the result of the combined treatment resulted in XBP1s protein accumulation that was not seen with either of the single agent treatments. Hyper activation of the IRE1

pathway suggests that antagonizing the crosstalk between the two pathways enhances ER stress signaling. The splicing of XBP1 is associated with the adaptation response by IRE1 [5]. IRE1 also regulates an alarm response through activation of the JNK pathway [5]. Parallel to the observed XBP1 splicing, disruption of the feedback between the FAS and proteasome pathways resulted in a more rapid and robust activation of JNK signaling (Figure 5).

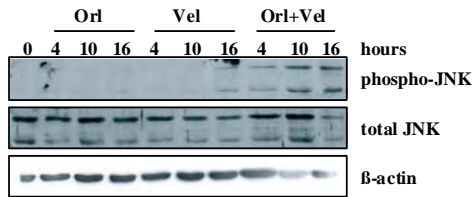


Figure 5. JNK activation in FAS inhibitor/Velcade treated cells. PC-3 cells were treated with orlistat (25 μ M), Velcade (10 nM) or the combination for the indicated times. Lysates were collected and p-JNK and total JNK were detected by western blot.

To evaluate the ultimate consequence of combining FAS inhibitors with Velcade cell death was also evaluated (Figure 6). After 16 hours, neither of the FAS inhibitor or Velcade treated cells demonstrated overt signs of apoptosis. On the other hand, when the FAS inhibitors were combined with Velcade there was a dramatic increase in the levels of cleaved caspase-3 and cleaved PARP, two canonical markers of apoptosis. Combining FAS inhibitors with Velcade also lead to a significant reduction in the clonogenic survival of prostate tumor cells compared to treatment with the corresponding single agents. The difference in cell killing between orlistat combined with Velcade and C75 combined with Velcade appears to correlate with the level of inhibition of FAS by the corresponding inhibitor (data not shown). A further cell death analysis to identify

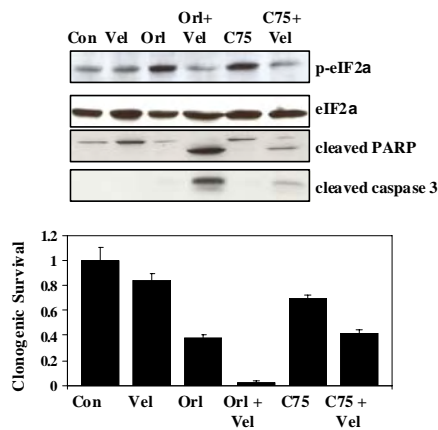


Figure 6. Increase in ER stress-dependent cell death. Cells were treated with Velcade (10nM), C75 (10 μ M), orlistat (25 μ M) or the combination of Velcade with FAS inhibitors for 16 hours. Lysates were collected and probed for cleaved PARP, cleaved caspase 3, p-eIF2 α and total eIF2 α (top). Clonogenic survival was also determined after the same treatment. Colonies were grown for approximately 14 days and stained with crystal violet and quantified.

synergy between the two agents is currently being undertaken. Preliminary evidence suggests that there is some level of synergy between the two classes of ER stressing agents (data not shown).

Because JNK was activated in cells treated with orlistat, Velcade or both, we asked what would happen if JNK were inhibited. Co-treatment with a JNK inhibitor decreased activation of caspase-3 and cleavage of PARP, indicating reduced apoptosis (Figure 7). In conjunction with this, JNK inhibition also reduced CHOP expression, consistent with the association between CHOP and apoptosis. Surprisingly, though, JNK inhibition reduced clonogenic survival in cells treated with orlistat, Velcade or both (not shown). The discrepancy between the clonogenic survival data and the western blots demonstrating reduced apoptosis forced further interpretation. Recent literature suggests that ER stress and autophagy can act in concert to protect cells against cellular stresses [12]. Therefore we hypothesized that FAS inhibitors might also be inducing autophagy. Indeed, early experiments demonstrate that FAS inhibitors induce the processing of LC3, one

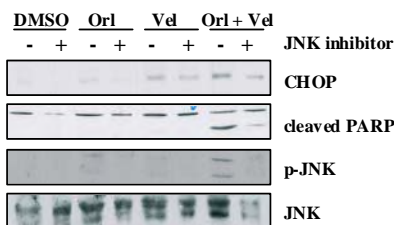


Figure 7. Inhibition of JNK reduces hallmarks of apoptosis. Cells treated with orlistat (25 μ M), Velcade (10 nM) or the combination, with or without JNK inhibitors (20 μ M). Lysates were collected after 24 hours and probed for CHOP, cleaved PARP and JNK activation

hallmark of autophagy (preliminary data not shown)[12]. These experiments are still in the early stages and require further analysis of the autophagy response. Collectively, the data

demonstrate how cells respond to depleted intracellular fatty acid levels and to instances when stress is further

amplified by proteasome inhibition. The crosstalk between the FAS and proteasome pathway will be valuable in understanding the interplay between ER stress, autophagy and cell death.

Specific aim 2. To analyze the molecular basis for FAS inhibition by orlistat. The FAS-TE domain (residues 2200-2502) was expressed as a His-tag fusion in *E. coli* and purified using a combination of nickel-NTA affinity and DEAE anion exchange chromatography. Following removal of the His-tag by biotinylated thrombin, the purified protein was incubated with an excess of Orlistat to ensure complete complex formation. The uncomplexed Orlistat was removed by passage through a Superdex 200 column. We have shown for the first time that the enzymatic action of FAS-TE on Orlistat results in the cleavage of the β -lactone ring, formation of a stable acyl-enzyme intermediate, and inhibition of the enzyme. The molecular weight of the FAS-TE:Orlistat complex (MW= 35116), as determined by electrospray mass spectrometry, is consistent with the addition of one Orlistat molecule (MW= 496 Da) to FAS-TE (MW= 34620 Da). Crystals of the complex were grown using the hanging-drop, vapor diffusion method using sodium dihydrogen phosphate and polyethylene glycol 3350 as the buffer and precipitant, respectively. Several data sets of the Orlistat complex have been collected under cryo-cooling conditions with ethylene glycol as the cryo-protectant at the National Synchrotron Light Source, Brookhaven National Laboratory. The structures were solved using the molecular replacement method using the original 2.6 Å structure as the search model within the program PHASER. These structures have been fully refined and a manuscript describing the results is in preparation. A brief summary of our findings is given below.

A 2.3 Å dataset collected from a crystal grown over a two week period clearly shows the covalent adduct between Ser2308 and the C1 carbonyl group of the former β -lactone ring of Orlistat (**please see Figure 1D in the Appendix, manuscript #3 published in *Nature Structural and Molecular Biology* [13]**). This complex ($R_{\text{work}}/R_{\text{free}} = 22.5/27.3\%$) provides the first rationale for the site of attack on the β -lactone ring and the unusual stability of the acyl-enzyme intermediate that results in inhibition. The attack of Ser2308 on the C1 atom over the C3 atom of Orlistat is consistent with the observed reactions with pancreatic lipase and chemical reactivity considerations. The stability of the intermediate appears to come from the extensive contacts of the lipophilic components of Orlistat to the enzyme surface (Figure 1F, manuscript #3 [13]). As a result the C3 hydroxyl oxygen atom and the nitrogen atom of the N-formyl moiety hydrogen bond to Glu2251. These interactions are of particular importance since they most likely prevent the activation of a water molecule by His2481 that under normal catalysis would readily hydrolyze the intermediate. Moreover, this binding mode is different from the typical interactions of a carbonyl group within the oxyanion hole of α/β hydrolases including serine proteases. If the FAS-TE:Orlistat complex followed the latter contexts, the ester group would interact with the backbone nitrogen atoms of Ile2250 and Tyr2309.

A 1.95 Å structure of the Orlistat complex has also been determined from crystals that took several months to grow. In this complex the acyl-intermediate was hydrolyzed ($R_{\text{work}}/R_{\text{free}} = 19.2/24.7\%$). Hydrolysis of the ester bond resulted in a carboxyl group at C1. This group interacts through a water molecule to the backbone nitrogen atoms of Ile2250 and Tyr2309 and the O γ atom of Ser2308. Together these changes cause a subtle shift of the inhibitor to the left when compared to the covalent adduct (Figure 3B, manuscript #3). Palmitate was proposed to partially bind in the interface cavity. In contrast, we observed that the fatty acid portion of Orlistat (extends off the C5 position) binds to what we have coined the ‘specificity channel’ and the N-formyl-Leu binds within interface cavity. Several conserved residues from subdomain B (e.g. Phe2423, Tyr2424, and Leu2427) line the channel. The binding of Orlistat to this channel is consistent with the observation that loop insertions in the α/β hydrolase fold in this region often modulate or mediate substrate binding.

The new FAS-TE:Orlistat structures are a significant advance for the structure-based design of compounds to inhibit FAS for cancer treatment. For example, the N-formyl-Leu moiety does not completely fill the interface cavity (Fig. 2, Manuscript #3 [13]). One can easily envision extending the chain further and to install functional groups that maximize interactions with the mixed hydrophobic/hydrophilic character of this

cavity. Another intriguing possibility would be to modify the 6-carbon group that extends off of the C2 position of Orlistat. We have already shown that treatment with Ebelactone B (EbeB) and not Ebelactone A (EbeA) leads to significant inhibition of FAS [4]. EbeB contains an ethyl group at C2 while EbeA contains a methyl group. Therefore, it is clear that a minimum of a 2-carbon chain is necessary at this position. Moreover, modifying the C2 group to maximize a charge-charge interaction with Glu2431 and/or a pi-stacking interaction with Tyr2343 may be extremely beneficial for improving the selectivity of the compound for FAS.

In summary, our work to date has resulted in several key observations. (1) Orlistat is in fact a substrate of FAS-TE. (2) FAS-TE is inhibited by the formation of a stable acyl-enzyme intermediate. (3) Hydrolysis of the intermediate occurs slowly. (4) Upon hydrolysis the binding mode of Orlistat shifts with respect to the catalytic triad. (5) The palmitate-like 16 carbon structure of Orlistat binds to an elongated hydrophobic channel that is exclusively generated by subdomain B. (6) This binding mode is in stark contrast to the proposed binding mode of palmitoyl-CoA based on a previous modeling/mutagenesis study.

Palmitoyl-CoA complex with FAS-TE. As described above, the Orlistat-FAS-TE complexes have led to a proposal for the binding of substrate into the specificity channel and not the interface cavity. One of the other goals of Specific Aim 2 was to determine the crystal structure of FAS-TE in complex with palmitoyl-CoA, a standard substrate mimetic. A determination of this structure will test the specificity channel binding hypothesis. Our efforts to determine this structure have been more challenging. Our initial efforts were to incubate pre-grown, wild-type FAS-TE crystals with a several fold molar excess of palmitoyl-CoA. All of the data sets collected on these crystals did not show the presence of the ligand in the active site. We feel that this phenomenon is most likely due to enzymatic activity of the domain which resulted in the hydrolysis of palmitoyl-CoA to palmitate and CoA. Therefore, we have mutated the active site Ser residue, Ser2308, to Ala to inactivate the enzyme. This mutant was pre-incubated with palmitoyl-CoA and then re-screened for crystallization conditions. Several crystallization conditions different from the Orlistat complex conditions have been identified. Diffraction data collected on these crystals has revealed that palmitoyl-CoA is bound in the active site within the specificity channel. However, the current electron density is still quite weak, indicating the ligand is only partially occupied. Additional optimization experiments have found that by slowly increasing the pH of the solution bathing the crystals (pH 5.5 to 7.5) increases the occupancy. We have also optimized the crystal growth and cryo-cooling conditions and are now routinely able to collect 2 Å data in house. Further optimization experiments are in progress and include varying the time of incubation and concentration of palmitoyl-CoA. Based on our Orlistat work, it may also be that stearic-CoA may prove to be more tightly bound since FAS-TE can also hydrolyze this substrate which is two carbons longer in length. The experiments highlighted here will be continued under the NIH R01 award secured with the DoD funding.

Specific aim 3. To select and characterize novel FAS inhibitor scaffolds using peptide phage display. Because of our success with the experiments related to Aims 1 and 2 we have not proceeded further with Aim 3.

Key Research Accomplishments:

- Fatty acid synthase inhibitors induce endoplasmic reticulum stress (manuscript 2).
- There is crosstalk between the fatty acid synthesis pathway and the proteasome pathway: FAS inhibitors induce accumulation of ubiquitin-modified proteins and proteasome inhibitors induce fatty acid synthesis.
- FAS inhibitors when combined with the FDA-approved proteasome inhibitor Velcade induce enhanced ER stress signaling and ER stress-associated cell death.
- FAS inhibitors induce autophagy.
- Determination of crystal structure of human FAS-TE domain in a covalent complex with Orlistat (manuscript 3)

- Determination of crystal structure of FAS-TE in complex with the hydrolyzed form of Orlistat (manuscript 3)
- Development of a model for β -lactone inhibition of FAS-TE
- Development a novel model of substrate binding to FAS-TE
- Generated initial crystals of the FAS-TE palmitoyl-CoA complex for further optimization.
- First demonstration that FAS is overexpressed in glioblastoma (manuscript 1).

Reportable Outcomes:

Manuscripts

1. Weiling Zhao, **Kridel, S.J.**, Thornburn, A., Little, J.L., Hebbar, S., and Robbins, M.E. Fatty acid synthase: a novel target for anti-glioma therapy. (2006) *British Journal of Cancer*, 95(7): 869-78.
2. Little, J.L., Wheeler, F., Koumenis, C., and **Kridel S.J.**, Inhibition of Fatty Acid Synthase Induces Endoplasmic Reticulum Stress in Tumor Cells (2007). *Cancer Research*, 67(3):1262-9.
3. Pemble, C.W., Johnson, L.C., **Kridel, S.J.**, and Lowther W.T., Crystal structure of the thioesterase domain of human fatty acid synthase inhibited by Orlistat. (2007) *Nature Structural and Molecular Biology* 14(8): 704-709. (**Article of the month highlight**)
4. Chen Y.Q., Edwards, I.J., **Kridel, S.J.**, Thornburg, T., and Berquin, I.M. Dietary fat-gene interactions in cancer. (2007) *Cancer and Metastasis Reviews* DOI 10.1007/s10555-007-9075-x (Invited Review)
5. **Kridel, S.J.**, Lowther, W.T., and Pemble, C.W. Fatty acid synthase inhibitors: new directions for oncology. *Expert Opinion on Investigational Drugs* (2007) 16(11): 1817-29 (Invited Review)

Abstracts

1. Little, J.L., Fels D.R., Koumenis, C., and **Kridel, S.J.**, (2005) Orlistat induces ER stress in prostate tumor cells. American Association for Cancer Research (AACR). Anaheim, California.
2. Little, J.L., Wheeler, F.B., Fels, D.R., Koumenis, C., and **Kridel, S.J.** Inhibition of fatty acid synthase induces ER stress-dependent cell death. American Association for Cancer Research (AACR), Washington, D.C. April 1-5, 2006.
3. Pemble, C.P., **Kridel, S.J.**, Lowther, W.T., Thioesterase domain of human fatty acid synthase: structural insights into chain-length selectivity. Meeting of the American Crystallographic Association, Honolulu, Hawaii, July 22-26, 2006.
4. Little, J.L., Wheeler F., Fels, D.R., Koumenis C. and **Kridel, S.J.**, Fatty acid synthase inhibitors induce ER stress-dependent cell death, 11th Prouts Neck Meeting in Prostate Cancer, Prouts Neck, Maine, November 2-5, 2006.
5. Little, J.L., Wheeler, F., Fels, D.R., Koumenis, C., and **Kridel, S.J.** Fatty acid synthase inhibitors induce ER stress-dependent cell death. American Association for Cancer Research (AACR) Innovations in Prostate Cancer Research, December 6-9, 2006, San Francisco, CA.
6. Pemble, C.W., Johnson, L.C., **Kridel, S.J.**, Lowther, W.T. β -lactone mediated inhibition and substrate selectivity revealed from the crystal structure of the thioesterase domain of human fatty acid synthase complexed with Orlistat. Virginia Tech Crystallography Symposium, Blacksburg, VA., May 2007.
7. Pemble, C.W., Johnson, L.C., **Kridel, S.J.**, Lowther, W.T. Crystal structure of the thioesterase domain of human fatty acid synthase inhibited by Orlistat. 37th Mid-Atlantic Macromolecular Crystallography Meeting, Charlottesville, VA, June 2007.
8. Pemble, C.W., Johnson, LC., **Kridel, S.J.**, Lowther, WT. Human fatty acid synthase complexed with the anti-tumor compound Orlistat.
9. Little, J.L., Wheeler, F., Fels, D.R., Koumenis, C., and **Kridel, S.J.** ER Stress

Upregulates the Fatty Acid Synthesis Pathway: Implications for Anti-Tumor Therapy.

American Association for Cancer Research (AACR), Los Angeles, CA, 2007.

10. Little, J.L., Wheeler, F.B., Fels, D.R., Koumenis, C.m and **Kridel S.J.** Crosstalk between the fatty acid synthesis and proteasome pathways enhance UPR signaling and cell death. Department of Defense, Prostate Cancer Research Program IMPACT meeting, Atlanta, GA September 5-8, 2007.

Awards:

Pemble, C.W., RCSB Protein Data Bank National Poster Prize, Meeting of the American Crystallographic Association, Honolulu, Hawaii, July 2006

Presentations

1. Novartis, Basel, Switzerland, July 2005. Title: Identification and characterization of novel fatty acid synthase inhibitors.
2. Molecular Genetics Graduate program, Wake Forest University School of Medicine, December 2006, Targeting fatty acid synthesis in tumor cells.
3. W. Todd Lowther, Molecular and Cellular Biology of Lipids, Gordon Research Conference, Waterville Valley Resort, NH, Crystal structure of the thioesterase domain of human fatty acid synthase inhibited by Orlistat, July 22-27, 2007
4. 10th International Conference on Bioactive Lipids in Cancer, Inflammation and Related Diseases, Montreal, Canada, September 16-19, 2007. Title: The role of *de novo* fatty acid synthesis in tumors.

Funding received, based on this award

CA411401-01	Kridel (PI, 4.2 months)	9/01/0 - 8/31/11	\$177,500
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NIH/NCI

Fatty acid synthase inhibitors and prostate cancer.

The goal of the proposal is to determine the role of the ER stress response in mediating the anti-tumor effects of fatty acid synthase inhibitors and to use x-ray crystallography to better understand FAS inhibitor binding to the thioesterase

PC060524	Kridel (PI, 3.6 months)	12/01/06-11/30/09	\$125,000
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DOD (PCRP)

Fatty acid synthase inhibitors engage the cell death program through the endoplasmic reticulum.

The goal of this proposal is to determine how calpains and ER generated ROS and Ca²⁺ activate cell death in prostate cancer cells. A second goal is to determine whether FAS inhibitor inhibit prostate tumor growth in a spontaneous mouse model of prostate cancer.

Conclusion

As detailed in the body of this final report, several novel contributions have resulted from this funded New Investigator award. First, we demonstrated that FAS inhibitors induce ER stress, specifically in tumor cells. In addition, in our most recent studies, we have preliminary evidence to suggest that FAS inhibitors also induce autophagy. Because FAS expression and activity is almost uniquely limited to tumor tissue, the FAS enzyme has become an attractive target for therapeutic development. Several FAS inhibitors have been identified, including the focus of this proposal, Orlistat. The comprehensive analysis employed for the work in this proposal demonstrated that orlistat, two other FAS inhibitors, and siRNA knockdown of FAS all induce the ER stress response in tumor cells. These findings will be important in the development and translation of next generation FAS inhibitors into the clinic. The finding that FAS inhibitors can be combined with the FDA-approved proteasome inhibitor Velcade to enhance ER stress signaling and cell death further highlights the importance of the ER stress response in mediating the therapeutic activity of FAS inhibitors. The second major finding resulting from this funded proposal is that FAS inhibitors also induce autophagy. A small but

growing body of literature suggests that ER stress and autophagy are intimately linked. Because FAS inhibitors induce both ER stress and autophagy, we also think the two pathways are intimately linked. Work in the coming months will be geared toward addressing this issue.

The third, and perhaps most impactful, contribution from this funded proposal was determining the structure of orlistat bound to the thioesterase domain of FAS. The significance of this finding is several fold. First, the FAS-orlistat structure is the first, and only, published structure of any human FAS domain bound to a ligand. Second, orlistat appears to bind the active site as a substrate mimetic. That is, the fatty acyl-like chain of orlistat resembles the natural substrate of FAS, palmitate. Third, the crystal structure provides a framework, or blueprint, for the development of novel FAS inhibitors. Although orlistat is FDA approved and an effective drug against tumor cell *in vitro*, it has poor bioavailability. As a result, orlistat will not likely be effective as an anti-tumor agent for prostate cancer in its current formulation. The data from our crystal structure provides us a mechanism for the development of novel FAS inhibitors that target the thioesterase domain. This is a line of investigation that is currently underway in the laboratory.

So what does this body of knowledge contribute? Several academic laboratories and pharma companies are developing inhibitors against FAS. The work presented in this report highlight multiple novel findings about the action of FAS inhibitors. This will contribute to the development of FAS inhibitors and provide an avenue toward the translation of FAS inhibitors into the clinic for potential use in treating men with prostate cancer.

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Fatty acid synthase: a novel target for anti-glioma therapy

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High levels of fatty acid synthase (FAS) expression have been observed in several cancers, including breast, prostate, colon and lung carcinoma, compared with their respective normal tissue. We present data that show high levels of FAS protein in human and rat glioma cell lines and human glioma tissue samples, as compared to normal rat astrocytes and normal human brain. Incubating glioma cells with the FAS inhibitor cerulenin decreased endogenous fatty acid synthesis by approximately 50%. Cell cycle analysis demonstrated a time- and dose-dependent increase in S-phase cell arrest following cerulenin treatment for 24 h. Further, treatment with cerulenin resulted in time- and dose-dependent decreases in glioma cell viability, as well as reduced clonogenic survival. Increased apoptotic cell death and PARP cleavage were observed in U251 and SNB-19 cells treated with cerulenin, which was independent of the death receptor pathway. Overexpressing Bcl-2 inhibited cerulenin-mediated cell death. In contrast, primary rat astrocytes appeared unaffected. Finally, RNAi-mediated knockdown of FAS leading to reduced FAS enzymatic activity was associated with decreased glioma cell viability. These findings suggest that FAS might be a novel target for anti-glioma therapy.

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In 2006, 18 820 individuals living in the US will be diagnosed with primary brain cancer (Jemal *et al*, 2006). The most common and most lethal primary brain tumour in adults is glioblastoma multiforme. Glioblastoma multiforme represents one of the most challenging of all cancers to treat successfully, characterised not only by aggressive proliferation and expansion but also by inexorable tumour invasion into distant brain tissue (Kew and Levin, 2003). Improvements in multimodality treatments including surgery, radiotherapy, and the recent addition of temozolomide have improved median survival, but this is still only approximately 14 months (Stupp *et al*, 2005) and long-term survival is dismal. The development of novel biological therapies for primary brain tumours remains an urgent need. Current cancer treatment is rapidly evolving in parallel with an understanding of tumour biology (Weiss, 2000; Karpati and Nalbantoglu, 2003). Genetic abnormalities and differential gene expression between normal and cancer cells can provide novel targets for anticancer therapy (Lang *et al*, 1999; Bansal and Engelhard, 2000; Grill *et al*, 2001).

Fatty acid synthase (FAS) is a 270 kDa cytosolic multifunctional polypeptide and is the primary enzyme required for catalysing the conversion of dietary carbohydrates to fatty acids (Wakil, 1989). Normal cells preferentially use circulating dietary fatty acids for the synthesis of new structural lipids. Thus, FAS expression is generally low to undetectable in normal human tissues, other than the liver and adipose tissue. In contrast, FAS is overexpressed in many human tumours, including carcinoma of the breast (Milgraum *et al*, 1997; Wang *et al*, 2001b), prostate (Epstein

et al, 1995; Swinnen *et al*, 2002), colon (Rashid *et al*, 1997), ovary (Gansler *et al*, 1997), endometrium (Pizer *et al*, 1998b), mesothelium (Gabrielson *et al*, 2001), lung (Piyathilake *et al*, 2000), thyroid (Vlad *et al*, 1999), and stomach (Kusakabe *et al*, 2002). Abnormally active endogenous fatty acid metabolism appears to be important for cancer cell proliferation and survival (Ookhtens *et al*, 1984). Moreover, overexpression of FAS in breast, prostate, and thyroid cancers has been associated with more aggressive malignancies (Epstein *et al*, 1995; Gansler *et al*, 1997; Vlad *et al*, 1999; Swinnen *et al*, 2002). The preferential expression of FAS in cancer cells suggests that FAS could be a promising target for antitumour therapy (Kuhajda, 2006).

Inhibitors of FAS have been used to study the loss of FAS function in tumour cells. The first identified 'specific' inactivator of FAS, cerulenin, (2R, 3S)-2,3-epoxy-4-oxo-7,10-trans,trans-dodecadienamide, is a natural antibiotic product of the fungus *Cephalosporium ceruleans* (Omura, 1976). Cerulenin irreversibly inhibits FAS by binding covalently to the active site cysteine thiol in the β -ketoacyl-synthase domain (Funabashi *et al*, 1989). Cerulenin is selectively cytotoxic to a number of established human cancer cell lines, including breast (Gabrielson *et al*, 2001), colon (Pizer *et al*, 1998a), and prostate (Furuya *et al* 1997; Pizer *et al*, 2001). Kuhajda *et al* (2000) reported that cerulenin inhibited fatty acid synthesis in tumour cells in a dose-dependent manner; the cytotoxic effect of cerulenin to human breast tumour cells generally paralleled the level of endogenous fatty acid synthesis. Fatty acid synthase inhibition by cerulenin leads to apoptotic cell death in breast, prostate, and colon cancer cells (Furuya *et al*, 1997; Huang *et al*, 2000; Li *et al*, 2001). However, cerulenin's chemical instability renders it inappropriate as a systemic anticancer agent. C75, a potent derivative of cerulenin and a more stable form of FAS inhibitor, has been tested recently for its anti-breast tumour effects

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(Kuhajda *et al*, 2000; Pizer *et al*, 2000). *In vivo* and *in vitro* studies have confirmed the selective toxicity of C75 against tumour cells. C75-mediated inhibition of FAS increases malonyl-CoA levels and inhibits CPT-1 activity, preventing the oxidation of newly synthesised fatty acids. High levels of malonyl-CoA and CPT-1 inhibition might represent a mechanism, whereby FAS inhibition leads to tumour cell death (Pizer *et al*, 2000). C75 treatment of mesothelioma and prostate cancer xenografts in nude mice led to significant inhibition of tumour growth (Gabrielson *et al*, 2001; Pizer *et al*, 2001). Subcutaneous xenografts of MCF7 breast cancer cells in nude mice treated with C75 showed fatty acid synthesis inhibition, apoptosis, and inhibition of tumour growth to less than 12.5% of control volumes, without comparable toxicity in normal tissues (Pizer *et al*, 2000).

More recently, an activity-based proteomics strategy revealed *in vitro* and *in vivo* antitumour activity for Orlistat, an FDA-approved drug used for treating obesity. Orlistat is a novel inhibitor of the thioesterase domain of FAS that inhibited prostate cancer cell proliferation, induced apoptosis, and inhibited the growth of PC-3 human tumour xenografts implanted in nude mice (Kridel *et al*, 2004).

To date, there is little information regarding FAS expression in brain tumours. Slade *et al* (2003) reported that significantly larger amounts of FAS protein were detected in the neuroblastoma cell line SK-N-SH compared with that in the human fibroblast cell line Hs27. We hypothesised that FAS would be highly expressed in glioma cells and that inhibition of FAS would lead to glioma cell death. In this paper, we show that this hypothesis appears to be correct. For the first time, we show upregulation of FAS in glioma cells and human glioma tissue, dose- and time-dependent decreases in glioma cell viability, and clonogenic survival with FAS inhibition, as well as increased apoptotic cell death. These studies identify FAS as a potential target for glioma therapy.

MATERIALS AND METHODS

Cell culture

Human U373, U118, U87, and U138 as well as rat C6 glioma cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). The ethyl nitrosourea-induced rat 36B10 glioma cell line was obtained from Dr Vincent Traynelis, Division of Neurosurgery, Department of Surgery, University of Iowa (Spence & Coates, 1978) and routinely maintained in high glucose DMEM containing 5% bovine calf serum, 2 mM L-glutamine, 100 IU ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, and 0.1 mM non-essential amino acids (all from GIBCO, Gaithersburg, MD, USA) at 37°C with 5% CO₂ in air. Cells were trypsinised and reseeded at a 1:5 dilution every 3 days. Human U251 and SNB-19 glioma cell lines were a kind gift of Dr Sue Hess (Department of Radiation Oncology, WFUSM) and were maintained in DMEM/F12 and F10 media supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 100 IU ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin. Primary rat astrocytes were isolated from 1- to 2-day-old Sprague-Dawley rat pups as described previously (Murphy, 1990). Cortices were removed from the rat pups; the meninges were stripped and homogenised. After incubation with trypsin for 15 min in a 37°C shaking water bath, the homogenate was centrifuged and the pellet was resuspended in a trypsin inhibitor/DNAse solution, triturated, and layered over a bovine serum albumin solution. The cell pellet was resuspended and maintained in MEM medium with 1% L-glutamine, 10% FBS, and 6g l⁻¹ glucose at 37°C in a humidified atmosphere of 5% CO₂ until ready to use.

Human glioma and normal brain tissue

Human brain tissue lysates were prepared by homogenisation in modified RIPA buffer (150 mM sodium chloride, 50 mM Tris-HCl

(pH 7.4), 1 mM phenyl methyl sulphonyl fluoride, 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% sodium dodecyl sulphate (SDS), 5 µg ml⁻¹ aprotinin, and 5 µg ml⁻¹ leupeptin). The tissue lysates were centrifuged for 10 min at 10 000 r.p.m. to remove debris and the supernatants were stored at -80°C until use.

Fatty acid synthesis

To test whether cerulenin inhibits FAS activity in glioma cells, lipid synthesis was determined using ¹⁴C-labelled acetic acid methods as described previously (Rashid *et al*, 1997). Briefly, 5 × 10⁵ SNB-19, U251, and C6 cells were plated into 24-well plates for 24 h (three replicates per group). After incubation with 5 µg ml⁻¹ cerulenin for 2 h at 37°C, the cells were washed and were then incubated with ¹⁴C-labelled acetate for 4 h. Total lipids were extracted and ¹⁴C counts determined using liquid scintillation counting.

Western blot analysis

Western blot analysis was performed as described previously (Zhao *et al*, 2001). Cells were lysed using lysis buffer containing 50 mM Tris (pH 7.0), 1 mM EDTA, 150 mM NaCl, 1 mM PMSF, 1 µg ml⁻¹ aprotinin, 1 µg ml⁻¹ leupeptin, 1 mM Na₃VO₄, and 1 mM NaF, and stored in aliquots at -70°C until use. Ten micrograms of cell lysate was mixed with an equal volume of sample buffer containing 62.5 mM Tris/HCl (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 2–3 drops of saturated bromophenol solution, denatured by boiling, and separated in a 7.5% polyacrylamide mini-gel at a constant voltage of 120 V for 2 h. The proteins were transferred by electrophoresis at 100 V for 1 h to ECL nitrocellulose membrane (Amersham, Arlington Heights, IL, USA). The membranes were blocked for 1 h at room temperature in 5% (wt vol⁻¹) non-fat dry milk in TTBS containing 20 mM Tris-HCl (pH 7.0), 137 mM NaCl, and 0.05% (vol vol⁻¹) Tween-20. After washing in TTBS for 2 × 10 min, the membranes were then blocked with 5% non-fat milk and probed with either primary mouse anti-FAS (Pharmingen, San Diego, CA, USA), mouse anti-Bcl-2 (Santa Cruz, Santa Cruz, CA, USA), or rabbit anti-poly(ADP-ribose) polymerase (PARP) antibody (Athens Biotech, Athens, GA, USA). After washing three times in TBST for 10 min each, the blots were incubated with anti-mouse or anti-goat IgG horseradish peroxidase conjugate (1:10 000 dilution, Sigma, St Louis, MO, USA) for 1 h at room temperature. Antigen was detected using standard chemical luminescence methodology (ECL; Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Cell viability assay

Cell viability was determined using a modified MTT assay (Griscelli *et al*, 2000). Briefly, 1000–5000 cells well⁻¹ were plated in 24-well plates and incubated overnight. Cells were then treated with 0.5–10 µg ml⁻¹ cerulenin for 24–144 h. At the end of the follow-up period, MTT in PBS was added and the cultures were incubated for 4 h at 37°C. The dark crystals formed were dissolved by adding to the wells an equal volume of SDS/dimethylformamide (DMF) extraction buffer (20% SDS and 50% N, N-DMF (pH 4.7), in PBS). Subsequently, plates were incubated overnight at 37°C. A 100 µl aliquot of the soluble fraction was transferred into 96-well microplates, and the absorbance at 570 nm was measured using an ELISA plate reader.

Clonogenic survival assay

Clonogenic survival assays were performed as described previously (Vartak *et al*, 1998). Two hundred and fifty to 2000 cells were plated in 60 mm dishes and incubated overnight. Cells were then treated with 0–10 µg ml⁻¹ cerulenin and cultured until colonies had formed (10–14 days). Cells were then fixed for 10 min in a

solution containing 10% acetate and 10% methanol. The cells were then stained with 0.4% crystal violet for another 10 min. The crystal violet was removed and cells were washed with tap water until clear. Colonies containing ≥ 50 cells were counted. For each experiment, four culture dishes were performed and experiments were carried out in triplicate. Surviving fraction was calculated from the number of colonies formed in the cerulenin-treated dishes compared with the number formed in the untreated control, where plating efficiency is defined as the percentage of cells plated that form colonies, and surviving fraction = number of colonies formed/(number of cells plated \times plating efficiency).

Irradiation

Cells were irradiated with a range of single doses of 0–8 Gy of γ rays using a ^{137}Cs self-shielded irradiator at a dose rate of 4.0 Gy min $^{-1}$. All irradiations were performed at room temperature; control cells received sham irradiation. After irradiation, the culture plates were returned to the incubator and maintained at 37°C for 96 h. Cell viability was assessed using the MTT assay described above.

Flow cytometric analysis of cell cycle status

Flow cytometric evaluation of the cell cycle was performed using a modified protocol (Amant *et al*, 2003). Briefly, 5×10^5 cells were plated on 100 mm dishes in medium complemented with 10% FBS overnight. The culture medium was then replaced using serum-free medium for 24 h and the cells treated with 0–10 $\mu\text{g ml}^{-1}$ cerulenin for 24 h. Cells were then trypsinised and collected into ice-cold PBS, and fixed with ice-cold 70% ethanol in PBS for at least 24 h at 4°C. Cells were then stained using 1 ml propidium iodide (PI) solution (20 mg l $^{-1}$ PI and 20 mg l $^{-1}$ RNase in PBS) for 3 h and read on a flow cytometer (Beckman-Coulter, Fullerton, CA, USA).

Fatty acid synthase shRNA constructs

The nucleotide targets of the FAS coding sequence were selected based on previously published experiments (De Schrijver *et al*, 2003) and computer-based target analysis using siRNA Target Finder (Ambion Inc., Austin, TX, USA). Two 19 base pair siRNA oligonucleotides were used in our study according to siRNA selective guidelines (Ambion Inc.). The first oligonucleotide was AACCTGAGATCCCAGCGCTG, corresponding to the nucleotides 1212–1231 of human FAS; the second oligonucleotide was AAGCAGGCACACACGATGGAC, corresponding to the nucleotides 329–348. The two target sequences were aligned to the human genome in a BLAST search to eliminate those with significant homology to other genes. A scrambled nucleotide sequence was designed and used as the negative control. The loop sequences and overhangs were added to form the short hairpin constructs. Then, the short hairpin RNA (shRNA) encoding oligonucleotides were cloned into the *Bam*H1 and *Hind*III restriction sites downstream of the H1 promoter in pSilencer 3.0-H1 according to the manufacturers' instructions (Invitrogen, Grand Island, NY, USA).

Transient transfection

Transient transfections were performed using Lipofectamine according to the manufacturer's instructions (Invitrogen). Briefly, 5×10^6 cells were plated in a 60 mm dish supplied with 5 ml media for 24 h. Cells were then washed twice with serum- and antibiotic-free DMEM medium. Four micrograms of FAS RNAi plasmids/scrambled negative control plasmid and 20 μl Lipofectamine were premixed for 20 min and applied to cells in 4 ml serum- and antibiotic-free DMEM medium. After 4 h, the serum- and antibiotic-free DMEM medium was replaced with 5 ml of complete medium.

Stable transfection

U251 cells were grown on 60 mm dishes for 24 h and were then transfected with 2 μg of the endoplasmic reticulum (ER)-targeted GFP-Bcl-2 expression vector, wild-type (WT) GFP-Bcl-2 expression vector, or empty vector (gift of Dr Clark W Distelhorst, Case Western Reserve University Medical School and University Hospitals of Cleveland, Cleveland, OH, USA) using Lipofectamine plus reagent according to manufacturer's protocol (Life Technologies). The positive transfected cells were selected in the presence of the neomycin-analogue G418 (800 $\mu\text{g ml}^{-1}$).

Dominant-negative FADD expression

Recombinant doxycycline (Dox)-regulated YFP and dominant-negative YFP-FADD-DN adenoviruses were made using the AdenoX Tet-off kit from Clontech (Palo Alto, CA, USA). Viruses were produced according to the manufacturer's instructions. Dominant-negative adenovirus FADD-DN or adenovirus YFP (empty vector) were co-infected into U251 and SNB-19 glioma cells with a Tet repressor virus. Once FAD-DN was expressed at high levels, as demonstrated by robust YFP fluorescence, cells were treated as required.

Statistical analysis

Statistical analysis was carried out using one-sample Student's *t*-test to compare differences between the treated cells and their appropriate controls. A *P*-value of <0.05 was considered significant.

RESULTS

Fatty acid synthase is overexpressed in human and rat glioma cell lines as well as in human glioma tissue samples

To determine if there is differential expression in glioma cells compared with normal brain cells, we measured FAS protein levels in six human glioma cell lines, U251, U373, U138, U118, U87, and SNB-19 and in two rat glioma cell lines, C6 and 36B10, as well as in primary rat astrocytes. Cells were cultured and lysed in 500 μl lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, and 1 mM of EDTA, PMSF, Na₃VO₄ and NaF, and proteinase inhibitors. Fatty acid synthase protein levels were analysed by Western blotting. As shown in Figure 1A, a low level of FAS protein was detected in normal primary astrocytes. In contrast, markedly elevated levels of FAS protein were observed in the entire human and rat glioma cell lines. Densitometric analysis revealed 4- to 20-fold increases in FAS expression in the glioma cell lines in comparison with that seen in primary rat astrocytes (Figure 1B). Elevated levels of FAS protein, ranging from 2- to 3.5-fold increases, were also observed in lysates of human glioma tissue samples compared with those obtained from normal human brain (Figure 1C and D).

Treating glioma cells with the FAS inhibitor cerulenin leads to inhibition of fatty acid synthesis

Cerulenin, a potent noncompetitive pharmacological inhibitor of FAS, binds covalently to the active site of the condensing enzyme region, inactivating a key enzyme step in fatty acid synthesis. To confirm the inhibitory effect of cerulenin on FAS in glioma cells, fatty acid synthesis activity was measured using the ^{14}C -labelled acetic acid method (Rashid *et al*, 1997). U251 and SNB-19 human glioma as well as C6 rat glioma cells were incubated with or without cerulenin for 2 h. After labelling with ^{14}C -actinic acid, the total lipid was extracted and the amount of ^{14}C incorporated in the extracted total lipids was determined using liquid scintillation

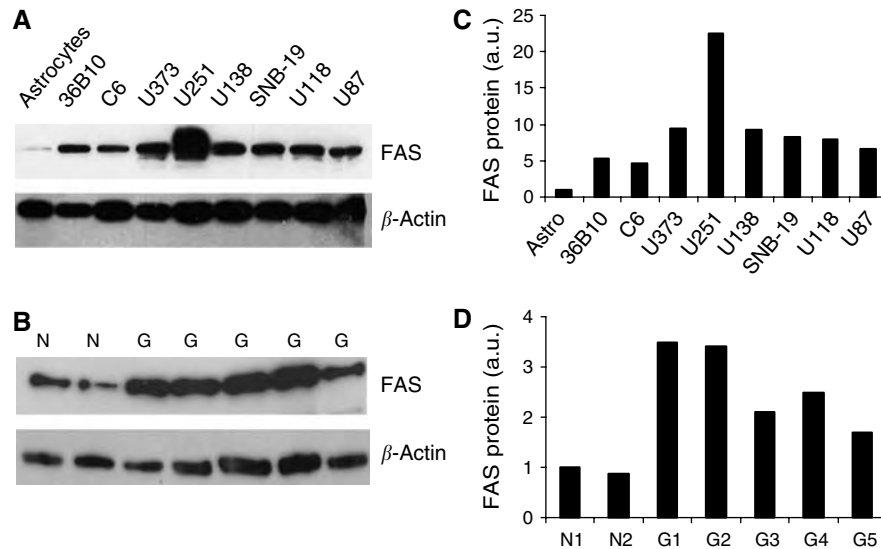


Figure 1 Fatty acid synthase is overexpressed in human and rat glioma cell lines and in human glioma tissue compared with primary rat astrocytes and normal human brain tissue, respectively. U138, U251, U373, SNB-19 human, and C6 rat glioma cells, as well as primary rat astrocytes were grown on 100 mm dishes until 90% confluent. The protein level of FAS was analysed by Western immunoblotting using a monoclonal mouse anti-FAS antibody; β -actin protein level was used as a loading control. **(A)** A representative Western Blot indicating the differential expression of FAS in rat and human glioma cell lines as well as primary rat astrocytes. **(C)** The mean densitometric values obtained from two independent experiments. **(B)** A Western blot of lysates obtained from normal human brain as well as several human glioma tissue samples. **(D)** The densitometric analysis of these blots.

counting. As shown in Figure 2, endogenous fatty acid synthesis in cerulenin-treated glioma cells decreased by approximately 50% in SNB-19, U251, and C6 cells compared with untreated controls. Fatty acid synthase activity was not detectable in primary rat astrocytes (data not shown).

Incubating glioma cells with cerulenin leads to selective time- and dose-dependent decreases in glioma cell viability and survival; normal astrocytes appear unaffected

To demonstrate whether cerulenin is selectively cytotoxic to glioma cells, we measured the effects of cerulenin on glioma cells and primary rat astrocytes using the MTT and/or clonogenic assay. U251, SNB-19, U87, U118 human, and C6 rat glioma cells were treated with 0–15 $\mu\text{g ml}^{-1}$ cerulenin for 24 and 96 h. We observed rounded up cells following incubation with higher doses (Figure 3A and B) in 24 h and cell death. As shown in Figure 3C, the mean IC_{50} values for U251 cells were 4.7 and 2.3 $\mu\text{g ml}^{-1}$ at 24 and 96 h, respectively. SNB-19 glioma cells showed a similar cytotoxic response to treatment with cerulenin; the mean IC_{50} values for SNB-19 cells were 5.3 and 2.6 $\mu\text{g ml}^{-1}$ at 24 and 96 h, respectively (Figure 4A). Moreover, treatment with cerulenin led to a significant reduction in glioma cell clonogenic survival; incubating SNB-19 cells with 3 $\mu\text{g ml}^{-1}$ cerulenin reduced clonogenic cell survival by 97% compared to non-treated controls (Figure 4B). We also compared the cytotoxicity of cerulenin to C6 rat glioma cells and to normal primary rat astrocytes. After treatment with 10 $\mu\text{g ml}^{-1}$ of cerulenin for 24 h, glioma cell viability was reduced to less than 10%; the mean IC_{50} value was 6.5 $\mu\text{g ml}^{-1}$ (Figure 4C). In contrast, primary astrocytes appeared resistant to cerulenin treatment. Incubating astrocytes with 0.5–7.5 $\mu\text{g ml}^{-1}$ cerulenin for 24 h failed to affect cell viability (Figure 4D).

Cerulenin-induced cell death occurs via an apoptotic mechanism

Poly(ADP-ribose) polymerase, an enzyme catalysing the poly(ADP-ribosyl)ation of various nuclear proteins with NAD, has been

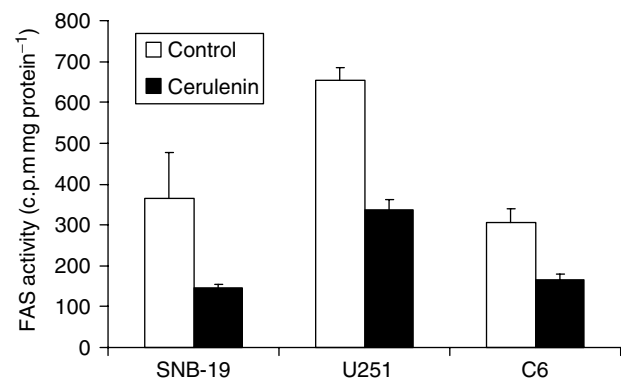


Figure 2 Treating glioma cells with the FAS inhibitor cerulenin leads to inhibition of fatty acid synthesis. U251 and SNB-19 human and C6 rat glioma cells were treated with 5 $\mu\text{g ml}^{-1}$ cerulenin for 2 h. Fatty acid synthesis activity was then measured using the ^{14}C -labelled acetate assay. Lipids were extracted and cerulenin-induced inhibition of labelled acetate incorporation into lipid was measured using liquid scintillation counting. Mean \pm s.e.; $n = 3$; * $P < 0.05$.

suggested to contribute to cell death by depleting the cell of NAD and ATP (Berger *et al*, 1983). Poly(ADP-ribose) polymerase cleavage is a well-established marker for apoptosis. To determine whether apoptosis is involved in cerulenin-induced cell cytotoxicity, we measured PARP cleavage as well as assessing morphological changes. As noted above, cell morphology was changed; cells appeared rounded up after incubation with cerulenin, resulting in cell death. To determine PARP cleavage, U251 and SNB-19 cells were treated with 0–10.0 $\mu\text{g ml}^{-1}$ cerulenin for 24 h and Western blot analysis was used to study the alterations in protein level of PARP in treated and non-treated glioma cells. Treatment with cerulenin led to a dose-dependent increase in cleaved PARP protein in both glioma cell lines (Figure 5).

Overexpressing Bcl-2 leads to inhibition of cerulenin-mediated cytotoxicity

Overexpression of the antiapoptotic protein Bcl-2 has been shown to inhibit cell death induced by several apoptotic signals and/or

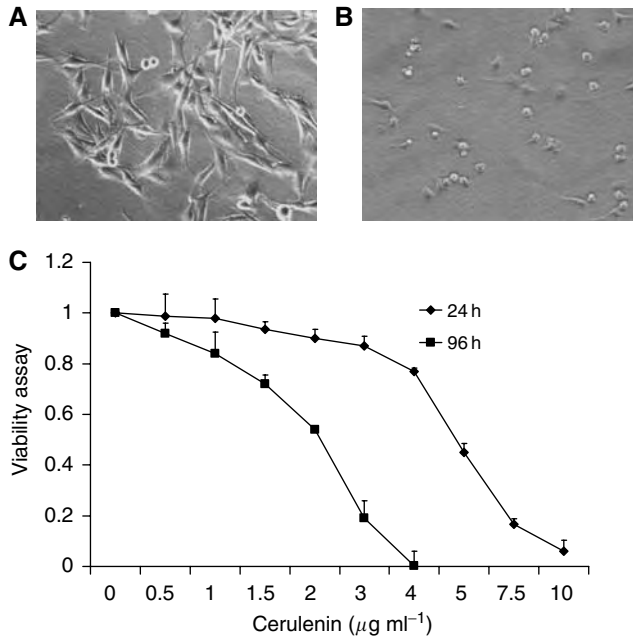


Figure 3 Cerulenin reduces U251 cell viability in a time- and dose-dependent manner. 5×10^4 or 5000 U251 human glioma cells were seeded into 24-well plates and treated with 0–10 $\mu\text{g ml}^{-1}$ cerulenin for 24 and 96 h, respectively. Cell viability was assessed using the MTT assay. (A) The morphological appearance of DMSO-treated control cells observed 24 h after treatment. (B) Incubating U251 glioma cells with cerulenin for 24 h resulted in the cells adopting a rounded up appearance. (C) Incubating U251 glioma cells with cerulenin cells led to a time- and dose-dependent reduction in cell viability. Mean \pm s.e.; $n = 3$.

mediators (Wang *et al*, 2001a). To determine if overexpressing Bcl-2 would result in a similar inhibition of cerulenin-mediated apoptosis, U251 cells were stably transfected with either WT Bcl-2, ER-targeted Bcl-2, or empty vector. As shown in Figure 6A, transducing cells with Bcl-2 led to a marked increase in protein levels compared with the vector-transduced cells. U251 cells were then treated with 5 $\mu\text{g ml}^{-1}$ of cerulenin for 24 h, and cell viability assessed using the MTT assay. As noted previously, incubating U251 cells stably transfected with vector control alone with 5 $\mu\text{g ml}^{-1}$ cerulenin was associated with a reduction in cell viability to 55.5 \pm 3.4% of controls. Overexpressing ER/Bcl-2 and WT/Bcl-2 increased cell viability to 71.0 \pm 3.2 and 85.9 \pm 2.8%, respectively ($P < 0.05$; Figure 6).

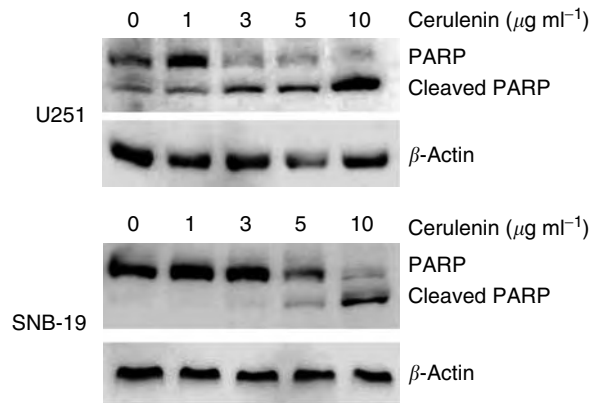


Figure 5 Cerulenin induces PARP cleavage in human glioma cells. U251 and SNB-19 cells were treated with 0–10 $\mu\text{g ml}^{-1}$ cerulenin for 24 h and then collected and lysed using lysis buffer. Poly(ADP-ribose) polymerase, cleaved PARP, and β -actin protein levels were analysed using Western blot. Representative Western blots show that cerulenin treatment was associated with dose-dependent increases in cleavage of PARP in both U251 (A) and SNB-19 (B) glioma cells. Data represent results of two independent experiments.

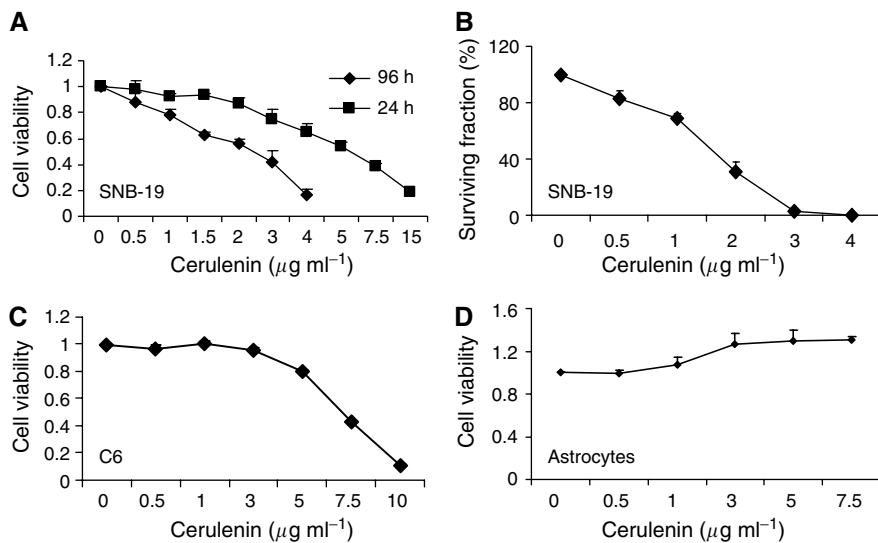


Figure 4 Cerulenin selectively reduces glioma cell viability and clonogenic survival; astrocytes are unaffected. 5×10^4 and 5000 SNB-19 human glioma cells were seeded into 24-well plates and treated with 0–10 $\mu\text{g ml}^{-1}$ cerulenin for 24 and 96 h, respectively. Cell viability was assessed using the MTT assay. To determine clonogenic survival, 500–2000 cells were seeded into 60 mm dishes and treated with 0–10 $\mu\text{g ml}^{-1}$ cerulenin for 10–15 days. Cells were fixed and stained with violet blue and colonies were counted using a colony counter. (A) Incubating SNB-19 cells with cerulenin led to a time- and dose-dependent reduction in cell viability. (B) A dose-dependent cerulenin-mediated reduction in clonogenic survival. (C) Incubating C6 rat glioma cells with cerulenin led to a time- and dose-dependent reduction in cell viability. In contrast, (D) shows that incubating primary rat astrocytes with 0.5–7.5 $\mu\text{g ml}^{-1}$ cerulenin for 24 h did not affect cell viability.

Translational Therapeutics

The death receptor FADD pathway is not involved in cerulenin-induced apoptosis

FADD-DN is a dominant-negative form of the FADD protein that blocks all known death receptor-induced apoptotic pathways. Activation of the death receptor pathway has been implicated in the mechanism of various chemotherapeutic agents that can work by upregulating death receptor levels or by raising autocrine production of death ligand. To test if such pathways contribute to cerulenin-induced apoptosis, U251 and SNB-19 cells were co-infected with adenoviruses expressing YFP or YFP-tagged FADD-DN. Fluorescence could be observed in infected cells (Figure 7B). After co-infection for 48 h, uninfected control cells, YFP-expressing cells,

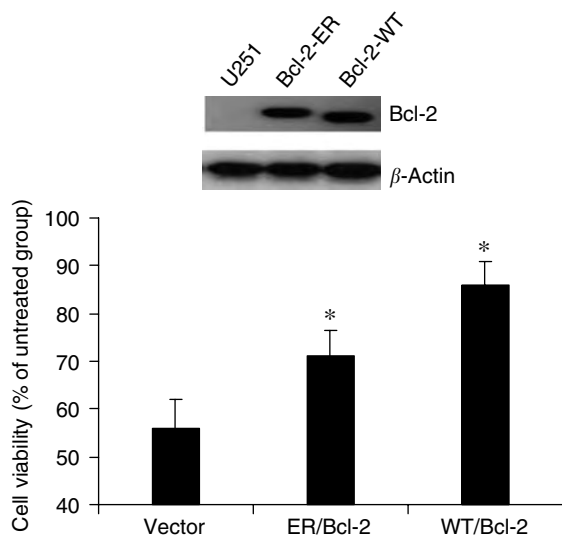


Figure 6 Bcl-2 overexpression protects U251 cells from cerulenin-induced cytotoxicity. U251 cells stably transfected with empty vector, ER-targeted vector, or WT Bcl-2 vector showed a marked increase in levels of Bcl-2 immunoreactive protein (A). Cells were treated with $5 \mu\text{g ml}^{-1}$ cerulenin for 24 h. Cell viability was then assessed using the MTT assay (B). Mean \pm s.e.; $n = 4$, $P < 0.05$.

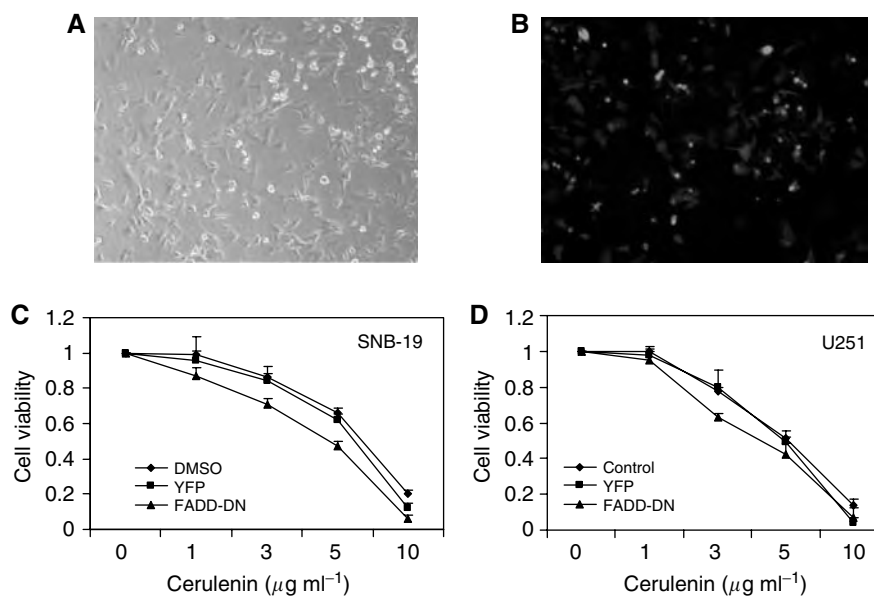


Figure 7 The death receptor FADD pathway is not involved in cerulenin-induced apoptosis. U251 and SNB-19 human glioma cells were co-infected with adenoviruses expressing YFP or YFP-tagged FADD-DN. (A) U251 cells treated with DMSO, (B) U251 cells co-infected with YFP-FADD-DN. Cells were then treated with $0-10 \mu\text{g ml}^{-1}$ cerulenin for 24 h and cell viability was then assessed using the MTT assay. Co-infection with YFP or YFP-FADD-DN failed to inhibit the cerulenin-mediated glioma cell cytotoxicity.

and YFP-FADD-DN-expressing cells were treated with $0-10 \mu\text{g ml}^{-1}$ cerulenin for 24 h and the MTT assay was performed to measure cell viability. As shown in Figure 7C and D, no significant differences in cell viability were found in the various groups, indicating that the death receptor pathway is not associated with FAS inhibition-induced cell apoptosis.

Treating human glioma cells with cerulenin leads to an S-phase cell cycle block

We hypothesised that the cerulenin-induced cell growth inhibition and death might involve cell cycle arrest. To test our hypothesis, we performed flow cytometry analysis. U251 and SNB-19 human glioma cells were treated with DMSO, 1 or $3 \mu\text{g ml}^{-1}$ cerulenin for 24 h. The cells were then collected and stained with PI for flow cytometry analysis. Representative cell cycle profiles are shown in Figure 8. After treatment with cerulenin for 24 h, the number of U251 cells in S phase increased markedly to 58% of the total cell population compared with controls (23%). A similar increase in the % of cells in S phase was seen 24 h after treatment of SNB-19 cells with cerulenin (Figure 8). These results suggest that cerulenin-mediated FAS inhibition induced an S-phase block in glioma cells.

Pretreating glioma cells with cerulenin does not alter radiosensitivity

Cell radiosensitivity is cell cycle dependent; cells present in the S phase are relatively radioresistant as compared with cells in other phases of the cell cycle (Pawlik and Keyomarsi, 2004). As treating glioma cells with cerulenin induced an S-phase block, we hypothesised that irradiating glioma cells in the presence of cerulenin would lead to either no effect or increased radioresistance. As shown in Figure 9A, there was no difference in viability between glioma cells treated with $0.5-4.0 \mu\text{g ml}^{-1}$ of cerulenin 2 h before irradiation with a single dose of 5 Gy of ^{137}Cs γ rays when compared with glioma cells treated with radiation alone. Increasing the time the cells were treated with cerulenin to 8 h before irradiation similarly failed to alter glioma cell radiosensitivity. Irradiating cerulenin-treated cells with single doses of

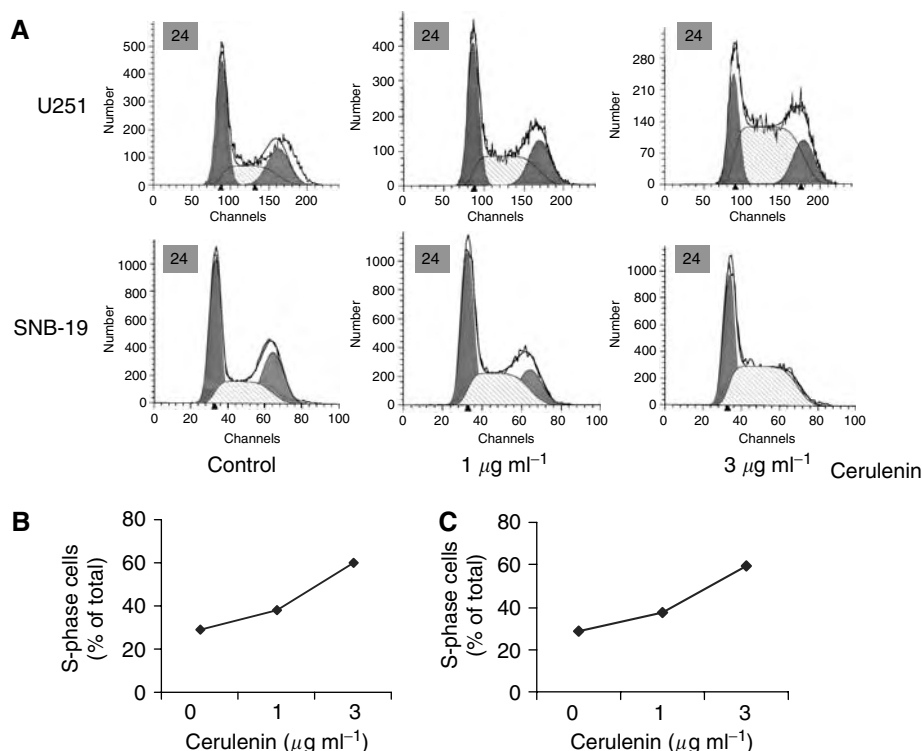


Figure 8 Treating human glioma cells with cerulenin leads to a dose-dependent accumulation of cells in S phase. U251 and SNB-19 human glioma cells were treated with DMSO, 1 $\mu\text{g ml}^{-1}$ cerulenin, or 3 $\mu\text{g ml}^{-1}$ cerulenin for 24 h. The cells were then harvested, fixed, stained with PI, and analysed by flow cytometry. **(A)** A representative flow cytometry experiment; **(B)** and **(C)** the mean data obtained from the results of two independent experiments. These data indicate that incubating U251 and SNB-19 cells with cerulenin led to a dose-dependent increase in S-phase cell accumulation.

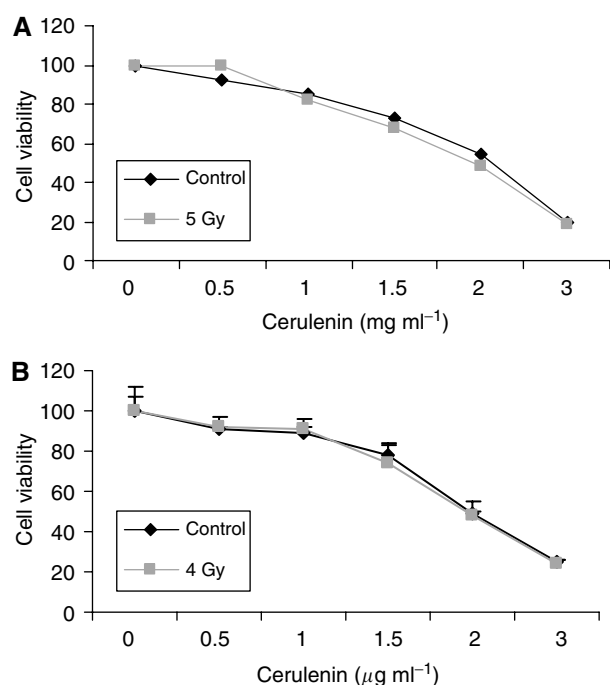


Figure 9 Treating human glioma cells with cerulenin fails to alter their radiosensitivity. SNB-19 human glioma cells were treated with 0.5–4.0 $\mu\text{g ml}^{-1}$ cerulenin for 2 h, control cells received DMSO. The cells then received either γ irradiation (single dose of 4 or 5 Gy γ rays [■]) using a ¹³⁷Cs irradiator or sham irradiation (◆), incubated for 96 h, and then viability was assessed using the MTT assay.

2, 4, or 8 Gy had no effect on glioma cell radiosensitivity. Figure 9B shows data for glioma cells treated with cerulenin 8 h before a single dose of 4 Gy; a similar response was seen after doses of 2 and 8 Gy (data not shown).

Fatty acid synthase shRNA transfection leads to downregulation of FAS expression and enzymatic activity that is associated with decreased glioma cell viability

To confirm that the effects of cerulenin were due to inhibition of FAS, we used RNAi to knock down FAS protein levels. To identify effective RNAi against FAS expression, we tested two different plasmid-based hairpin constructs of FAS RNAi; a scrambled sequence was used as the negative control. The shRNAs encoding oligonucleotides are shown in Figure 10B. They were cloned into the *Bam*H1 and *Hind*III restriction sites downstream of the H1 promoter in pSilencer 3.0-H1 (Figure 10A). U251 and SNB-19 cells were seeded into 60 mm dishes for 24 h and transfected with 2 and 4 μg FAS shRNA DNA for a further 72 h. Cells were then lysed and FAS protein expression analysed using Western blot analysis. As shown in Figure 10C, scrambled shRNA (control) had no effect on FAS expression. In contrast, FAS shRNA1 and FAS shRNA2 reduced significantly FAS protein levels. Moreover, this reduction in FAS protein was associated with a significant ($P < 0.05$) reduction in FAS enzymatic activity (Figure 10D). To test the effects of RNAi-mediated FAS silencing on glioma cell growth, SNB-19 cells were transiently transfected with a range of concentrations of plasmid DNA of scrambled shRNA and FAS shRNA for 72 h. Cytotoxicity was determined using the MTT assay. As shown in Figure 10E, cell viability was dose-dependently reduced in glioma cells transfected with FAS RNAi. These data confirm the effect of cerulenin as being FAS-specific.

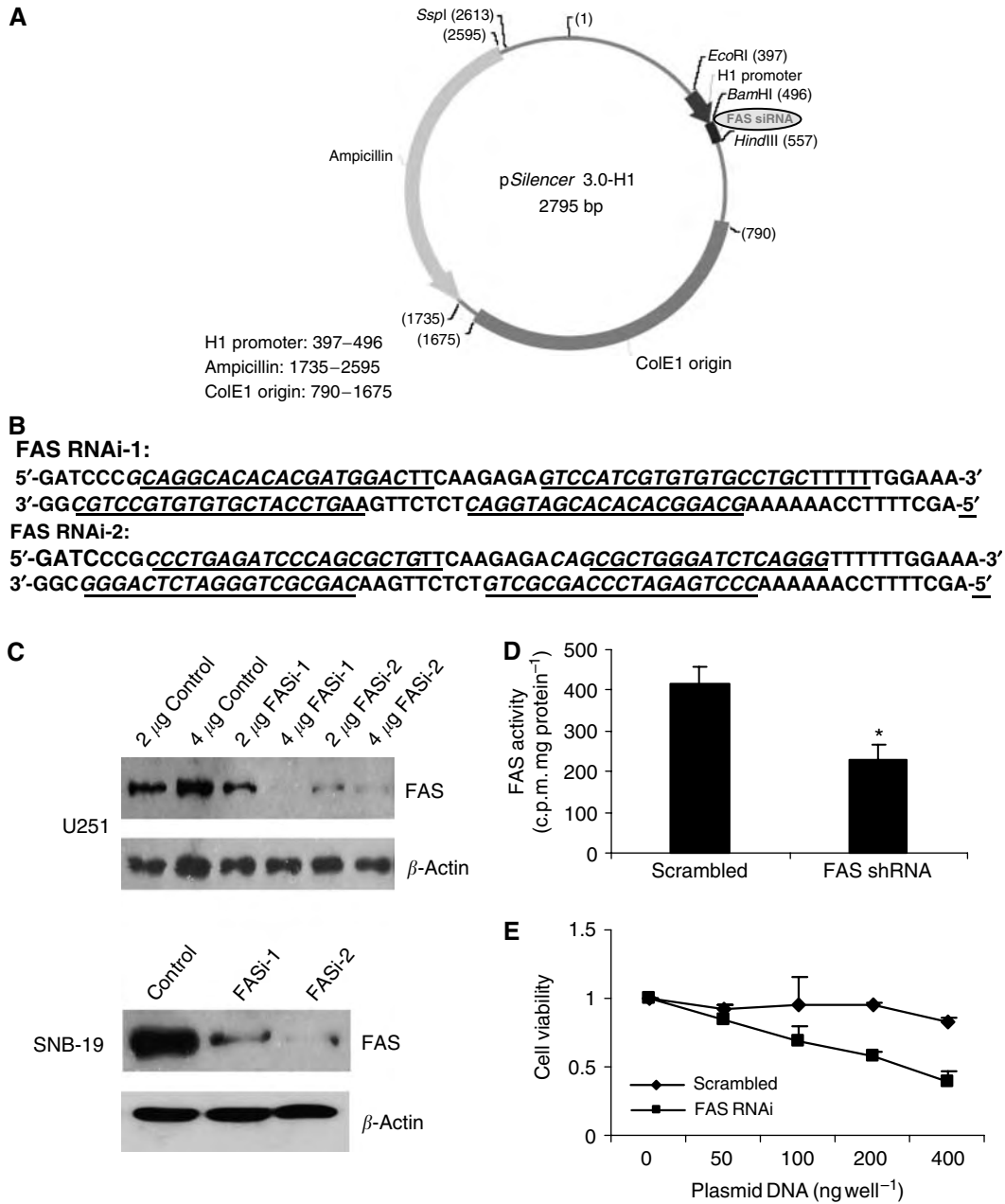


Figure 10 Fatty acid synthase shRNA transfection downregulates FAS expression and inhibits glioma cell growth. Sense and antisense oligonucleotides encoding the shRNAs (**B**) were cloned into the *Bam*HI and *Hind*III restriction sites downstream of the H1 promoter in pSilencer 3.0H-1 (**A**). U251 and SNB-19 cells were seeded into 60 mm dishes for 24 h and transfected with 2 and 4 μ g FAS shRNA DNA for a further 72 h. Cells were then lysed and FAS protein expression analysed using Western blot analysis. As shown in (**C**), FAS shRNA1 and FAS shRNA2 reduced markedly FAS protein levels; FAS enzymatic activity was also significantly reduced (**D**). Control cells transfected with a scrambled shRNA showed no change in FAS expression. As shown in (**E**), cell viability was significantly reduced in U251 glioma cells transfected with FAS RNAi.

DISCUSSION

Our data reveal, for the first time, that the levels of FAS expression observed in a variety of rat and human glioma cell lines and several human glioma tissue samples are higher compared to their normal cell counterpart, the astrocyte, as well as normal human brain tissue, respectively. Mammalian FAS is a multifunctional enzyme complex that catalyses the synthesis of palmitate from acetyl-CoA and malonyl-CoA with NADPH as a provider of reducing equivalents (Kuhajda, 2006). Humans take up large amount of proteins and lipids through their daily diet. Therefore, the levels of FAS in most tissues are low. In human tissue, FAS is distributed in cells involved in lipid metabolism and in hormone-sensitive cells,

such as adipocytes, hepatocytes, sebaceous glands, and type II alveolar cells (Weiss *et al*, 1986). High FAS activity was observed in foetal brain tissue and gradually declined following aging (Salles *et al*, 2002). FAS mRNA level in 20-day-old rat brain was about 20% compared with that measured in 5-day-old brain (Garbay *et al*, 1997; Muse *et al*, 2001). In the adult rat brain, immunohistochemical analysis for FAS revealed positive staining cells in cortical neurons of the frontooccipital lobes; only weak staining was observed in astrocytes (Kusakabe *et al*, 2000).

Glioma cell FAS expression has not been reported previously. We found a marked differential expression of FAS in glioma and normal astrocytes. A similar upregulation of FAS expression was noted in lysates of human glioma samples compared with lysates of

normal human brain tissue. Administration of the FAS inhibitor cerulenin led to significant time- and dose-dependent decreases in glioma cell viability and survival. Low doses of cerulenin caused cell cycle arrest, whereas higher doses led to cytotoxicity. In contrast, normal astrocytes were unaffected. These findings suggest that FAS might be a novel target for anti-glioma therapy.

The mechanism(s) underlying malignant cell cytotoxicity following FAS inhibition has been studied in several cancer cell lines. FAS inhibition can cause accumulation of malonyl-CoA, which leads to inhibition of carnitine palmitoyltransferase-1 and, indirectly, the fatty acid oxidation pathway (Thupari *et al*, 2001). Rapid accumulation of malonyl-CoA was observed after FAS inhibition (Kuhajda *et al*, 2000). Blocking the accumulation of malonyl-CoA using 5-(tetradecyloxy)-2-furoic acid, an inhibitor of acetyl-CoA carboxylase, reduced significantly cerulenin- and C75-induced cytotoxicity and inhibition of breast tumour growth. Apoptosis appeared to play a major role in this cell death. In our experiments, we demonstrated clearly that PARP cleavage, an apoptotic marker, was induced in cerulenin-treated U251 and SNB-19 cells; this was associated with significant cytotoxicity. Heiligtag *et al* (2002) reported that cerulenin is an effective inducer of apoptosis in WT or mutant p53 neuroblastoma, melanoma, and colon carcinoma cell lines; normal human cells were resistant to cerulenin-induced apoptosis. Further, apoptosis was mediated both by overexpression of proapoptotic Bax, in a p53-independent manner, and by a rapid release of mitochondrial cytochrome *c*, leading to activation of caspase 3 and 9. Data presented in the current studies confirm a P53-independent apoptotic response in glioma cells following FAS inhibition; similar glioma cell cytotoxicity was observed in either P53 WT (U87 and U118) or in P53 mutant glioma cell lines (U251, SNB-19, U138, and C6).

Bcl-2, a protooncogene that enhances cell survival by inhibiting apoptosis (Vaux *et al*, 1988), is localised in the ER, the outer mitochondrial membrane, and the nuclear envelope (Akao *et al*, 1994). Stable expression of Bcl-2 or Bcl-2 targeted to the ER has been shown to inhibit apoptosis (Wang *et al*, 2001a). Similarly, we observed that stable expression of Bcl-2-targeted to the ER or WT Bcl-2 expressed not only in the ER but also in the mitochondria and nuclei, reduced cerulenin-mediated glioma cell death. These data provide additional support for the hypothesis that cerulenin-mediated toxicity is due, in part, to an increase in apoptotic cell death. To investigate further the apoptotic response, the role of the death receptor pathway in cerulenin-mediated apoptosis was evaluated by infecting glioma cells with a FADD-DN adenovirus. In agreement with the previously suggested mechanism involving cytochrome *c* release (Heiligtag *et al*, 2002) FADD-DN, which inhibits all the known death receptor pathways, had no effect on cerulenin-induced glioma cell death.

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Cerulenin- and C75-mediated alterations in cell cycle progression have been observed in a variety of cancer cell lines. Although FAS inhibition can lead to a block in the cell cycle before G₁ (Kuhajda *et al*, 2000), there are data that support an S-phase arrest in breast cancer cells (Zhou *et al*, 2003) and in colon carcinoma cells (Pizer *et al*, 2001). In the current study, we demonstrated a marked, dose-dependent S-phase block at 24 h after treatment. The mechanism(s) of FAS inhibition-induced cell cycle arrest is still not clear. *De novo* fatty acid synthesis by tumour cells accounted for more than 93% of total lipid fatty acids in an experimental tumour model, indicating that endogenous fatty acid synthesis could be a significant source of fatty acids for tumour cell growth and survival (Ookhtens *et al*, 1984). Most of the fatty acids produced by tumour cells are incorporated into membrane phospholipids, and phospholipid synthesis is inhibited when fatty acid synthesis is inhibited (Pizer *et al*, 1996; Jackowski *et al*, 2000). Phospholipid biosynthesis is greatest during the G₁ and S phases, with doubling of the membrane mass occurring during S phase in preparation for cell division (Jackowski, 1994). Thus, cells in S phase should be most sensitive to changes in phospholipid metabolism. In contrast, cells in S phase are relatively radioresistant; the expected outcome would be either no effect or increased radioresistance. Indeed, as predicted, combining cerulenin treatment with ionising radiation did not alter glioma cell radioresistance.

We used selective gene silencing by siRNA (Zamore *et al*, 2000) to confirm the role of FAS inhibition in glioma cell cytotoxicity. RNAi-induced knockdown of FAS that led to a significant reduction in FAS enzymatic activity resulted in a dose-dependent reduction in glioma cell viability, confirming similar gene silencing approaches with RNAi described in human LNCaP prostate cancer cells (De Schrijver *et al*, 2003).

In conclusion, our studies demonstrate that (1) human and rat glioma cell lines express high levels of FAS compared with normal brain astrocytes; high levels of FAS were also seen in human glioma tissue compared with normal human brain, (2) FAS inhibition is selectively cytotoxic to glioma cells; normal astrocytes are unaffected; and (3) both cytotoxic and cytostatic effects contribute to the cerulenin-mediated effects in glioma cells. These findings support the hypothesis that FAS might be a novel target for anti-glioma therapy.

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Inhibition of Fatty Acid Synthase Induces Endoplasmic Reticulum Stress in Tumor Cells

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Abstract

Fatty acid synthase (FAS), the cellular enzyme that synthesizes palmitate, is expressed at high levels in tumor cells and is vital for their survival. Through the synthesis of palmitate, FAS primarily drives the synthesis of phospholipids in tumor cells. In this study, we tested the hypothesis that the FAS inhibitors induce endoplasmic reticulum (ER) stress in tumor cells. Treatment of tumor cells with FAS inhibitors induces robust PERK-dependent phosphorylation of the translation initiation factor eIF2 α and concomitant inhibition of protein synthesis. PERK-deficient transformed mouse embryonic fibroblasts and HT-29 colon carcinoma cells that express a dominant negative PERK (Δ C-PERK) are hypersensitive to FAS inhibitor-induced cell death. Pharmacologic inhibition of FAS also induces the processing of X-box binding protein-1, indicating that the IRE1 arm of the ER stress response is activated when FAS is inhibited. Induction of ER stress is further confirmed by the increased expression of the ER stress-regulated genes *CHOP*, *ATF4*, and *GRP78*. FAS inhibitor-induced ER stress is activated prior to the detection of caspase 3 and PARP cleavage, primary indicators of cell death, whereas orlistat-induced cell death is rescued by coinubation with the global translation inhibitor cycloheximide. Lastly, FAS inhibitors cooperate with the ER stress inducer thapsigargin to enhance tumor cell killing. These results provide the first evidence that FAS inhibitors induce ER stress and establish an important mechanistic link between FAS activity and ER function. [Cancer Res 2007;67(3):1262–9]

Introduction

Fatty acid synthase (FAS) is a multifunctional enzyme that catalyzes the terminal steps in the synthesis of the 16-carbon fatty acid palmitate in cells (1, 2). In normal tissue, the FAS expression levels are relatively low because fatty acid is generally supplied by dietary fatty acids. On the other hand, FAS is expressed at significantly higher levels in many tumors including those of the prostate, breast, colon, ovary, and others (3–5). This expression profile suggests that tumors require higher levels of fatty acids than can be supplied from the circulation. Several reports have shown that FAS expression levels correlate with tumor progression, aggressiveness, and metastasis (5–7). In fact, FAS expression levels

are predictive of the progression from organ-confined prostate cancer to metastatic prostate cancer (6), indicating that FAS provides a metabolic advantage to tumor cells. Because of the strong link between FAS expression and cancer, FAS has become an attractive target for therapeutic intervention.

The functional connection between FAS and tumor progression has been provided by the discovery and design of small molecule drugs that inhibit the catalytic activity of FAS (8, 9). Cerulenin and C75, which target the keto-acyl synthase domain of FAS, were the first small molecules to be described as inhibitors of FAS activity in human tumor cells. These pharmacologic agents inhibit FAS activity and induce cell death in many tumor cell lines *in vitro* (5, 7). The compounds are also effective at inhibiting the growth of human tumor xenografts *in vivo* and have chemopreventive abilities (10–12). We were the first to describe orlistat as an inhibitor of the thioesterase domain of FAS (13). Orlistat inhibits FAS activity and induces cell death in a variety of tumor cell lines and is able to effectively inhibit the growth of prostate tumor xenografts in mice (13–15). The data linking FAS function and tumor cell survival emphasizes the relevance of FAS as an attractive antitumor target. The importance of fatty acid synthesis in tumor cells is further underscored by data demonstrating that pharmacologic and genetic inhibition of two upstream enzymes in the fatty acid synthesis pathway, ATP citrate lyase and acetyl CoA carboxylase, also induces cell death in tumor cell lines (16–18).

Because FAS is a target for therapeutic intervention, it is important to fully understand the role of FAS in tumor cells as well as the antitumor effects of FAS inhibitors. Given that the endoplasmic reticulum (ER) is the major site for phospholipid synthesis in cells, it is not surprising that previous studies have identified a link between pathways that regulate lipid synthesis and the ER stress response (19–21). Fatty acid synthesis in general, and FAS activity in particular, drives phospholipid synthesis which primarily occurs in the ER (22). Because of the direct connection between FAS activity and phospholipid synthesis, we tested the hypothesis that pharmacologic blockade of FAS activity might induce ER stress in tumor cells (22). The data presented herein shows for the first time that inhibition of FAS induces ER stress specifically in a variety of tumor cells and not in normal cells. Importantly, we also show that FAS inhibitors cooperate with a known ER stress inducer, thapsigargin, to induce cell death. The data also provide evidence that FAS inhibitors might be combined with PERK inhibitors to more effectively treat cancer. The evidence suggests that increased FAS expression in tumor cells is important for ER function to maintain membrane biogenesis and suggests a role for ER stress in the antitumor effects of FAS inhibitors.

Materials and Methods

Materials. The PC-3, DU145, HT-29, HeLa, and FS-4 cell lines were obtained from American Type Culture Collection (Manassas, VA). Cell culture medium and supplements were from Invitrogen (Carlsbad, CA).

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Antibodies against eIF2 α , phospho-eIF2 α , cleaved caspase 3, and cleaved PARP were from Cell Signaling Technologies (Beverly, MA). Antibody against FAS was from BD Transduction Labs (San Diego, CA). Antibody against β -tubulin was from NeoMarkers (Fremont, CA). TRIzol was from Invitrogen. Avian myeloblastosis virus–reverse transcriptase and Taq Polymerase were from Promega (Madison, WI). ^{35}S -Methionine and ^{14}C -acetate were purchased from GE Healthcare (formerly Amersham Biosciences, Piscataway, NJ). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA), except for those designed for short interfering RNA (siRNA), which were synthesized by Dharmacon (Lafayette, CO). All other reagents were purchased from Sigma (St. Louis, MO), Calbiochem (San Diego, CA), or Bio-Rad (Hercules, CA).

Cell culture and drug treatments. Prostate tumor cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C and 5% CO $_2$. Wild-type and *PERK*^{-/-} mouse embryonic fibroblasts (MEF), obtained from David Ron (Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, NY), HeLa cervical cancer cells, and FS-4 human foreskin fibroblasts were maintained in DMEM-high glucose supplemented with 10% fetal bovine serum. HT-29 colon carcinoma cells were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum. HT-29 cells expressing the pBabe-puro empty vector or the pBabe-puro- ΔC -PERK construct were maintained with 1 $\mu\text{g}/\text{mL}$ of puromycin and supplemented with 20% fetal bovine serum, nonessential amino acids, and 2-mercaptoethanol. Cells were treated for the indicated times and drug concentrations as indicated. Orlistat was extracted from capsules in ethanol as described previously and stored at -80°C (13). Further dilutions were made in DMSO.

Generation of ΔC -PERK–expressing cells. To generate human tumor cells with deficient PERK signaling, HT-29 cells seeded in six-well plates were transfected with 1 μg of pBabe-puro or pBabe-puro- ΔC -PERK using LipofectAMINE (Invitrogen). These plasmids have been described previously (23). Stable populations of each construct were selected by incubating transfected cells with 3 $\mu\text{g}/\text{mL}$ of puromycin for 48 h. The transfected cell populations were then maintained in 1 $\mu\text{g}/\text{mL}$ of puromycin for subsequent experiments in the medium described above.

Immunoblot analysis. Cells were harvested after the indicated treatments, washed with ice-cold PBS, and lysed in buffer containing 1% Triton X-100 and a complete protease, kinase, and phosphatase inhibitor cocktail. Protein samples were electrophoresed through 7.5%, 10%, or 12% SDS-polyacrylamide gels and transferred to nitrocellulose, except for blots to detect phospho-eIF2 α and eIF2 α , which were transferred to Immobilon-P membrane (polyvinylidene difluoride). Immunoreactive bands were detected by enhanced chemiluminescence (Perkin Elmer Life Sciences, Inc., Boston, MA).

Metabolic labeling of protein and fatty acid synthesis. To measure fatty acid synthesis, 1×10^5 cells per well were seeded in 24-well plates. Cells were treated with C75 (10 $\mu\text{g}/\text{mL}$), orlistat (25, 50 $\mu\text{mol}/\text{L}$), or cerulenin (5, 10 $\mu\text{g}/\text{mL}$) for 2 h. ^{14}C -Acetate (1 μCi) was added to each well for 2 h. Cells were collected, washed, and lipids were extracted and quantified as previously described (13). To measure new protein synthesis, PC-3 cells were seeded in six-well plates. Orlistat (50 $\mu\text{mol}/\text{L}$) and thapsigargin (1 $\mu\text{mol}/\text{L}$) were added for the indicated times. After incubation with orlistat or thapsigargin, the cells were switched to methionine-deficient medium although maintaining the drug concentrations. Methionine-deficient medium supplemented with 100 $\mu\text{Ci}/\text{mL}$ of ^{35}S -methionine was added to the cells for 30 min to label newly synthesized proteins. After the labeling period, cells were washed, lysed, and samples were resolved by electrophoresis through a 10% SDS-polyacrylamide gel. The gel was then stained with Coomassie, dried, and the relative protein synthesis of each sample was quantified after scanning with a Typhoon 9210 (Amersham) using ImageQuant software.

Clonogenic survival assays. Cells were plated in six-well plates at a low density depending on the individual cell type. PC-3 cells were plated at a density of 800 cells per well, except for the experiment combining C75 with thapsigargin, for which PC-3 cells were plated at 3,000 cells per well. HT-29 and MEF cells were plated at a density of 400 cells per well. Human tumor cells were plated 48 h prior to each experiment, whereas MEFs were plated

24 h prior to treatment. Fresh medium containing the indicated drugs was added at the indicated concentrations for 12 to 20 h as indicated. The medium was then removed, the wells were washed and fresh medium was added. Plates were incubated until macroscopic colonies formed. To visualize colonies, the wells were washed twice with ice-cold PBS and fixed for 10 min with a 10% methanol/10% acetic acid solution. Colonies were stained with a 0.4% crystal violet/20% methanol solution for 10 min. The crystal violet solution was removed, the wells were washed with water to remove excess dye, and dried at room temperature overnight. Colonies were quantified by counting and by solubilization in 33% acetic acid followed by spectrophotometric analysis at 540 nm. The survival of treated cells was normalized relative to vehicle-treated cells and statistical significance was determined by two-tailed Student's *t* tests.

Detection of X-box binding protein-1 splicing and ATF4, GRP78, CHOP, and GADD34 expression. Cells were exposed to the various drug treatments or transfected with siRNA for the indicated times. Total RNA was isolated from cells using TRIzol according to the manufacturer's directions. cDNA was generated from 2 μg of total RNA using Avian myeloblastosis virus–reverse transcriptase. X-box binding protein-1 (XBP-1) was amplified by PCR with Taq polymerase using the oligonucleotides AACAGAGTAGCAGCTCAGACTGC (sense) and TCCTTCTGGGTA-GACCTCTGGGAG (antisense). The XBP-1 products were resolved on 2% Tris acetate-EDTA agarose gels and imaged on the Typhoon 9210 at 610 nm. The expression of CHOP, ATF4, GRP78, and GADD34 was determined by semiquantitative PCR using RNA collected as described above. Multiple cycles were tested for each gene to determine the optimum cycles in the linear range. The oligonucleotide sequences used were: CHOP, CAGAACCAGCAGAGGTCACA and AGCTGTGCCACTTTCCTTTC; GRP78, CTGGGTACATTTGATCTGACTGG and GCATCTGGTGGCTTCCAGC-CATTC; ATF4, CTTACGTTGCCATGATCCCT and CTTCTGGCGGTACC-TAGTGG; and GADD34, GTGGAAGCAGTAAAAGGAGCAG and CAGCAACTCCTTCTCCTCG. The CHOP, GRP78, and ATF4 products were resolved on 1% Tris acetate-EDTA agarose gels and imaged on the Typhoon 9210 at 610 nm.

Suppression of FAS expression with siRNA. A paired siRNA oligonucleotide against the *FAS* gene (*FAS1* sense, GUAGGCCUCCACUC-CUAUU) and one siRNA against luciferase as a negative control (*Luc* sense, CUUACGUGAUACUUCGAUU) were designed and synthesized by Dharmacon. The individual siRNAs (30 nmol/L) were transfected into cells upon plating with siPORT *NeoFX* transfection reagent (Ambion, Austin, TX) according to the instructions of the manufacturer. Cells were collected at the indicated times after transfection and then harvested for RNA to perform reverse transcription-PCR (RT-PCR) or protein for immunoblot analysis.

Results

Pharmacologic inhibition of FAS induces phosphorylation of eIF2 α in tumor cells. Several studies have shown that lipid composition is important for maintaining ER function (19–21, 24, 25). Other studies have shown that FAS drives phospholipid synthesis in tumor cells (22). Because of this fact, we hypothesized that FAS inhibitors might induce ER stress. One hallmark of ER stress is the PERK-dependent phosphorylation of the translation initiation factor eIF2 α . We first examined the phosphorylation status of eIF2 α in cells treated with three different pharmacologic inhibitors of FAS (Fig. 1A). PC-3 cells were treated with orlistat (12.5–50 $\mu\text{mol}/\text{L}$, *left*) or cerulenin (5 or 10 $\mu\text{g}/\text{mL}$, *middle*) for 16 h, or C75 (10 $\mu\text{g}/\text{mL}$) for 8 to 24 h (*right*). Each FAS inhibitor induced robust phosphorylation of eIF2 α at each concentration after 16 h of treatment, as did thapsigargin (data not shown). Similarly, all three inhibitors induced eIF2 α phosphorylation regardless of tumor cell type tested (data not shown). Likewise, fatty acid synthesis was inhibited to similar degrees by each treatment, as measured by ^{14}C -acetate incorporation into total cellular lipids (Fig. 1B, *left*). Because the phosphorylation of eIF2 α leads to the inhibition of

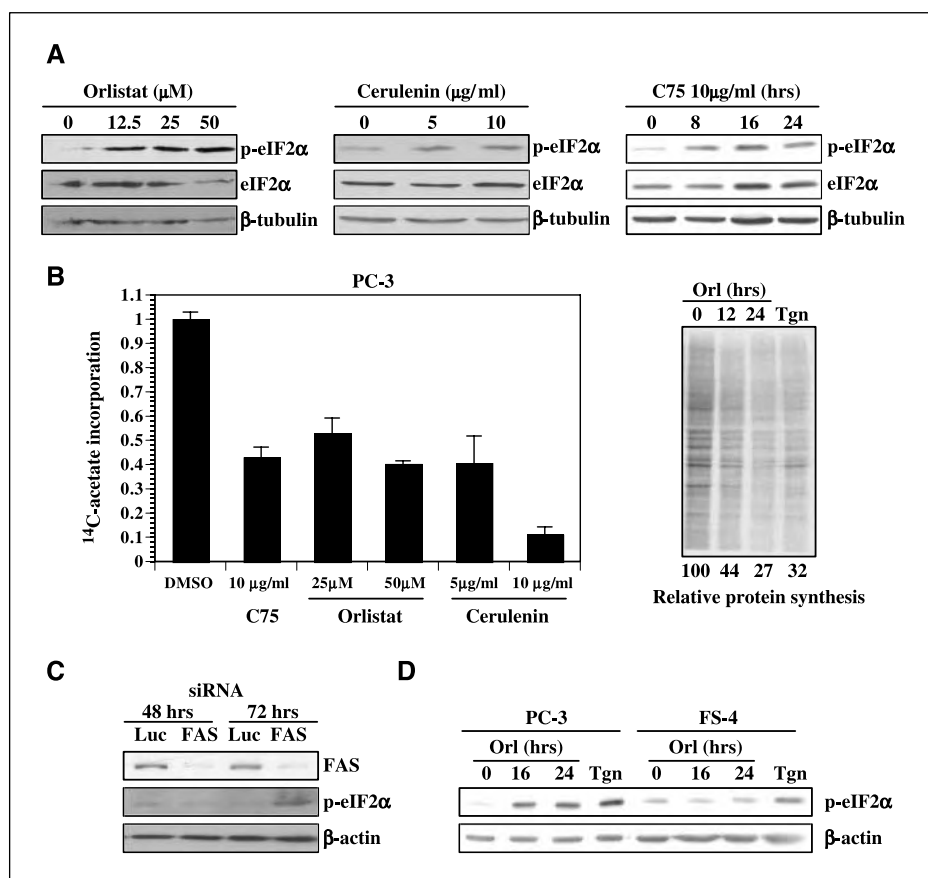


Figure 1. FAS inhibitors induce phosphorylation of eIF2 α in tumor cells. **A**, PC-3 cells were treated with the indicated concentrations of orlistat (*left*) or cerulenin (*middle*) for 16 h, or C75 (10 μ g/mL) for 8 to 24 h (*right*). Samples were resolved by SDS-PAGE, transferred to polyvinylidene difluoride and the membrane was probed with antibodies specific for phospho-eIF2 α , total eIF2 α , and β -tubulin. **B**, PC-3 cells were treated for 2 h with C75 (10 μ g/mL), orlistat (25 and 50 μ mol/L), or cerulenin (5 and 10 μ g/mL), then incubated with 14 C-acetate (1 μ Ci) for 2 h. Cells were collected, washed, and lipids were extracted and quantified relative to vehicle-treated control (*left*). PC-3 cells were treated with orlistat (50 μ mol/L) for the indicated times or thapsigargin (Tgn, 1 μ mol/L) for 1 h and then pulsed with 10 μ Ci of 35 S-methionine for 30 min. Protein aliquots were then resolved by SDS-PAGE and new protein synthesis was quantified by scanning on a Typhoon 9210. Quantification was relative to vehicle-treated controls (*right*). **C**, PC-3 cells were transfected with siRNA against FAS or luciferase (Luc) for the indicated times and analyzed by immunoblot. **D**, FS-4 normal foreskin fibroblasts were treated with orlistat (25 μ mol/L) for 24 h or Tgn (1 μ mol/L) for 1 h side by side with PC3 cells and prepared for immunoblot analysis.

protein synthesis, we did a 35 S-methionine labeling experiment to measure the levels of newly synthesized proteins in cells treated with orlistat. PC-3 cells were treated with orlistat (50 μ mol/L) for 12 and 24 h or thapsigargin (1 μ mol/L) as a positive control for 1 h (Fig. 1B, *right*). Orlistat treatment reduced protein synthesis by 56% after 12 h and by 73% at 24 h, similar to treatment with thapsigargin. Therefore, orlistat treatment is sufficient to induce the phosphorylation of eIF2 α and, subsequently, inhibit protein synthesis. To further confirm our findings, a genetic approach was also used to inhibit FAS expression. PC-3 cells were transfected with FAS-specific siRNA or siRNA against luciferase as a negative control. Immunoblot analysis showed a nearly 70% reduction of FAS protein in the samples 48 h after transfection, which continued through 72 h (Fig. 1C). Consistent with our findings using pharmacologic inhibitors, the reduction of FAS expression levels resulted in the detection of significant levels of phosphorylated eIF2 α at 72 h (Fig. 1C). Conversely, treatment of normal human foreskin FS-4 fibroblasts with the FAS inhibitor orlistat did not result in the phosphorylation of eIF2 α (Fig. 1D). Collectively, these data indicate that eIF2 α phosphorylation induced by FAS inhibition is, indeed, specific to both FAS and tumor cells.

PERK mediates eIF2 α phosphorylation in response to orlistat treatment. There are four known eIF2 α kinases: PERK, GCN2, PKR, and HRI; however, PERK is the kinase that phosphorylates eIF2 α during the ER stress response (26, 27). To determine whether PERK is the kinase responsible for the phosphorylation of eIF2 α in response to FAS inhibition, wild-type and *PERK*^{-/-} MEFs transfected with *Ki-Ras*^{V12} were obtained and tested for their sensitivity to orlistat (27). The wild-type and

PERK^{-/-} MEFs were treated with orlistat (12.5 μ mol/L) for 8, 16, and 24 h (Fig. 2A) or treated with thapsigargin (1 μ mol/L) for 1 h (data not shown). In the wild-type MEFs, orlistat induced the phosphorylation of eIF2 α within 8 h (Fig. 2A), consistent with our findings in prostate tumor cell lines (Fig. 1A). On the other hand, no significant phosphorylation of eIF2 α was evident during the same time course of orlistat treatment in the *PERK*-deficient cells (Fig. 2A). As expected, thapsigargin only induced phosphorylation of eIF2 α in the wild-type and not the *PERK*^{-/-} MEFs (data not shown). These data indicate that FAS inhibition results in PERK-dependent phosphorylation of eIF2 α .

It has been shown that *PERK*-deficient cells are hypersensitive to ER stress-induced apoptosis (28). Because of this, we tested whether *PERK*^{-/-} MEFs were hypersensitive to orlistat-induced cell death using clonogenic survival assays. Wild-type and *PERK*^{-/-} MEFs were treated with vehicle, orlistat (25 μ mol/L), or thapsigargin (100 nmol/L) for 16 h (Fig. 2B). As expected, the *PERK*^{-/-} MEFs were hypersensitive to thapsigargin-induced cell death as shown by a 3-fold decrease in clonogenic survival ($P < 0.005$). Similarly, the *PERK*^{-/-} MEFs showed hypersensitivity to orlistat treatment, showing reduced clonogenic survival of wild-type-transformed MEFs to 70% of vehicle-treated cells. On the other hand, clonogenic survival was decreased nearly 4-fold to <20% ($P < 0.005$) in the *PERK*^{-/-} MEFs following orlistat treatment. These data indicate that the inhibition of FAS activity induces ER stress which is exacerbated by the loss of PERK.

To further support the results obtained in MEFs, we generated stable populations of HT-29 colon carcinoma cells transfected with a dominant negative PERK construct that lacks the kinase domain

(Δ C-PERK) or the corresponding empty vector (23). These cells were seeded at a low density and treated with C75 (9 μ g/mL), orlistat (25 μ mol/L), or thapsigargin (10 nmol/L) to assess clonogenic survival. As expected, the HT-29 Δ C-PERK cells were hypersensitive to thapsigargin as shown by a nearly 3-fold reduction in clonogenic survival (Fig. 2C, $P < 0.005$). Similarly, the HT-29 Δ C-PERK cells were also hypersensitive to both orlistat and C75 compared with the empty vector-transfected cells. Clonogenic survival was reduced >2-fold in C75-treated cells and nearly 4-fold in orlistat-treated cells (Fig. 2C, $P < 0.005$). These results confirm that PERK function is important for an adaptive response in tumor cells when FAS activity is inhibited.

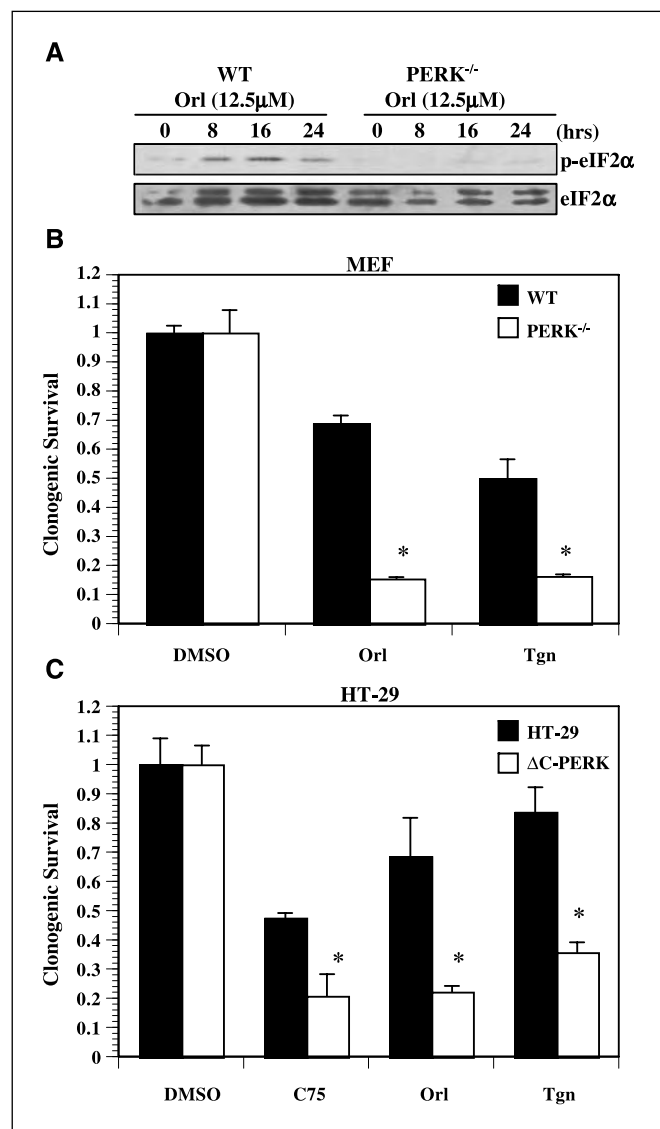


Figure 2. PERK phosphorylates eIF2 α in response to, and protects cells against, orlistat treatment. **A**, wild-type (WT) and *PERK*^{-/-} MEFs transformed with with *Ki-Ras*^{V12} were treated with orlistat (12.5 μ mol/L) for the indicated times. Samples were analyzed with antibodies specific for phospho-eIF2 α and total eIF2 α . **B**, clonogenic survival assays were done in WT and *PERK*^{-/-} MEFs, treated with vehicle, orlistat (25 μ mol/L), or Tgn (100 nmol/L) for 16 h, in triplicate. The surviving fraction was normalized relative to vehicle-treated controls. **C**, HT-29 cells and HT-29/ Δ C-PERK cells were treated with vehicle, orlistat (25 μ mol/L), C75 (9 μ g/mL), or Tgn (10 nmol/L) for 16 h, in triplicate. Clonogenic survival was normalized relative to vehicle-treated controls and statistical significance was determined by two-tailed Student's *t* tests.

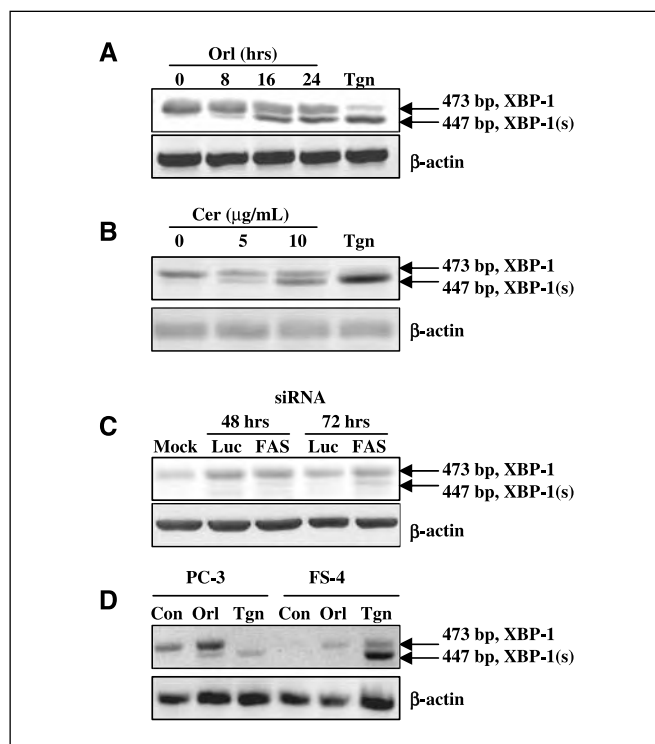


Figure 3. FAS inhibitor treatment activates processing of XBP-1. **A**, PC-3 cells were treated with orlistat (50 μ mol/L) for the indicated times or Tgn (500 nmol/L) for 8 h. **B**, PC-3 cells were treated with the indicated concentrations of cerulenin for 16 h or Tgn (500 nmol/L) for 8 h. **C**, HeLa cells were transfected with siRNA against FAS or luciferase (*Luc*) for the indicated times. **D**, PC-3 and FS-4 cells were treated with orlistat (50 μ mol/L) for 16 h or Tgn (1 μ mol/L) for 1 h. Total RNA was collected and RT-PCR was done as described in experimental procedures. XBP-1 is indicated by the 473 bp product and XBP-1(s) is indicated by the 447 bp fragment.

Treatment with orlistat induces processing of XBP-1. In addition to the PERK-regulated arm, the ER stress response can also be mediated by IRE1, a kinase with endonuclease activity that facilitates the splicing of XBP-1 mRNA to yield the splice variant XBP-1(s) during ER stress (29). To determine whether pharmacologic inhibition of FAS also activates the IRE1 pathway, the status of XBP-1 mRNA was assessed by RT-PCR using oligonucleotides which flank the splice site in the XBP-1 mRNA. Total RNA was collected from PC-3 cells treated with orlistat (50 μ mol/L) for 8 to 24 h (Fig. 3A) or with cerulenin (5 or 10 μ g/mL) for 16 h (Fig. 3B). Thapsigargin treatment resulted in a loss of the 473 bp product associated with unspliced XBP-1 and the appearance of the processed 447 bp form associated with XBP-1(s) that is produced only in response to ER stress (Fig. 3A). Similarly, orlistat treatment resulted in the appearance of the 447 bp XBP-1(s) product in as few as 8 h with maximum processing by 16 h (Fig. 3A). Cerulenin also induced the processing of XBP-1 (Fig. 3B). To further confirm the role of FAS inhibition in the processing of XBP-1 to XBP-1(s), HeLa cells were transfected with FAS-specific siRNA or siRNA against luciferase as a negative control and collected for RT-PCR (Fig. 3C). The 447 bp fragment that corresponds to the ER stress-specific XBP-1(s) product was detected at 72 h in the FAS-specific siRNA samples. Although PC-3 cells exhibit XBP-1 splicing after both orlistat and thapsigargin treatment, FS-4 cells exhibited only minor induction of XBP-1 and no splicing in response to orlistat (Fig. 3D). These results suggest that the IRE1 pathway of the ER stress

response is activated in parallel to the PERK pathway when FAS is inhibited in tumor cells.

Inhibition of FAS activity induces expression of ER stress-regulated genes. Activation of the ER stress response induces the expression of a number of genes associated with adaptation and cell death, including *CHOP*, *GRP78*, and *ATF4*. *CHOP* has been implicated in ER stress-dependent apoptosis and *CHOP*^{-/-} cells are mildly resistant to apoptosis following treatment with ER-stressing agents (30). The mRNA expression of *CHOP* is induced in DU145 cells treated with orlistat (50 $\mu\text{mol/L}$) or C75 (9 $\mu\text{g/mL}$; Fig. 4A). Similar effects were seen in PC-3 cells (data not shown). *CHOP* mRNA expression was also induced following siRNA-mediated knockdown of FAS expression in PC-3 cells (Fig. 4B). Increased mRNA expression of *CHOP* may indicate that ER stress could play a role in cell death induced by FAS inhibition (30). The mRNA expression of *GRP78*, an ER stress-regulated chaperone, is also induced in orlistat-treated cells (Fig. 4C; ref. 31). Furthermore, mRNA expression of the transcription factor *ATF4*, which is dependent on eIF2 α phosphorylation, was also induced by orlistat treatment (Fig. 4C). Collectively, the data in Figs. 1–4 show that FAS inhibitors induce the ER stress response.

ER stress is an early event in cells treated with FAS inhibitors. To determine whether phosphorylation of eIF2 α and subsequent indications of the ER stress response events coincide or precede cell death induced by FAS inhibition, we analyzed the temporal phosphorylation of eIF2 α and markers of caspase activity and cell death. In PC-3 cells treated with orlistat, phosphorylation of eIF2 α was evident at 16 h of treatment, whereas significant cleavage of caspase 3 and PARP are not detectable until 24 and 48 h, respectively, consistent with previous reports (13). These data

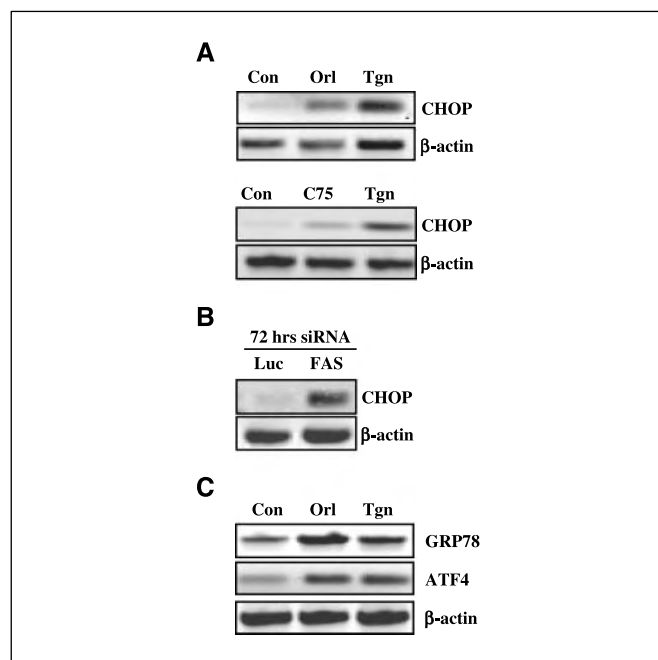


Figure 4. Inhibition of FAS activity induces mRNA expression of ER stress-regulated genes. *A*, DU145 cells were treated with vehicle control, orlistat (50 $\mu\text{mol/L}$, top), C75 (9 $\mu\text{g/mL}$, bottom), or Tgn (500 nmol/L) for 16 h. *B*, PC-3 cells were transfected with siRNA against FAS or luciferase (*Luc*) for 72 h. *C*, PC-3 cells were treated with vehicle control or orlistat (25 $\mu\text{mol/L}$) for 24 h or Tgn (1 $\mu\text{mol/L}$) for 1 h. Total RNA was collected with TRIzol and semiquantitative RT-PCR was done with oligonucleotides specific for *CHOP* (*A* and *B*), *GRP78* and *ATF4* (*C*), or β -actin (*A–C*).

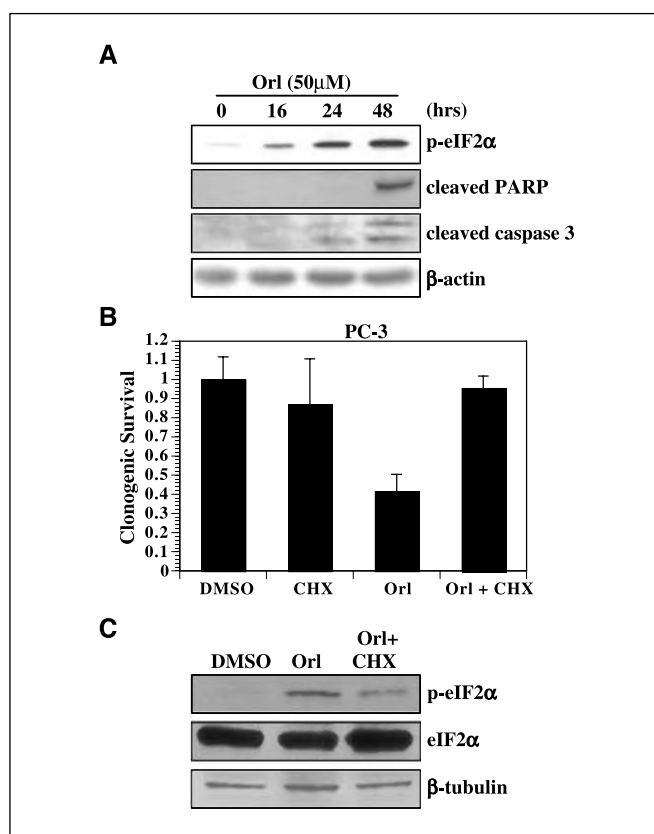
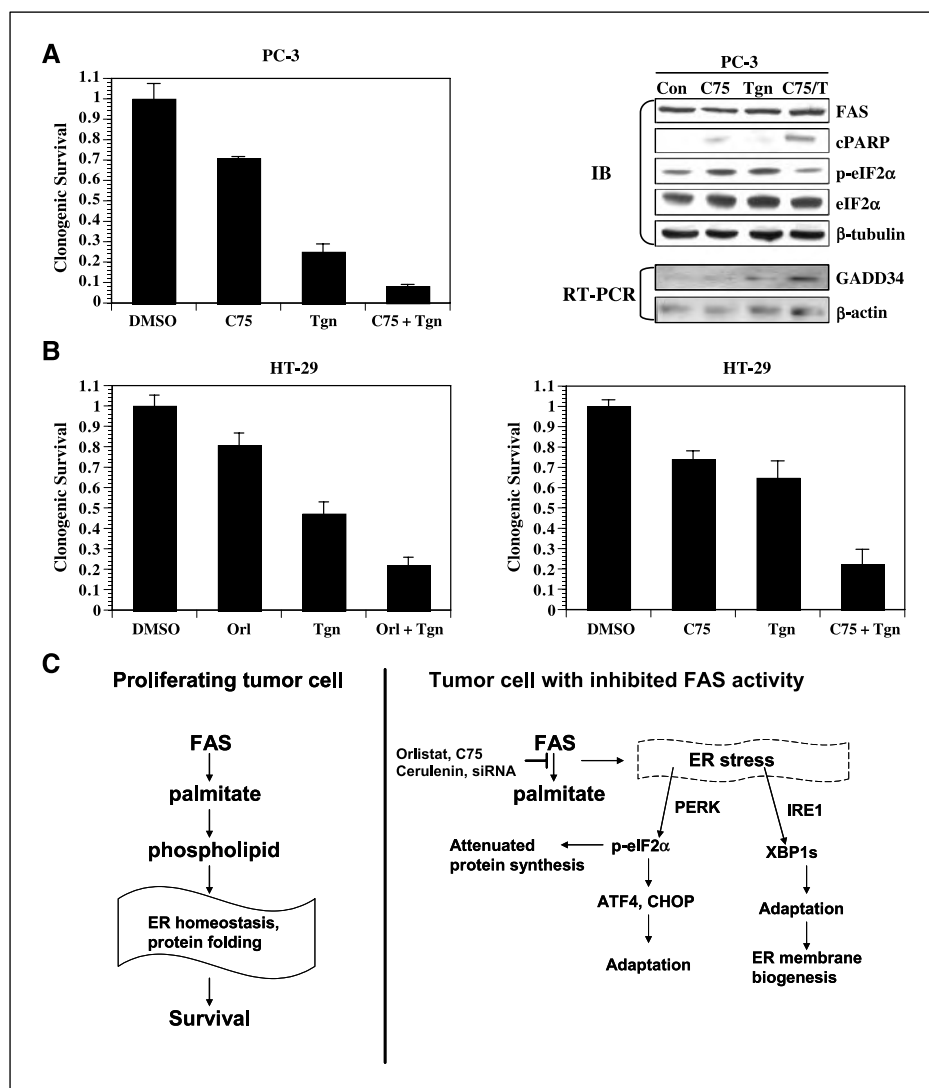


Figure 5. Phosphorylation of eIF2 α is an early event in cells treated with FAS inhibitors. *A*, PC-3 cells were treated with 50 $\mu\text{mol/L}$ of orlistat for the indicated times and samples were collected for immunoblot analysis and probed with antibodies specific for phospho-eIF2 α , cleaved PARP, cleaved caspase 3, and β -actin. *B*, PC-3 cells were treated with DMSO, cycloheximide (CHX, 1 $\mu\text{g/mL}$), orlistat (25 $\mu\text{mol/L}$), or orlistat with cycloheximide for 16 h. Clonogenic survival was normalized relative to vehicle-treated controls. *C*, PC-3 cells were treated with DMSO, cycloheximide (1 $\mu\text{g/mL}$), orlistat (25 $\mu\text{mol/L}$), or the combination of orlistat and cycloheximide for 16 h. Samples were collected for immunoblot analysis and probed with antibodies specific for phospho-eIF2 α , total eIF2 α , and β -tubulin.

indicate that the ER stress response is induced prior to caspase 3 activation and PARP cleavage. These data are further supported by the timeline of XBP-1 processing following orlistat treatment (Fig. 3A). Previous studies have shown that inhibition of protein translation with cycloheximide can ameliorate the effects of ER stress, likely by decreasing protein burden on the ER (28). Clonogenic survival assays and immunoblot analysis were done in PC-3 cells treated with vehicle control, cycloheximide (1 $\mu\text{g/mL}$), orlistat (25 $\mu\text{mol/L}$), or orlistat with cycloheximide (Fig. 5B and C). Cycloheximide treatment rescued clonogenic survival of PC-3 cells treated with orlistat (Fig. 5B), and also inhibited orlistat-induced phosphorylation of eIF2 α (Fig. 5C). These data are consistent with previous reports on the protective effect of cycloheximide on cells under ER stress and suggest that reduced protein burden on the ER or reduced expression of a specific proapoptotic factor could be responsible.

Cooperation between FAS inhibitors and thapsigargin. At least one study has shown that ER stress can have a negative effect on the activity of chemotherapeutic drugs (32). To determine the effect of ER stress on FAS inhibitor-induced cell death, clonogenic survival assays, immunoblot, and RT-PCR analysis were done on tumor cells treated with orlistat or C75 in combination with the ER

Figure 6. Pharmacologic FAS inhibitors cooperate with thapsigargin. **A**, PC-3 cells were treated with DMSO, C75 (9 $\mu\text{g}/\text{mL}$), Tgn (25 nmol/L), or the combination of each for 12 h and clonogenic survival was normalized relative to vehicle-treated controls (*left*). Times and doses of these various experiments were selected to achieve minimal cell death from single agents, so that the effect of the combination would be most clear. PC-3 cells were treated with DMSO, C75 (9 $\mu\text{g}/\text{mL}$), Tgn (25 nmol/L), or the combination of both for 20 h and samples were subjected to immunoblot analysis (*top right*), or RNA was collected and semiquantitative RT-PCR was done using primers specific for GADD34 or β -actin (*bottom right*). **B**, HT-29 were treated with DMSO, orlistat (25 $\mu\text{mol}/\text{L}$), Tgn (25 nmol/L), or the combination of each for 12 h (*left*). Cells were treated with DMSO, C75 (9 $\mu\text{g}/\text{mL}$), Tgn (25 nmol/L), or the combination of each for 12 h (*right*). Clonogenic survival was normalized relative to vehicle-treated controls. **C**, model demonstrating that in a proliferating tumor cell, FAS contributes to ER function by driving phospholipid synthesis (*left*). When FAS is inhibited (*right*), ER stress is induced, which activates a series of downstream events that mediate adaptation and promote ER membrane biogenesis.



stress agent, thapsigargin (Fig. 6A and B). Lower doses of FAS inhibitors and thapsigargin were used to achieve reduced cell killing by either single agent in order to maximize the effect of the combination of the two drugs. PC-3 cells were seeded at a low density and treated with C75 (9 $\mu\text{g}/\text{mL}$), thapsigargin (25 nmol/L), or the combination of both for 12 h and assayed for clonogenic survival. The clonogenic survival of cells treated with the combination of drugs was significantly reduced compared with either agent alone (Fig. 6A, *left*). This coincides with immunoblot data demonstrating that levels of cleaved PARP are highest in lysates from cells treated with both drugs (Fig. 6A, *top right*). Interestingly, whereas both C75 and thapsigargin induced the phosphorylation of eIF2 α separately, the level of phosphorylated eIF2 α was significantly reduced in cells treated with the two agents combined, with no change in total eIF2 α levels (Fig. 6A, *top right*). This data suggests that the combined agents facilitated a more rapid progression of the ER stress response and induction of the GADD34 feedback loop (33). In support of this, mRNA expression of GADD34 was increased in PC-3 cells treated with both agents, as compared with either agent alone (Fig. 6A, *bottom right*). Confirming that the effects of this combination of drugs are not cell type-specific, clonogenic survival was assessed in HT-29

cells treated with one of two combinations: either (a) orlistat (25 $\mu\text{mol}/\text{L}$), thapsigargin (25 nmol/L), or the combination of both for 12 h (Fig. 6B, *left*); or (b) C75 (9 $\mu\text{g}/\text{mL}$), thapsigargin (25 nmol/L), or the combination of both for 12 h (Fig. 6B, *right*). Although clonogenic survival only indicates an additive interaction, these data, importantly, show that ER stress does not inhibit the actions of FAS inhibitors and may actually enhance their efficacy.

Discussion

The ER stress response is a choreographed series of cellular events activated by specific insults that result in altered ER function (26, 34). The combined effect of this response is the activation of genes that are specifically expressed to engage an adaptation protocol. Upon prolonged stress, the adaptation mechanism of the ER stress response is saturated, thus, activating cell death. Several studies have developed important connections between lipid synthesis pathways and the ER stress response (19–21, 35). Inhibition of phospholipid synthesis, especially that of phosphatidylcholine, induces ER stress-related pathways (20). Similarly, altering phospholipid metabolism by manipulation of phospholipase activity amplifies the ER stress response in β -cells

(36). In addition, the accumulation of the G_{M1}-ganglioside activates the ER stress response in neurons, indicating that sphingolipid levels are also important for regulating ER function (35). Recent studies have shown that cholesterol-induced apoptosis in macrophages is triggered by ER stress induction, and small molecule inhibitors of cholesterol synthesis activate the integrated stress response (24, 25). There are also direct links between the individual ER stress components and lipid synthesis pathways. For instance, overexpression of the ER stress-specific XBP-1(s) expands ER volume by increasing the activity of enzymes responsible for phosphatidylcholine synthesis (19). Moreover, mice that are null for *XBP-1* have underdeveloped ER (37, 38). Collectively, these data show that lipid and sterol levels are important for maintaining ER function.

Because FAS inhibitors are being developed as antitumor agents, it is important to understand the effects these drugs have on both normal and tumor cells. The evidence here shows that FAS inhibitors induce the ER stress response in a variety of tumor cells, but not in normal cells. A model summarizing these data is presented in Fig. 6C. Our results suggest that in a proliferating tumor cell, FAS activity drives phospholipid synthesis, which facilitates ER homeostasis and function. When FAS activity is inhibited in tumor cells, the result is PERK-dependent phosphorylation of eIF2 α , a concomitant attenuation of protein synthesis and the IRE1-mediated processing of XBP-1. Interestingly, phosphorylation of eIF2 α persists for as long as 48 h with no attenuation (Fig. 5A), suggesting that protein phosphatase 1 activity is not activated or that GADD34 is not induced as is evidenced by the lack of expression in Fig. 6A. Downstream of PERK and IRE1 activation, inhibiting FAS activity also induces mRNA expression of canonical markers of the ER stress response pathway including CHOP, GRP78, and ATF4. The precise role of these players has not been determined in cells that have been treated with FAS inhibitors. However, it has previously been shown that ATF4 acts to protect cells from ER-generated reactive oxygen species (40). Another report showed that siRNA-mediated knockdown of FAS or acetyl CoA carboxylase in breast cancer cells results in cell death that is mediated by reactive oxygen species and is attenuated by supplementation with the antioxidant vitamin E, which suggests that the ER stress response in general, and ATF4 expression specifically, may be a response to changes in the redox status of FAS inhibitor-treated cells (18). These data are consistent with the hypothesis that the ER stress response initially acts to protect cells from FAS inhibitors, but do not rule out that ER stress could also facilitate cell death after prolonged stress.

It is interesting to note that several indicators of ER stress, including phosphorylation of eIF2 α , inhibition of protein synthesis, and XBP-1 processing are detected well before the canonical

hallmarks of apoptosis, cleaved caspase 3 and cleaved PARP. This indicates that the ER may be an early sensor of fatty acid and phospholipid levels in tumor cells. When the ER stress response is unable to fully restore ER function perturbed by FAS inhibition, it is possible that this could lead to the initiation of a cell death program. Consistent with this notion, cycloheximide is able to inhibit the orlistat-induced cell death and phosphorylation of eIF2 α . These data do not conflict with previous reports demonstrating that FAS inhibitors activate the intrinsic cell death pathway and that ceramide accumulation contributes to FAS inhibitor-induced cell death (34, 41). In fact, a previous study showed that ceramide accumulation was also associated with thapsigargin-induced ER stress and apoptosis (36). It is possible that FAS inhibitor-induced ER stress may result in ceramide accumulation which is important for cell death; however, the relationship between FAS inhibition, ER stress, and subsequent downstream events remain to be determined. Given the importance of phospholipid synthesis during S phase of the cell cycle, showing that FAS inhibitors induce ER stress in tumor cells also compliments a previous study which established that FAS inhibitors induce apoptosis during S phase (42, 43). Collectively, the data presented herein fill a critical gap in our understanding of how endogenous fatty acid synthesis is required to maintain proper ER integrity and function.

We have shown that inhibiting FAS activity in tumor cells induces an ER stress response. Based on these findings, we propose that one teleologic explanation for high FAS levels in tumors is to provide support for a dynamic ER in rapidly proliferating cells. Furthermore, we hypothesize that the ER acts as a sensor of FAS activity and resulting phospholipid levels. In addition, we show that orlistat and C75 cooperate with the ER stressing agent thapsigargin to enhance cell death *in vitro*. Although thapsigargin is highly toxic and not a likely candidate for tumor therapy, these data imply that tumor microenvironment-induced ER stress will not hinder the efficacy of FAS inhibitors (44). The data also suggests that FAS inhibitors might be combined with PERK inhibitors to enhance tumor cell cytotoxicity. In summary, these data provide the first evidence that FAS inhibitors induce ER stress, which may explain some antitumor effects of FAS inhibitors, and they also establish an important mechanistic link between FAS and ER function in tumor cells.

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Crystal structure of the thioesterase domain of human fatty acid synthase inhibited by Orlistat

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Human fatty acid synthase (FAS) is uniquely expressed at high levels in many tumor types. Pharmacological inhibition of FAS therefore represents an important therapeutic opportunity. The drug Orlistat, which has been approved by the US Food and Drug Administration, inhibits FAS, induces tumor cell-specific apoptosis and inhibits the growth of prostate tumor xenografts. We determined the 2.3-Å-resolution crystal structure of the thioesterase domain of FAS inhibited by Orlistat. Orlistat was captured in the active sites of two thioesterase molecules as a stable acyl-enzyme intermediate and as the hydrolyzed product. The details of these interactions reveal the molecular basis for inhibition and suggest a mechanism for acyl-chain length discrimination during the FAS catalytic cycle. Our findings provide a foundation for the development of new cancer drugs that target FAS.

The human FAS protein is specifically upregulated in many tumors, including, but not limited to, those from prostate and breast tissue¹. FAS expression also correlates with poor prognosis, suggesting that FAS activity is advantageous to cancer cells. The natural product cerulenin and its synthetic analog C75 have been shown to inhibit FAS activity, induce apoptosis and inhibit tumor growth in xenograft and transgenic models of cancer^{2–5}. These studies and RNA interference experiments have verified FAS as a key anticancer target⁶. Orlistat, a US Food and Drug Administration (FDA)-approved β -lactone-containing drug, inhibits the thioesterase domain of FAS, induces endoplasmic reticulum stress and tumor cell death, inhibits tumor growth and prevents angiogenesis^{7–9}. Therefore, we set out to determine how Orlistat inhibits the FAS thioesterase, in an effort to provide a basis for the development of new therapeutic agents.

The thioesterase is the seventh functional domain of the 270-kDa FAS polypeptide (**Fig. 1a**)^{10,11}. The remaining domains of the FAS homodimer coordinate to primarily synthesize *de novo* the 16-carbon (C₁₆) fatty acid palmitate (**Supplementary Fig. 1** online). The thioesterase domain catalyzes the termination step by hydrolyzing the thioester bond between palmitate and the 4'-phosphopantetheine moiety of the acyl-carrier protein (ACP) domain^{12–14}. The release of the thioesterase from FAS by limited proteolysis, however, results in the production of fatty acids containing 20 to 22 carbons¹⁵. Thus, the thioesterase domain is essential in regulating the length of the fatty acid chain.

The structural origins behind the FAS reaction and substrate specificity have been investigated by several groups. Chemical cross-linking, complementation and cryo-EM studies have led to proposals

of the domain interactions within FAS^{10,11,16,17}. The recent crystal structure of porcine FAS has resolved many of the discrepancies between these proposals and provided remarkable insight into the shuttling of the growing fatty acid chain between the domains¹⁸. Notably, the ACP and thioesterase domains are not visible in the porcine FAS structure, suggesting that they are inherently flexible relative to the core of FAS.

The crystal structure of the recombinant thioesterase domain has previously been solved in the absence of ligands¹⁹. We herein describe the 2.3-Å-resolution crystal structure of the thioesterase in complex with Orlistat (**Fig. 1b**) in two forms: an unusually stable acyl-enzyme intermediate and the hydrolyzed product. These structures show that Orlistat is a substrate of the thioesterase domain, which is comprised of two subdomains (**Fig. 1c**), with the Ser2308-His2481-Asp2338 catalytic triad located in subdomain A. A surface loop within this canonical α/β -hydrolase fold is extended to form the unique, α -helical subdomain B. An analysis of site-directed mutants and molecular modeling using a substrate-analog inhibitor have also suggested that only about four to six carbon atoms at the terminus of palmitate fit into a cavity at the interface of the two subdomains¹⁹. In the present study, the C₁₆ core of Orlistat is reminiscent of palmitate and binds almost exclusively to a hydrophobic surface channel generated by subdomain B and not the interface cavity. These observations provide a clear mechanism for Orlistat inhibition, a rationale for substrate specificity and a description of possible ACP domain interactions. The resulting molecular blueprint will enable future biochemical studies and the design of anticancer compounds with improved potency, selectivity and bioavailability.

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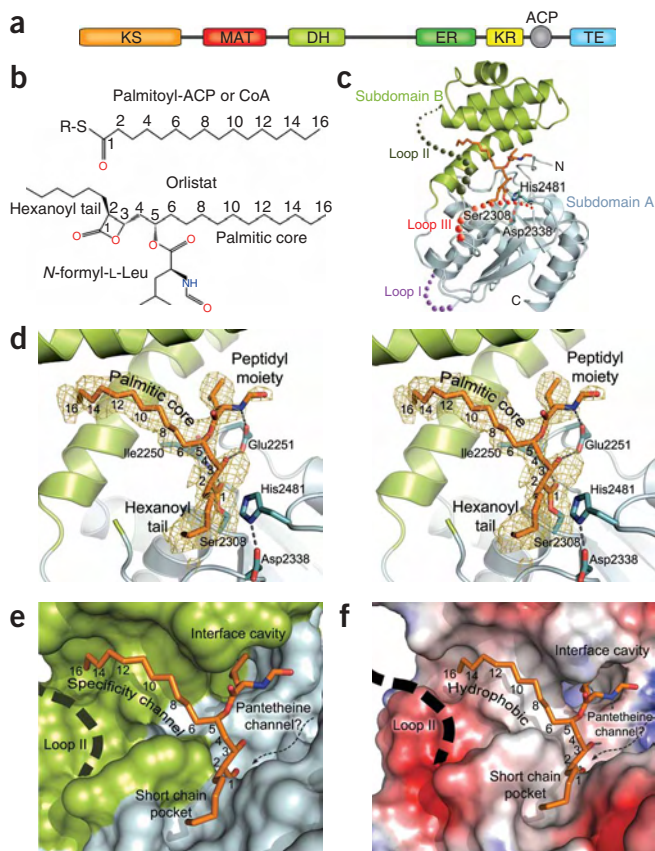


Figure 1 FAS and the covalent thioesterase–Orlistat complex. **(a)** Domain organization of FAS and its seven catalytic activities: β -ketoacyl synthase (KS), acetyl/malonyl-CoA transferase (MAT), β -hydroxyacyl dehydratase (DH), enoyl reductase (ER), β -ketoacyl reductase (KR), acyl-carrier protein (ACP) and thioesterase (TE). **(b)** Structural comparison between FAS substrates and Orlistat. R-S represents the thioester-linked 4'-phosphopantetheine moiety attached to either the ACP domain or CoA. **(c)** Overall fold of the thioesterase domain with Orlistat bound. The topology of the thioesterase within the Orlistat complex shares similarities with the apoenzyme structure¹⁹; superposition of the C α carbon atoms results in an r.m.s. deviation of 3.5 Å. **(d)** Orlistat bound in the active site of chain A. The Orlistat scaffold is divided into three fragments: peptidyl moiety (*N*-formyl-L-leucine substituent extending off the C5 carbon atom), palmitic core and hexanoyl tail (C2 substituent). Shown covering Orlistat and Ser2308 is the $F_o - F_c$ simulated-annealing omit electron density contoured at 3 σ . **(e, f)** Molecular **(e)** and electrostatic **(f)** surface representations colored to reflect the subdomain division (as in **c**) and electrostatic potential, respectively. The potential contours in **f** are shown on a scale from +130 (blue) to -40 $k_B T e^{-1}$ (red); white indicates no charge.

tion at all four chiral centers is consistent with a nucleophilic attack at the carbonyl C1 position and not the C3 position of the β -lactone ring. These observations are in agreement with previously reported mass spectrometry data for Orlistat hydrolyzed by porcine pancreatic lipase^{20,21}.

In addition to the Ser2308 adduct, the various chemical moieties of Orlistat (**Fig. 1b**) extensively interact with the surface of the thioesterase (**Fig. 1e, f**). The *N*-formyl-L-leucine moiety (or peptidyl moiety) interacts with a cavity at the interface that is also evident in the apoenzyme structure¹⁹. The 16-carbon palmitic core binds in a hydrophobic channel that we term the specificity channel, and the hexanoyl tail binds in a pocket that we term the short-chain pocket. These extensive interactions correlate well with the observed potency of Orlistat against the recombinant thioesterase domain and FAS in tumor cells^{7,22}.

The peptidyl moiety of Orlistat binds in the interface cavity, which is generated by residues from both subdomains (**Fig. 2a**). The L-leucine component interacts primarily with residues on the rim of the cavity, including Leu2222, Ile2250, Gln2374, Phe2370, Phe2371, Phe2375 and Phe2423. Glu2251 forms a hydrogen bond to the nitrogen atom of the *N*-formylamide group. The distal chamber of the cavity is filled with several water molecules held in place by a hydrogen-bonding network that includes Lys2426. The palmitic core of Orlistat binds a predominantly hydrophobic specificity channel (**Fig. 1f** and **Fig. 2b**) comprised of residues almost exclusively from subdomain B: Ala2363, Glu2366, Ala2367, Phe2370, Phe2423, Tyr2424, Leu2427, Arg2428 and Glu2431. Residues Ile2250 and Tyr2309 from subdomain A are the exceptions. The majority of the former residues are conserved among FAS homologs (**Supplementary Fig. 3** online). The main chain nitrogen atoms of the latter two residues form the oxyanion hole and weakly hydrogen-bond with the C1 carbonyl oxygen atom of Orlistat. Ala2363 and Tyr2424 provide a constriction at the end of the channel. Glu2431 and Arg2428 are located at the far end of the channel and provide an electrostatic barrier near the terminal carbon atom of the C₁₆ acyl chain of Orlistat. The hexanoyl tail, which extends off the C2 carbon atom of Orlistat, interacts with Gly2339, Thr2342, Tyr2343 and Tyr2462 (**Fig. 2c**). Moreover, this functional group packs against His2481 of the catalytic triad.

Hydrolyzed Orlistat

The electron density for hydrolyzed Orlistat in chain B of the complex (**Fig. 3a**) indicates that the acyl-enzyme intermediate has collapsed to

RESULTS

Thioesterase domain–Orlistat structure overview

The human thioesterase domain–Orlistat complex crystallized with two molecules (chains A and B) present in the asymmetric unit (**Table 1**), stabilized by a DTT molecule and several water molecules bound at the interface (**Supplementary Fig. 2** online). Each thioesterase molecule contained a different form of Orlistat: a covalent acyl-enzyme intermediate or the hydrolyzed product. The monomeric thioesterase domain is divided into subdomains A and B (**Fig. 1c**). Subdomain A contains a central, mostly parallel β -sheet made up of seven strands. Flanking the half barrel sheet are four α -helices, which complete the canonical α/β -hydrolase fold. Like other α/β -hydrolases, the thioesterase domain contains a loop insertion near the catalytic Ser2308–His2481–Asp2338 triad. In this case, however, the loop insertion is an entire domain (subdomain B) that adopts a unique, helical tertiary structure. Three additional loop regions had poor electron density and were not included in the model: loop I (residues 2326–2328, missing in chain A only) connects α -helix 4 (α_4) to β -strand 5 (β_5), forming a surface loop on the underside of the α/β domain, away from the active site; loop II (residues 2344–2360) bridges subdomain A and subdomain B; and loop III (residues 2450–2460) is near the catalytic triad linking β_6 to β_7 .

Orlistat acyl-enzyme intermediate

The electron density for chain A (**Fig. 1d**) shows that Orlistat binds in an extended conformation with covalent attachment to Ser2308 of the catalytic triad within subdomain A. A closer inspection of the Orlistat adduct indicates that it is a stabilized acyl-enzyme intermediate (that is, a stable ester linkage). Moreover, the retention of the S configura-

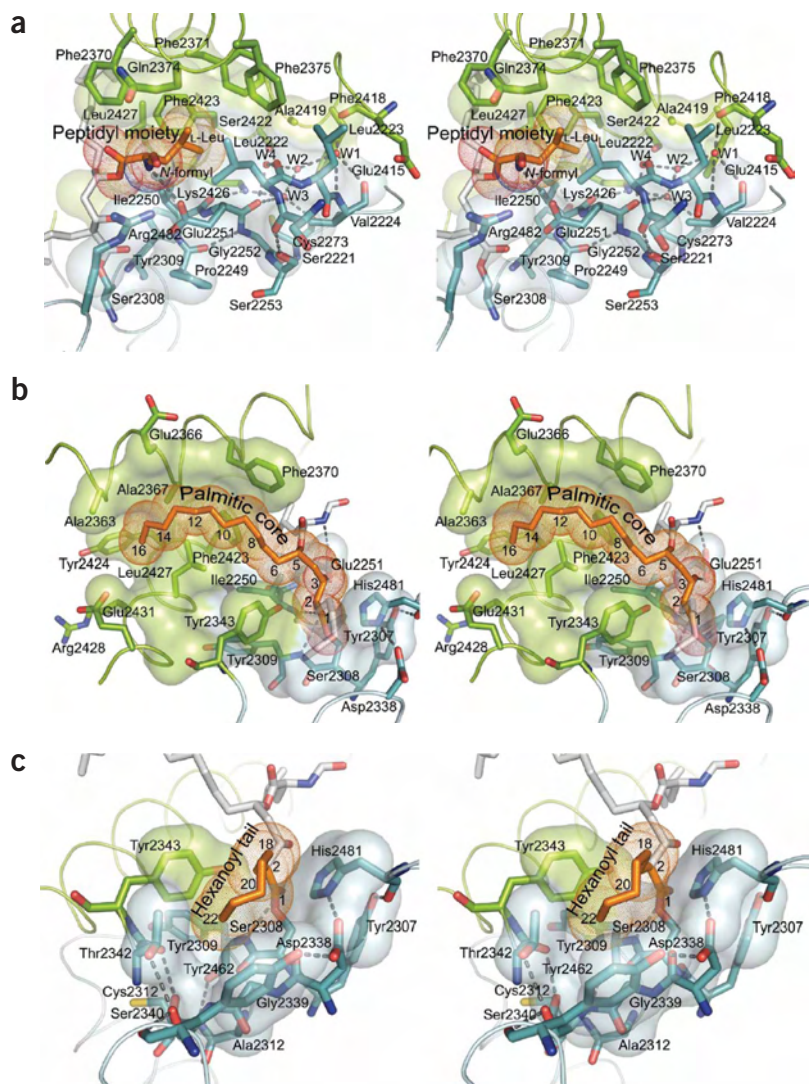


Figure 2 Detailed interactions between the thioesterase domain and Orlistat chemical moieties. Shown are atomic surfaces of only those residues that contribute to Orlistat binding in AREAIMOL calculations. (a) Stereo view (oriented 90° from Fig. 1d–f) of the bound peptidyl moiety within the interface cavity. Gray dashed lines denote putative hydrogen bonds. Water molecules are labeled W1–W4. The nitrogen atom of the *N*-formyl group is 3.2 Å from the O ϵ 1 atom of Glu2251. (b) Stereo view of the palmitic core packing onto the hydrophobic specificity channel. (c) Stereo view of the hexanoyl tail binding into the hydrophobic short-chain pocket.

with FAS inhibition^{7–9}. The possibility of targeting the thioesterase domain of FAS with Orlistat or other compounds prompted us to characterize the molecular basis for this important interaction. Our observations provide structural confirmation that nucleophilic attack occurs at the C1 carbonyl atom of Orlistat (Fig. 1b), leading to the formation of an unusually stable acyl-enzyme intermediate (Fig. 1d). Therefore, the Orlistat covalent complex will serve as a model for the interactions and reactivity of presumably all β -lactone-containing compounds designed for the thioesterase domain^{22,23}. Moreover, the C₁₆ hydrocarbon backbone of Orlistat mimics palmitate, enabling a rationalization of substrate specificity.

A previous study has docked a molecular model of the C₁₆-containing inhibitor hexadecyl sulfonyl fluoride onto the apoenzyme form of the thioesterase domain¹⁹. The authors proposed that only about four to six carbon atoms at the terminus of a C₁₆ or C₁₈ substrate fit into the interface cavity. In contrast, we found that the C₁₆ palmitate-like moiety of Orlistat is bound in an extended conformation to a hydrophobic specificity channel ~23 Å in length (Fig. 1c–e), and the *N*-formyl-L-leucine moiety packs into the interface cavity. We also identified a new short chain-binding pocket, which surrounds the hexanoyl tail that extends off the C2 position of Orlistat. Notably, if one counts the number of carbons from the C1 atom of Orlistat to the end of the L-leucine side chain (Fig. 2a), the aliphatic chain is 11 carbons in length, and the limited volume and depth of the interface cavity (219 Å³ and ~10–14 Å, respectively) suggest that C₁₆ substrates cannot be accommodated. The reduced activity of some mutants in this region is most probably a consequence of prying the subdomains apart. For example, the mutation of Ile2250, Ala2419 and Phe2423 to larger amino acid residues, as described¹⁹, would be especially detrimental, as the spatial relationships between the catalytic triad, oxyanion hole and specificity channel would be changed. Furthermore, mutation of Ile2250 to a bulky residue probably occludes the oxyanion hole¹⁹. Electrostatic calculations (Fig. 1f) suggest that the presence of Lys2426 and a series of water molecules in the interface pocket (Fig. 2a) would also deter the binding of aliphatic substrates.

The binding of palmitoyl substrates to the hydrophobic specificity channel of the thioesterase domain is supported by two additional

form a product containing a carboxylic acid at the C1 position. This finding is most probably a consequence of the 3-week crystallization period, as there are minor structural differences between the thioesterase molecules and no apparent crystal-lattice effects. The C1 position of the product is also shifted ~4 Å (Fig. 3b) relative to the covalent adduct, and two intervening water molecules now occupy the oxyanion hole. The position of the peptidyl moiety is altered slightly, and the palmitic core has shifted in register by approximately two carbon units (Fig. 3c). This shift places the terminal carbon atom closer to the hydrophobic constriction and charge barrier created by Ala2363, Tyr2424, Glu2431 and potentially Arg2428. The hexanoyl C2 substituent has also moved considerably (~9 Å), and Tyr2343 of loop II has repositioned to form a hydrogen bond with the new C1 carboxylate group of Orlistat. The interactions of the covalent adduct and hydrolyzed Orlistat also suggest that the 4'-phosphopantetheine arm of the ACP domain docks into another channel adjacent to Ser2308 (Fig. 1e,f) lined with the conserved residues Val2256, Tyr2307, Arg2482 and Leu2485 (Supplementary Fig. 3).

DISCUSSION

Although Orlistat is currently approved by the FDA for obesity management, the antitumor effects of Orlistat are clearly associated

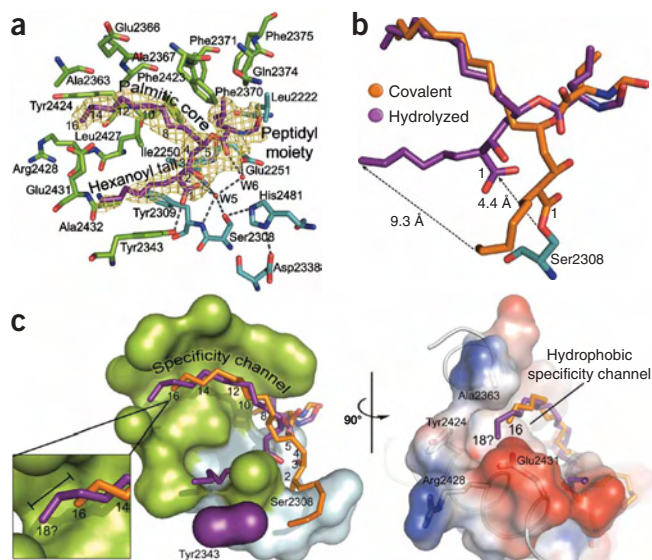


Figure 3 Metabolized Orlistat interactions and chain-length selectivity. (a) Hydrolyzed Orlistat within the active site of chain B, depicted as in **Figure 2**. Shown covering the Orlistat product is the $F_0 - F_c$ simulated-annealing omit electron density contoured at 3σ . (b) Superposition of the two metabolized forms of Orlistat. (c) Molecular (left) and electrostatic surface (right, as in **Fig. 1f**) representations of the covalent and hydrolyzed Orlistat complexes. Upon hydrolysis of the acyl-enzyme intermediate, Tyr2343 of loop II moves down to hydrogen-bond with the C1 carboxyl group of Orlistat. The resulting change in the surface is indicated in purple. A shift in register relative to the covalent intermediate extends the hydrolyzed product two carbon units (indicated by bar in inset) beyond C₁₆, mimicking the chain length of C₁₈.

observations. First, Orlistat is a substrate that forms an acyl-enzyme intermediate (**Fig. 1d**), which ultimately collapses to form the product (**Fig. 3a**). Second, the almost exclusive binding of the palmitic core to subdomain B is consistent with the observed structural plasticity of α/β -hydrolases in this region. Surface loop insertions into the common α/β core (**Fig. 1c**) have made the α/β -hydrolase superfamily among the most functionally diverse of protein families²⁴. Although catalysis occurs via the same chemistry in all α/β -hydrolases, a variety of substrates of different sizes are cleaved. Therefore, the thioesterase–Orlistat complexes provide evidence that the role of the inserted α -helical subdomain is to provide a hydrophobic surface channel for substrate selection. The presence of Glu2431 and Arg2428 and the constriction caused by Ala2363 and Tyr2424 (**Fig. 2b** and **Fig. 3c**) most probably generate the preference for C₁₆-containing substrates such as Orlistat. The translation of the Orlistat product complex that we observed is consistent with the ability of the thioesterase domain to catalyze the release of C₁₈ substrates^{12–14}.

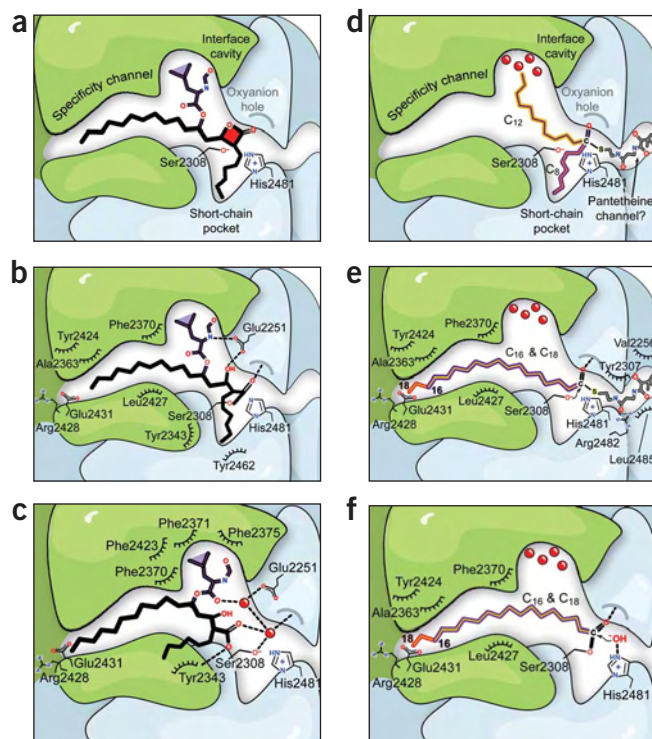
Figure 4 presents a model for the molecular basis of inhibition by β -lactone-containing compounds and of the hydrolysis of substrates. Although the binding mode of the β -lactone form of Orlistat is not known, we postulate that the oxygen atom of the C1 carbonyl group is bound in the oxyanion hole (**Fig. 4a**). The interactions with the specificity channel provide the correct register, leading to the attack of Ser2308 to form the acyl-enzyme intermediate and subsequent opening of the β -lactone ring (**Fig. 4b**). This intermediate is stabilized by the oxyanion hole and a hydrogen bond to Glu2251. Most

Figure 4 Molecular basis for Orlistat-mediated inhibition and substrate selectivity. (a) Proposed interactions with the intact β -lactone form of Orlistat. (b) The Orlistat acyl-enzyme intermediate. Semicircles with tick marks indicate van der Waals interactions. (c) Hydrolyzed Orlistat and its movements. (d) Hypothetical mechanism for chain-length sampling. Red spheres represent four water molecules involved in an intricate hydrogen-bonding network within the interface cavity (**Fig. 2a**); for simplicity, waters are omitted in **a–c**. The 4'-phosphopantetheine arm (gray stick model) of ACP is shown bound to the putative pantetheine channel. Representative growing acyl chains measuring 8 and 12 carbons in length (extrapolated from the Orlistat covalent complex) are shown binding transiently to the short-chain pocket and interface cavity, respectively. (e) Selection of C₁₆ and C₁₈ substrates and formation of the Michaelis complex. (f) Hydrolysis of the acyl-enzyme intermediate.

importantly, the hexanoyl tail that extends off the C2 position of Orlistat packs against His2481 (**Fig. 2c**). This interaction may prevent the activation of a water molecule that, for naturally occurring substrates, would result in the immediate hydrolysis of the intermediate.

Given time, however, the hexanoyl tail of Orlistat moves enough to allow water activation by His2481, and the Orlistat adduct undergoes deacylation (**Fig. 4c**). The peptidyl moiety seems to serve as a key determinant in anchoring the Orlistat product to the interface cavity, as only minor changes in this moiety were observed relative to the acyl-enzyme intermediate. In contrast, the hexanoyl-tail and palmitic-core fragments shift considerably to the left, which allows two intervening water molecules to bind, one of which is in the oxyanion hole. With these movements, the hexanoyl tail vacates the short-chain pocket and establishes new interactions with Tyr2309, Tyr2343 and Ala2432. Notably, the portion of loop II that is visible by crystallography undergoes a structural rearrangement (**Fig. 3c**) to form hydrophobic contacts and a hydrogen bond between the hydroxyl group of Tyr2343 and the new C1 carboxylate group of Orlistat.

Our thioesterase–Orlistat complexes also provide a rationale for chain-length selectivity during the fatty acid synthesis cycle



(Supplementary Fig. 1). During the FAS reaction, the acyl chain grows by two carbon units. One potential scenario for the selection of substrates is that the interactions of the thioesterase and ACP domains with the other domains of FAS influence the loading of substrate into the thioesterase domain. This domain can release palmitate and stearate (C₁₈) when attached to ACP or CoA^{10–14}. The proteolytic release of the thioesterase from FAS, however, results in the synthesis of C₂₀ and C₂₂ fatty acids¹⁵. Therefore, the thioesterase seems to be a crucial determinant of substrate selection. These observations suggest a second scenario, where FAS reaction intermediates sample the short-chain pocket and interface cavity (Fig. 4d). It appears that these regions are able to accommodate only short- to medium-length fatty acids (of 4 to 12 carbons). The thioester moiety of such potential thioesterase substrates is probably not positioned correctly for catalysis to occur²⁵. C₁₆ and C₁₈ substrates, however, would extend to the end of the specificity channel (Fig. 4e) like the palmitic core of Orlistat, thus positioning the scissile bond appropriately for catalysis. Ala2363, Tyr2424, Arg2428 and Glu2431 at the far end of this channel appear to be responsible for generating the barrier to longer substrates. In contrast to the slow hydrolysis of the Orlistat acyl-enzyme intermediate, His2481 facilitates the rapid hydrolysis of palmitoyl or stearoyl acyl-enzyme intermediates (Fig. 4f).

In this model, substrates that bind the specificity channel should be no shorter than 16 carbons, to correctly orient the thioester bond relative to Ser2308 for nucleophilic attack (Fig. 4e). In this context, the 4'-phosphopantetheine arm of the ACP domain or CoA may also help to position the substrate correctly for catalysis. We identified a putative binding channel adjacent to Orlistat and the catalytic triad. The proximity of the flexible loop II to the catalytic triad, short-chain pocket and specificity channel also suggests that loop II may participate in substrate loading within the macromolecular FAS dimer. These insights combined may be useful for orienting the ACP and thioesterase domains within the available EM structures and future crystal structures of intact FAS^{16,17}. We envision that the FAS thioesterase–Orlistat complexes will stimulate the design, synthesis, optimization and testing of new compounds for cancer therapy and additional functional studies concerning FAS biochemistry and substrate specificity.

METHODS

Protein expression and purification. The thioesterase domain of human FAS (residues 2200–2510) was cloned as previously described⁷. The domain was subcloned into the pET15b (Novagen) vector containing an N-terminal His₆ tag with an intervening thrombin cleavage site. The thioesterase was over-expressed in C41(DE3) *Escherichia coli* grown in a 10-l fermentor at 37 °C and induced by 0.5 mM IPTG at 25 °C when the cells reached an A₆₀₀ of 1. The cells were harvested after 4 h and lysed, and the supernatant was passed over a nickel–nitrilotriacetic acid affinity column (Qiagen). The desired fractions were immediately treated with 2 mM EDTA and 15 mM DTT and dialyzed overnight at 4 °C against 20 mM HEPES (pH 8.5), 1 mM EDTA and 1 mM DTT. The protein was further purified using a DEAE Macro-Prep column (Bio-Rad) with a linear gradient from 0 to 1 M NaCl in 20 mM HEPES (pH 8.5), 1 mM EDTA and 1 mM DTT. The thioesterase was concentrated to 2 mg ml⁻¹ and treated with 0.1 U mg⁻¹ biotinylated thrombin (Novagen) at 16 °C overnight to remove the His tag. His-tag cleavage was confirmed by MALDI-TOF mass spectrometry, and the biotinylated thrombin was removed by the addition of streptavidin agarose (Novagen). To obtain the preformed covalent thioesterase–Orlistat complex, a reaction mixture containing the thioesterase domain and a 60-fold molar excess of Orlistat (β-lactone form) was incubated at 20 °C for 1 h. The completion of adduct formation was confirmed by electrospray mass spectrometry. The resulting complex was purified away from excess Orlistat and buffer exchanged using a Superdex 200 gel-filtration column (GE Healthcare) equilibrated with 20 mM HEPES (pH 7.5), 100 mM NaCl and 1 mM DTT. The

Table 1 Data collection and refinement statistics

	Orlistat complexes
Data collection	
Space group	P2 ₁
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	41.9, 94.3, 69.7
α , β , γ (°)	90.0, 95.8, 90.0
Resolution (Å)	39.0–2.3 (2.38–2.30)
<i>R</i> _{merge}	0.06 (0.23)
<i>I</i> / σ <i>I</i>	17.0 (6.2)
Completeness (%)	96.0 (76.2)
Redundancy	7.1 (6.2)
Refinement	
Resolution (Å)	39.0–2.3
No. reflections	21,850
<i>R</i> _{work} / <i>R</i> _{free}	22.5 / 27.3
No. atoms	
Protein	4,016
Orlistat covalent	35
Orlistat hydrolyzed	36
DTT	8
Water	53
<i>B</i> -factors	
Protein	36.6
Orlistat covalent	60.2
Orlistat hydrolyzed	67.1
Water	30.6
R.m.s. deviations	
Bond lengths (Å)	0.01
Bond angles (°)	1.27

Data were collected from a single crystal. Values in parentheses are for the highest-resolution shell.

complex was concentrated to 17 mg ml⁻¹, flash-frozen in liquid nitrogen and stored at –80 °C.

Crystallization and structure determination. Crystals of the thioesterase–Orlistat complex were grown using hanging drop vapor diffusion. Equal volumes of protein (6 mg ml⁻¹ in 20 mM HEPES (pH 7.5), 100 mM NaCl and 30 mM DTT) and precipitant solution were mixed and equilibrated at 15 °C against well solutions containing 100 mM sodium dihydrogen phosphate (pH 4.25), 20%–26% (w/v) PEG 3,350 and 30 mM DTT. Crystals were transferred to a synthetic mother-liquor solution containing 50 mM sodium dihydrogen phosphate (pH 4.25), 32% (w/v) PEG 3,350, 30 mM DTT and 25% (v/v) of the cryoprotectant ethylene glycol before cryocooling to –170 °C. X-ray diffraction data ($\lambda = 1.0$ Å) were collected at the National Synchrotron Light Source, Brookhaven National Laboratory, on beamline X12C. Data were indexed and scaled to 2.3-Å resolution using the d*TREK suite²⁶ (Table 1). The structure was solved by molecular replacement using PHASER²⁷ with the native, uncomplexed structure of the thioesterase domain (PDB 1XKT)¹⁹ as a search model. The initial model was manually rebuilt using COOT²⁸. Iterative structure refinement was carried out using a combination of CNS²⁹ and REFMAC5 (ref. 30). DTT molecular topologies were obtained using the HIC-Up server³¹. The molecular coordinates and topologies of both the covalent and hydrolyzed forms of Orlistat were generated using the PRODRG2 server³². The final refined model of the thioesterase–Orlistat complex had *R*_{work} and *R*_{free} values of 22.5% and 27.3%, respectively. The structure was validated using the MOLPROBITY server³³, which reported 96.8% of the residues in the Ramachandran favored regions, 2.6% in the allowed regions and 0.6% as outliers. The thioesterase domain contains several loop regions that have intrinsic mobility. As a result, a protein model could not be built for the following disordered regions: residues 2326–2328 (loop I,

chain B only), 2344–2360 (loop II, both chains), 2450–2460 (loop III, both chains) and residues 2200–2220 and 2502–2510 on the N and C termini, respectively. AREAIMOL³⁴ was used to identify surface residues that interact with Orlistat through either hydrophobic or polar contacts. Other surface calculations were performed using CASTP³⁵.

Illustrations. All structural illustrations were generated using PyMOL³⁶.

Accession codes. Protein Data Bank: Coordinates and structure factors have been deposited with accession code 2PX6.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

C.W.P., S.J.K. and W.T.L. designed the experiments and interpreted the structure; S.J.K. generated the expression clone; C.W.P. and L.C.J. performed all protein expression, purification and crystallization; C.W.P. and W.T.L. contributed to structure solution and refinement; all authors contributed to manuscript preparation.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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Dietary fat–gene interactions in cancer

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Abstract Epidemiologic studies have suggested for decades an association between dietary fat and cancer risk. A large body of work performed in tissue culture and xenograft models of cancer supports an important role of various types of fat in modulating the cancer phenotype. Yet, the molecular mechanisms underlining the effects of fat on cancer initiation and progression are largely unknown. The relationships between saturated fat, polyunsaturated fat, cholesterol or phytanic acid with cancer have been reviewed respectively. However, few have considered the relationship between all of these fats and cancer. The purpose of this review is to present a more cohesive view of dietary fat–gene interactions, and outline a working hypothesis of the intricate connection between fat, genes and cancer.

Keywords Cancer · Dietary fat · Saturated fatty acids · Polyunsaturated fatty acids · Fatty acid synthase · Acylation · Lipid-mediated signal transduction · Isoprenoids · Cholesterol · Bile acids · Steroids

1 Introduction

Although a causal role of genetic alterations in human cancer is well established, epigenetic effects on cancer were not appreciated until recently [1]. Methylation, acetylation and other molecular mechanisms implicated in epigenetics are being studied intensively [2, 3], but environmental factors causing these changes are still unclear. Increasing evidence suggest that dietary fat–gene interactions play a critical role in initiation and progression of human cancer. Equally as important, alterations in the type and level of fats in diet and inhibition of *de novo* lipogenesis may hold a great promise in the prevention and treatment of cancers. Lipids belonging to several structural classes have been linked to cancer. Classification of lipids has recently been reviewed by an international group of investigators, dividing lipids into eight structural categories, namely fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides [4] (Table 1).

Lipids have many important biological functions, including energy storage, membrane synthesis, and multiple signaling pathways. Some lipids are obtained exclusively from diet, whereas others can also be synthesized *de novo*. A large number of gene products are involved in lipid biosynthesis and metabolism, and lipids exert various signaling functions which in turn modulate multiple genes (Fig. 1). Some of the most important dietary fats include glycerolipids (or triglycerides), which are metabolized to free fatty acids including saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acid (classified as omega-3 or omega-6), and methyl branched fatty acids. Saturated and monounsaturated fatty acids can be synthesized *de novo*. However, mammals lack the desaturases necessary for synthesizing omega-3 (n-3) and omega-6

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Table 1 Classes of lipids and selected examples [4]

Lipid classes
Fatty Acyls
Fatty Acids and Conjugates
Straight chain fatty acids (palmitate)
Unsaturated fatty acids
Monounsaturated fatty acids (oleate)
Polyunsaturated fatty acids (eicosapentaenoic acid, docosahexaenoic acid)
Methyl branched fatty acids (phytanic acid, pristanic acid)
Eicosanoids
Glycerolipids (triglycerides)
Glycerophospholipids (phosphoglycerides, phospholipids)
Glycerophosphocholines
Glycerophosphoethanolamines
Glycerophosphoserines
Glycerophosphoinositols
Sphingolipids
Sphingoid bases (sphingosine)
Ceramides (ceramide)
Sterol lipids
Sterols
Cholesterol
Steroids
C18 steroids (estrogens)
C19 steroids (androgens)
C21 steroids (gluco/mineralocorticoids, progestogens)
Secosteroids (Vitamin D)
Bile acids
Prenol lipids
Isoprenoids
Saccharolipids
Polyketides

(n-6) polyunsaturated fatty acids, therefore they must be obtained from diet. Their metabolism produces several classes of eicosanoids with key roles in signaling. Another important class of lipids is the sterol lipids, including cholesterol, steroid hormones, vitamin D and bile acids. Cholesterol is obtained from the diet as well as synthesized endogenously, and is the precursor for other sterol lipids, many of which regulate gene expression by binding to nuclear receptors. Fatty acids and intermediaries in cholesterol synthesis also serve a role in anchoring proteins to membranes, with crucial signaling consequences. Bile acid synthesis from cholesterol requires α -Methylacyl-CoA Racemase (AMACR), which is also critical for the metabolism of branched-chain fatty acids phytanic and pristanic acid in the peroxisome.

In this review, we will discuss the role of saturated fats, polyunsaturated fats, sterol lipids, and phytanic acid in cancer with an emphasis on proposed molecular mechanisms of action.

1.1 Saturated fats and cancer

1.1.1 *De novo* fatty acid synthesis in tumors

Although saturated fatty acids are readily available from dietary sources, in tumors the vast majority of them are synthesized *de novo* [5, 6]. Fatty acid synthase (FASN) catalyzes the synthesis of palmitate from acetyl-CoA and malonyl-CoA in eukaryotic cells using 14 ATP and 7 NADPH molecules for each palmitate molecule generated [7]. A number of other enzymes also participate in fatty acid synthesis. The conversion of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACC) is the rate limiting step. In addition, enzymes of the glycolytic and Krebs cycle, the pentose phosphate pathway, malic enzyme and ATP citrate lyase (ACLY) also contribute to the process (Fig. 2).

With the exception of liver and adipose, most normal adult tissues have little or no expression of FASN. In contrast, the expression of FASN is upregulated in many tumor types, including tumors of the prostate, breast, colon, and ovary [8]. It is clear that fatty acid synthesis is also required during development as the FASN and ACC knockout mice have lethal phenotypes [9, 10]. Interestingly, mice with liver-specific deletions of FASN or ACC have essentially normal liver function on typical diets [11, 12]. In liver and adipose tissues, FASN-generated fatty acids are stored as triglycerides, whereas in tumor cells, fatty acids are primarily segregated into phospholipids [13]. Thus, there appears to be a mechanistic distinction between fatty acid synthesis in the liver and in epithelial tumor cells.

The fact that fatty acid synthesis is energy consuming highlights the importance of the process to tumor cells and suggests that it provides an advantage for tumor cell survival and proliferation. FASN expression correlates with the aggressiveness of human prostate tumors, and is highest in androgen-independent metastases [14–16]. FASN expression is also an independent predictor of poor outcome in breast cancer patients [17]. Small molecules that target the keto-acyl synthase domain and the thioesterase domain of FASN are able to induce cell death specifically in tumor cell lines, and inhibit the growth of tumor xenografts [16, 18–20] or mammary tumor in neu-N transgenic mice [21]. Knockdown of FASN expression induces apoptosis in LNCaP cells [22]. The importance of *de novo* fatty acid synthesis in tumors is further underscored by several studies that demonstrate the requirement for other enzymes in the pathway. Inhibition of ATP citrate lyase (ACLY), the enzyme that converts citrate to acetyl-CoA, suppresses tumor cell growth [23] and knockdown of ACC induces cell death in prostate and breast cancer cells [24, 25]. Combined, these data demonstrate that fatty acid synthesis is required in tumors, and that enzymes in the lipogenic pathway are potential therapeutic targets [26, 27].

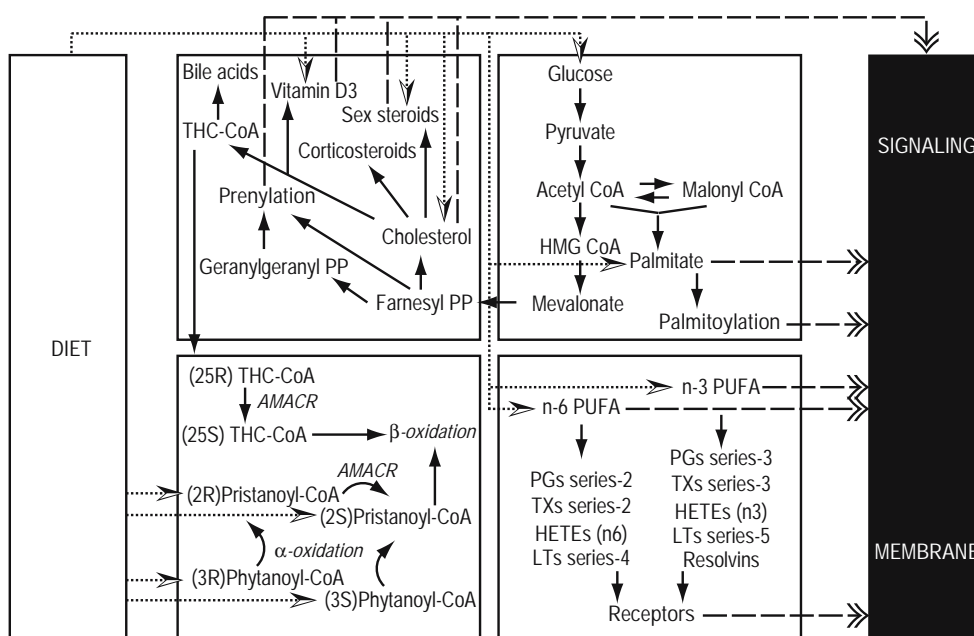


Fig. 1 Dietary and synthetic pathways of lipids and their inter-relationship. Lipogenic pathway and cholesterol/isoprenoids synthesis share a common precursor, i.e. acetyl-CoA. Cholesterol is the source of all steroids and bile acids. Bile acid synthesis and phytanic/pristanic acid oxidation both require AMACR activity. Saturated and monounsaturated fatty acids cannot be converted to n-6 or n-3 PUFA in

mammals, and PUFAs of the n-6 and n-3 series are not inter-convertible. However, dietary PUFA, mainly in form of linoleic acid for n-6 and α -linolenic acid for n-3, can be elongated and desaturated into AA and EPA which are the precursors for eicosanoids. Dietary intake of lipids is marked with *dotted lines*. Function of lipids is indicated with *broken lines*

1.1.2 Regulation of fatty acid synthesis in tumor

In liver and adipose, FASN is regulated by nutritional stimuli, primarily due to transcriptional regulation in response to insulin and other stimulatory molecules in a SREBP-1 dependent manner [5, 8]. In tumor cells, FASN appears able to bypass nutritional regulation. Instead, pathways that are important for tumor cell proliferation and survival drive FASN expression. FASN is reported to be regulated by androgen in prostate [28] and by progestins in breast cancer [29]. Treatment of LNCaP cells with the PI3 kinase inhibitor LY294002 significantly reduces FASN protein expression [30]. FASN levels also correlate with loss of Pten and nuclear localization of phosphorylated Akt in prostate tumor samples [31, 32]. Introduction of Pten into Pten-null cells or knockdown of Akt abrogates FASN expression [20, 30]. Transformation of MCF-10 breast cells with *H-ras* significantly increases, whereas treating breast cancer cells with a MAPK inhibitor decreases FASN expression [33]. These data demonstrate that the PI3k and Ras pathways serve as important regulators of FASN. An interesting paradox exists within these signaling axes. Several reports have demonstrated that inhibiting FASN activity in tumor cells decreases levels of phosphorylated Akt and *Her-2/neu*, indicating that a feedback loop exists within these pathways [20, 34]. In addition, there may be a cross-talk with another type of lipid as evidence suggests that polyunsaturated fatty acids suppress FASN synthesis [35, 36].

In addition to the transcriptional regulation described above, it has been reported that FASN can also be regulated by non-transcriptional mechanisms in prostate tumor cells. FASN levels are increased post-translationally by the isopeptidase USP2a that acts as a deubiquitinating enzyme to stabilize FASN proteins [37]. High FASN expression in prostate cancer may also be due to increased gene copy number as observed in LNCaP cells and paraffin-embedded tissue microarrays [38].

1.1.3 Cellular functions of fatty acids

As mentioned previously, in tumor cells FASN-generated fatty acids are primarily used to drive phospholipid synthesis and these phospholipids segregate preferentially to detergent-insoluble membrane domains [13]. Phospholipid synthesis occurs in the endoplasmic reticulum (ER), and a recent study demonstrates that inhibition of FASN by pharmacological agents or siRNA induced ER stress in tumor cells [39]. This provides a mechanistic connection between the activity of FASN and the function of the ER and cellular membrane. There is also indirect evidence showing that FASN activity is important for maintaining membrane integrity and function. Inhibition of FASN activity in ovarian cells reduces the phosphorylation status of AKT [20], albeit through an undefined mechanism. Considering that FASN activity is required for phospholipid synthesis, perhaps it is not surprising that phosphoinositide-

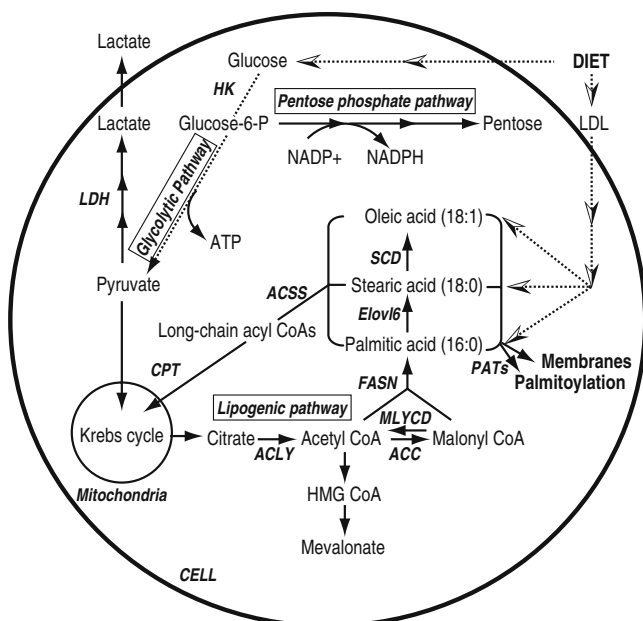


Fig. 2 Exogenous and endogenous sources of saturated fat. Saturated fatty acids such as palmitic acid can be obtained directly from diet or synthesized *de novo* from glucose. Glucose, through the glycolytic pathway, is converted to pyruvate that enters the Krebs cycle. Through the lipogenic pathway, palmitic acid is generated from acetyl-CoA and malonyl-CoA. Palmitic acid can then be elongated to stearic acid and desaturated to oleic acid. These fatty acids can modify critical signaling molecules such as Wnt and Hedgehogs through a process called palmitoylation, as well as provide membrane structural components necessary for rapid cell proliferation. *ACC* Acetyl-CoA carboxylase, *ACLY* ATP citrate lyase, *ACSS* acetyl-CoA synthase, *CPT* carnitine palmitoyltransferase, *Elovl6* ELOVL family member 6, *FASN* fatty acid synthase, *HK* hexokinase, *LDH* lactate dehydrogenase, *LDL* low density lipoprotein, *MLYCD* Malonyl-CoA decarboxylase, *PAT* palmitoyl acyl transferases (including DHHC, CRD and MBOAT family proteins), *SCD* stearoyl-CoA desaturase (delta-9-desaturase)

associated signaling is altered in cells treated with *FASN* inhibitors.

In addition to membrane biogenesis, fatty acids are also used for other processes in cells. Palmitate is an important precursor for the synthesis of sphingolipids including ceramide, and thus may be important for signaling processes [40]. Palmitate and other fatty acids are also added enzymatically as an important post-translational modification to proteins [41–43]. Fatty acid can be added to either cysteine or serine residues at the C- or N-termini of proteins or near transmembrane domains. Some of these proteins include Ras, Wnt, Hedgehog and small protein GTPases [43, 44]. The addition of palmitate to proteins increases hydrophobicity which leads to increased membrane association and targeting to facilitate efficient signaling processes. While these signaling molecules are important in a variety of cancers, there has been no direct demonstration that *de novo* fatty acid synthesis plays a role in protein palmitoylation. Future studies aimed at understanding the role of *de*

novo fatty acid synthesis in these processes will be important to fully appreciate how membrane dynamics and membrane associated signaling are regulated.

1.1.4 Dietary saturated fatty acids and cancer

As discussed above, a large body of evidence substantiates an important role of the *de novo* lipogenesis in cancer. The role of dietary saturated fat in cancer, however, is more controversial. Positive associations between saturated fat or animal fat consumption and cancer were found in some cohort studies [45] and in studies investigating cancer incidence in 20 countries [46]. Palmitic acid was significantly associated with an increase of breast cancer risk [47]. In general, inaccuracy in reporting dietary intake and difficulties in conducting mechanistic studies on human populations have hampered investigations on the role of dietary saturated fat in cancer development. Mechanistic studies, assessing the relative contribution of the endogenous *de novo* synthesis and the exogenous dietary source of palmitic acids to cancer, are needed.

1.2 Polyunsaturated fatty acids and cancer

Unlike saturated and mono-unsaturated fatty acids, both n-3 and n-6 polyunsaturated fatty acids (PUFA) are essential fatty acids that cannot be synthesized *de novo* by mammals, and are derived entirely from the diet. Terrestrial plants synthesize the first member of this series, linoleic acid (LA; 18:2n-6); within the body LA is metabolized by a series of alternating oxidative desaturation and elongation steps principally to arachidonic acid (AA; 20:4n-6). The n-3 PUFAs are found primarily in fish oils. Alpha linolenic acid (α LNA; 18:3n-3), synthesized by cold-water vegetation, is converted following ingestion by fish to two main constituents, eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3; Fig. 3). While all mammalian cells can interconvert the PUFAs within each series by elongation, desaturation and retroconversion, the two series are not interchangeable [48]. Human diet also provides α LNA. However, efficiency of its conversion to EPA and to DHA is very low [49]. In fact, studies have shown that dietary supplementation with α LNA or EPA does not result in a detectable increase in plasma DHA, particularly in males [50, 51]. Therefore, the main source of EPA and DHA in humans is through dietary intake of fish.

Fatty acids are present in the diet as triglycerides. Following dietary intake, triglycerides are packaged in the intestinal epithelium into chylomicrons, which are secreted into the lymphatic system and enter the circulation via the thoracic duct. Lipolysis of these particles, initially in tissues such as heart and lungs by the enzyme lipoprotein lipase, produces free fatty acids that quickly associate with serum

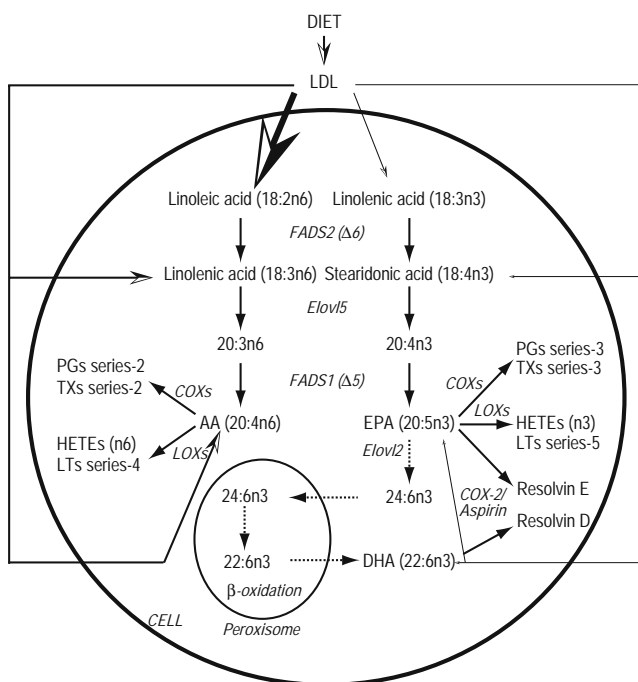


Fig. 3 Dietary intake of n-6 and n-3 PUFA and their metabolism in mammals. n-6 and n-3 PUFA are essential fatty acids. Mammals cannot synthesize them *de novo* but require them for survival. In addition, n-6 and n-3 series are not interchangeable. These PUFAs can be metabolized to corresponding series of eicosanoids, with opposing signaling effects. Another group of metabolites, the resolvins and protectins, are generated only from n-3 PUFAs. The relative dietary intake of lipids in a typical Western population is indicated by the *thickness of arrows*. Conversion of EPA to DHA requires several steps which occur at low efficiency in humans (*dotted arrows*). *FADS2* Fatty acid desaturase 2 (Delta 6 desaturase), *Elov1 2* and *5* ELOVL family member 2 and 5, *COX* cyclooxygenase, *LOX* lipoxygenase, *PG* prostaglandin, *HETE* hydroxyeicosatetraenoic acid, *TX* thromboxane, *LT* leukotriene, *EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid

albumin. Remnant particles return to the liver where their triglycerides are assembled with apolipoprotein B 100 (apoB) for secretion as very low density lipoprotein (VLDL) particles. In the circulation, lipolysis of VLDL produces LDL, which in turn is taken up by peripheral tissues. However, the molar concentration of fatty acids in LDL is an order of magnitude higher than that of fatty acid-albumin [52]. Furthermore, studies have shown that LDL receptors are upregulated in tumor cells [53]. Therefore, the LDL receptor mediated pathway is likely to be the major route for fatty acid delivery to peripheral tissues, especially tumor cells.

1.2.1 Dietary PUFA and cancer: human studies

In human population studies, an inverse relationship has been observed between breast cancer incidence and calories from fish oil [54, 55]. Data from 20 countries identified a positive correlation between breast cancer incidence and dietary intake of SFA and n-6 PUFA (but not MUFA) and a

negative relationship with fish oil consumption [46]. For prostate cancer, review of international and intranational epidemiological evidence indicates an emerging consensus of an inverse association between fish oil consumption and advanced/metastatic prostate cancer or prostate cancer mortality [56]. Moreover in the Health Professionals' Follow-up study, significant inverse relationships between intakes of marine fatty acids were strongest for metastatic cancers [57]. Epidemiological data for colorectal cancer from 24 European countries [58], Taiwan [59], and Japan [60] showed an inverse correlation between colorectal cancer risk/mortality and intake of fish. Nevertheless, current epidemiological literature on the association of marine PUFA and cancer remains controversial [61–63].

The population and ecological studies rely on data from self reported dietary fatty acid intakes or from estimates based on national consumption. These assessments correlate poorly with direct measurements of fatty acids in patient samples. The effect of n-3 PUFA depends on levels achieved in individuals [64]. Different species of fish not only vary in total fat content but display widely divergent n-3/n-6 PUFA content. Even for a given species of fish, the n-3 PUFA content depends on the location of its feeding grounds and the algae on which it feeds [65]. The EURAMIC study is one of the largest to use adipose tissue as a primary exposure measure for dietary fat intake [66]. In this study, a higher ratio of n-3/n-6 PUFA was detected in adipose tissue of control compared to breast cancer subjects. A comparison of serum fatty acids in patients with prostate cancer and benign prostatic hyperplasia (BPH) versus control individuals demonstrated EPA and DHA levels as well as n-3/n-6 PUFA ratio in the order of control > BPH > prostate cancer [67]. In prostate tissue, significantly reduced EPA and DHA levels and n-3/n-6 PUFA ratios were associated with cancer compared to BHP [68], and with advanced stage disease with seminal vesicle involvement compared to organ confined disease [69]. In a human case control study, reduced prostate cancer risk was associated with high erythrocyte phosphatidylcholine levels of EPA and DHA [70]. These studies, supported by direct measurement of fatty acids in patient samples, indicate that not only may increased intake of n-3 PUFA be protective against primary tumor development but also that dietary supplementation in cancer patients may delay or prevent metastases.

Both n-6 and n-3 PUFA can be metabolized to eicosanoids through the action of cyclooxygenases (COX) and lipoxygenases (LOX; Fig. 3). A case-control study of Singapore Chinese subjects found a statistically significant association between a COX-2 gene polymorphism and colon cancer risk among high consumers of dietary n-6 PUFA [71]. Interactions between dietary intake of n-3 PUFA and COX-2 gene polymorphisms were also reported for prostate [72] and colon cancer [73]. In addition, COX-2

polymorphisms appear to be associated with breast [74, 75], prostate [76], colon [77–81], lung [82–85], gastric [86] and other [87, 88] cancers. Association between genetic polymorphisms and cancer risk may exist for COX-1 [89], LOX-5 [90] and LOX-12 [91] as well.

1.2.2 Dietary PUFA and cancer: Animal studies

Animal studies strongly supported a promoting role of n-6 PUFA and a protective role of n-3 PUFA against cancer, due perhaps in part to a better control over dietary intake and less genetic heterogeneity as compared to human studies. In rodents, diets rich in SFA and n-6 PUFA are breast cancer-promoting, while those rich in n-3 PUFA are protective [92–98]. Study models include dietary supplementation in chemically induced rat carcinogenesis [92–94] and modulation of human breast cancer xenografts in athymic nude mice [96–98]. In azoxymethane-treated rats, the most widely used animal model of colon carcinogenesis, diets with high levels of n-3 PUFA reduce the incidence, multiplicity and size of colon tumors compared to diets rich in n-6 PUFA [99–111]. Similar findings were obtained in mouse models with transplantable colon tumors [112–117]. Some studies indicated that n-3 PUFA also reduced colon cancer metastasis [113, 118, 119]. Although consistent with results from breast and colon cancer studies, data on the dietary fatty acids in prostate cancer are relatively limited. It has been reported that a fish oil-enriched diet inhibited tumor growth compared to a diet enriched in corn oil [120], and that n-6 PUFA diet was tumor-promoting [121, 122]. Recently we used prostate-specific Pten knockout mice, an immune-competent, orthotopic prostate cancer model, and diets with defined polyunsaturated fatty acid levels. We found that omega-3 fatty acids reduced prostate tumor growth, slowed histopathological progression and increased survival, whereas omega-6 fatty acids had opposite effects. Introducing an omega-3 desaturase, which converts omega-6 to 3 fatty acids, into the Pten knockout mice reduced tumor growth similarly to the omega-3 diet [236].

Besides the use of n-3 PUFA in dietary prevention, their potential application in nutritional adjuvant therapy was also explored in several studies. In athymic nude mice with human breast cancer xenografts, lung metastases were inhibited by dietary supplementation with n-3 PUFA initiated either before or after surgical removal of the primary tumors [123]. Dietary n-3 PUFA was also shown to increase the efficacy of doxorubicin [124] and mitomycin C [125] in inhibiting tumor growth.

1.2.3 Mechanism of action of PUFAs in cancer cells

The promoting effect of n-6 PUFA and suppressive effect of marine n-3 PUFA on cancers are consistently observed in

animal studies, and largely supported by human population investigations. However, their molecular mechanisms remain ill-defined. Some of the proposed mechanisms of action are discussed below.

Competition for key metabolic enzymes Dietary linoleic acid (LA) is converted to arachidonic acid (AA), and α -linolenic acid (α LNA) to eicosapentaenoic acid (EPA; Fig. 3). Both AA and EPA can be metabolized to corresponding series of eicosanoids, including prostaglandins (PG), thromboxanes (TX), hydroxyeicosatetraenoic (HETE), and leukotrienes (LT; Fig. 3). n-3 PUFA may compete for COX-2 [126] or other enzymes to reduce the metabolism of AA. COX-2 is increased in many cancers and COX-2 inhibitors have been shown to slow the growth and progression of colon cancer in animal models [97–99] and in clinical trials [127]. Recently, an n-3 PUFA-induced decrease in the growth of prostate cancer xenografts was associated with decreased COX-2 and PGE₂ in the tumors [128]. In addition, AA-induced *in vitro* invasion of PC-3 cells towards human bone marrow stroma was inhibited by EPA through a PGE₂-dependent mechanism [129]. Thus n-3 PUFA may act as natural COX-2 inhibitors. On the other hand, n-3 PUFA-enriched diets effectively inhibited tumor formation by COX-2 deficient and COX-2 over-expressing colon cancer xenografts, suggesting a COX-2 independent pathway for n-3 PUFA tumor suppressing activity [115]. Combining low doses of COX-2 inhibitors with n-3 PUFA resulted in a synergistic inhibition of proliferation of HCA-7 human colon cells and induction of apoptosis, therefore this could be a useful approach for the prevention and treatment of colon cancer [130].

Opposing biological activities of eicosanoids Another mechanism may relate to the opposing biological activities of eicosanoids derived from n-6 and n-3 PUFAs [65]. n-3 species of eicosanoids lack the inflammatory and proliferative activity of n-6 species [126]. In addition, LOX and COX products of n-6 PUFA metabolism are pro-angiogenic whereas n-3 eicosanoids impair angiogenesis [131, 132]. Their suppressive activities may be attributed to down-regulation of protein kinase C [132–134], ras [135, 136], AP-1 [137], NF- κ B [138], all of which are upregulated by n-6 PUFA products. Because of these opposing effects, the ratio of n-3:n-6 PUFA present in a tissue is likely to be more critical than the absolute mass of the individual PUFA.

Besides eicosanoids, marine n-3 PUFA (EPA and DHA) can also be metabolized to resolvins and protectins [139, 140]. These compounds possess potent anti-inflammatory and immuno-regulatory actions [141]. Mounting evidence suggests that inflammation may play a critical role in the development of human cancer [142–144]. Therefore, one

of the possible mechanisms for inhibition of tumors by n-3 PUFA is by suppression of inflammation through resolvins.

Different properties of phospholipids Phosphoglycerides are made up of three subclasses, namely the ester-linked subclass 1-acyl-2-acyl-phospholipids, and ether-linked subclass 1-*O*-alkyl-2-acyl-phospholipids and 1-*O*-alk-1'-enyl-2-acyl-phospholipids. In mammals, the *sn*-1 position on the glycerol backbone of phospholipids is usually linked to a saturated fatty acid such as stearic acid, and the *sn*-2 position to an n-6 PUFA such as AA. Feeding cells or animals with n-3 PUFA results in replacement of n-6 with n-3 fatty acid at the *sn*-2 position [145, 236]. n-3 fatty acyl chains (EPA or DHA) in the *sn*-2 position are poor substrates for cytosolic phospholipase A₂ (cPLA₂) [146, 147], therefore incorporation of n-3 PUFA may reduce the availability of free AA and consequently n-6 series eicosanoids. In addition, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (PC) can be converted to 1-*O*-alkyl-2-lyso-PC and then to the platelet activating factor (PAF), 1-*O*-alkyl-2-acetyl-(PC). Incorporation of n-3 PUFA at the *sn*-2 position may also reduce the conversion from ether-linked PC to lyso-PC and consequently PAF. PAF has been shown to be angiogenic in several systems and is thought to be important in the neovascularization of human breast cancer [148] and Kaposi's sarcoma [149]. Studies also suggest that TNF α acts by stimulating the production and release of PAF by endothelial cells and monocytes [150], and vascular endothelial growth factor may act by inducing PAF synthesis and thus increase vascular permeability [151].

Different propensities for oxidation The propensity of fatty acids to undergo auto-oxidation is related to the number of double bonds. Long chain PUFAs have been proposed to promote lipid peroxidation and thus enhance carcinogenesis. However, studies have shown that the incorporation of n-3 PUFA into cell membranes leads to increased lipid peroxidation products that enhance apoptosis in the cells [152]. Moreover, the tumor inhibitory effect of n-3 PUFA in human breast and colon cancer cell lines was blocked in the presence of vitamins E or C [153–155], thus supporting a growth suppressing role for n-3 PUFA oxidation products.

Differential abilities to activate PPARs An emerging area of interest associated with PUFA and cancer is the ability of PUFA to regulate gene expression through peroxisome proliferators-activated receptors (PPARs). This family of nuclear transcription factors comprises three isoforms (α , β/δ and γ) that bind fatty acids to regulate lipid metabolism and homeostasis but differ in target gene regulation. Both PPAR α and PPAR β/δ have been shown

to stimulate the proliferation of cancer cells [156, 157] whereas a large body of evidence has shown that PPAR γ is growth inhibitory [158–160]. Loss-of-function mutations in PPAR γ have been associated with human colon cancer [161]. In breast and prostate cancer cell lines, DHA induces apoptosis through activation of PPAR γ [162] (Edwards et al., unpublished). EPA and LA were ineffective in this process. A major question that remains is why EPA and LA, both known ligands for PPAR γ , failed to activate the receptor to induce apoptosis.

The mechanisms of action of PUFAs discussed above were studied in various cancer cells and largely in culture. Therefore, the relative contribution of these or other yet unidentified mechanisms needs to be evaluated *in vivo* in a systematic manner.

1.3 Sterol lipids

1.3.1 Cholesterol and cancer

A potential link between cholesterol and cancer was suggested almost a century ago [163, 164]. Some recent studies have associated high cholesterol intake with high risk of certain cancers [165, 166], and suggested that long-term use of statins, cholesterol lowering drugs, may significantly reduce relative risk of colorectal and prostate cancers [167–169]. Evidence on short-term use of statins and protection of cancer is more controversial [170].

Cholesterol tends to localize in areas known as lipid rafts that are enriched in certain types of proteins involved in cell signaling including caveolin-1 [171, 172]. Cholesterol levels are elevated in some tumor cells, and an increase in membrane cholesterol may cause the fusion of smaller isolated rafts into larger raft structures while coalescing important signaling molecules such as GPI-anchored proteins and caveolin-1 [165]. Cholesterol also plays an interesting role in modification of signaling molecules. Proteins important in development such as hedgehog proteins require covalent attachment of cholesterol for activity [41, 42]. Cholesterol modification restricts the spread of a hedgehog concentration gradient [41, 42, 173]. As discussed earlier, hedgehog proteins are also palmitoylated. Hedgehog signaling is important not only in development [41, 42] but also in tumorigenesis [174–177] as well as other human diseases [174].

1.3.2 Protein prenylation

In addition to dietary sources, cholesterol can be synthesized *de novo* from acetyl-CoA (Fig. 4). Intermediates in cholesterol synthesis (farnesyl and geranyl pyrophosphates) can also be used for covalent modification of proteins [178]. Several groups of proteins known to play an

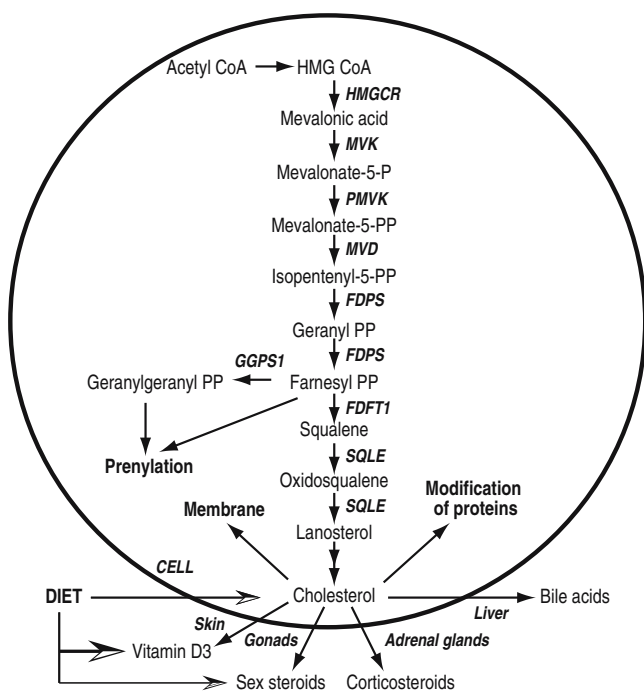


Fig. 4 Generation of isoprenoid, cholesterol, steroids and bile acids. Farnesyl pyrophosphate, geranylgeranyl pyrophosphate, and cholesterol are synthesized from acetyl-CoA. Farnesyl and geranylgeranyl pyrophosphate are important for cell signaling through prenylation of proteins. Cholesterol is critical for membrane function, modification of signaling molecules such as Hedgehogs, and serves the sole precursor for steroids and bile acids. Cholesterol is converted to sex steroids in the gonads, to corticosteroids in the adrenal glands, to bile acids in the liver, and to vitamin D3 in the skin. These metabolites exert important signaling functions by binding to families of nuclear receptors which regulate gene expression. *MVK* Mevalonate kinase, *HMGCR* 3-hydroxy-3-methylglutaryl-CoA reductase, *PMVK* phosphomevalonate kinase, *MVD* mevalonate (diphospho) decarboxylase, *FDPS*, farnesyl diphosphate synthase, *GGPS1* geranylgeranyl diphosphate synthase 1, *FDFT1* farnesyl-diphosphate farnesyltransferase 1, *SQLE* squalene epoxidase

important role in tumor initiating and progression require prenylation, including the Ras, Rho and Rab small G proteins [178]. Inhibitors of farnesyltransferase and other transferases responsible for prenylation are in development as chemotherapeutic drugs [179, 180] with some encouraging preclinical results [181]. HMG-CoA reductase inhibitor statins reduce the endogenous synthesis of cholesterol as well as well prenylation of proteins. Therefore, the effect of statins on cancer may be due to cholesterol reduction [167–169] and/or prenylation inhibition [182]. It is unclear, though, whether statins or isoprenoid transferase inhibitors will be more effective in cancer therapeutics. In addition, little is known regarding the effects of dietary changes that modify *de novo* lipogenesis might have on protein prenylation or other covalent modifications by fatty acids such as palmitate and myristate [43].

1.3.3 Bile acids and cancer

Cholesterol can be further modified in the liver to form bile acids (Fig. 4) which are secreted in bile and act to emulsify lipids to aid in their digestion. Bile acids are reabsorbed in the gut with about 95% efficiency and are essential in maintaining cholesterol homeostasis. An earlier class of cholesterol lowering drugs are bile acid sequestrants, which prevent reabsorption of bile acids by the intestine and result in excretion through the feces. However these agents are less effective than statins at reducing cholesterol levels [183] and therefore their use as hypolipidemic agent has decreased. Bile acids can be modified in the gut by intestinal flora to form secondary bile acids that may act as tumor promoters and carcinogens [184]. Mechanisms of action for these effects may be attributed to their ability to increase oxidative stress [184, 185]. Cancers of the colon, intestine, esophagus and stomach show increased incidence with increased levels of bile acids and the levels of potentially harmful bile acids is increased for persons eating a high fat diet in general [185]. Not all bile acids act as tumor promoters and some synthetic bile acids may even have potential for use as cancer therapeutic drugs [186, 187]. Interestingly, bile acid metabolism requires the AMACR gene which is over-expressed in prostate cancer (see next section).

1.3.4 Sex steroids and hormonally regulated cancers

Steroids are all derived from cholesterol (Fig. 4) and bind to nuclear receptors which regulate expression of a vast number of genes. The role of sex steroids is most studied in relation to hormonally regulated cancers such as these of the breast and prostate. In breast cancer, the estrogen and progesterone receptors are thought to play an important role in the tumor development [188, 189]. Hormonal therapy is often chosen as the first line therapy for estrogen receptor-positive breast cancer because it is usually well tolerated. However, most tumors eventually become refractory to hormonal therapy, and novel therapies targeting signaling pathways that are activated in hormone-resistant breast cancer are under intense development [190]. Ovarian synthesis of estrogens ends at menopause, and in post-menopausal women estrogens are derived primarily from adipose tissues through the action of aromatases [191]. Prolonged exposure to estrogens has been demonstrated to increase breast cancer risk, possibly due both to a carcinogenic effect of estrogen metabolites and the stimulation of proliferation via the estrogen receptor signaling pathways [188]. Similarly, prostate cancer cells rely on the androgen receptor for proliferation [192], and androgen ablation therapy is a standard treatment for advanced prostate cancer [193]. Androgen receptor mutations occur in advanced [194] and hormone-

refractory metastases [195, 196], and a germ-line androgen receptor mutation confers prostate cancer risk [197]. Mutations in the androgen receptor can lead to altered ligand binding specificity such that it binds progesterone, estradiol, or glucocorticoids [196, 198, 199]. In addition, prostate cancer risk may also be associated with genes encoding enzymes essential to androgen biosynthesis [200]. Thus, sex steroids play a significant role in hormonally regulated cancers. However, what effects dietary cholesterol and dietary fat levels may have on endogenous steroid synthesis is less clear. A pilot study demonstrated that changes in dietary fat intake could precipitate significant changes in serum cholesterol, total testosterone, and the free androgen index [201].

1.3.5 Vitamin D and analogs as anti-cancer agents

Vitamin D₃ can be synthesized from cholesterol in the skin exposed to sunlight (Fig. 4); however, individuals with limited sun exposure may require dietary supplementation. Vitamin D plays an important role in bone and mineral metabolism. In addition, it is now clear that numerous cell types, both normal and cancerous, possess a specific high-affinity vitamin D receptor (VDR) for 1,25(OH)₂D. The VDR is a ligand-dependent transcription factor of the steroid receptor superfamily [202].

Use of vitamin D and its analogs as anti-cancer agents has been investigated for decades. Many studies have associated vitamin D with a lower risk of developing prostate, colon and breast cancers, and more recently endometrial, skin, and pancreatic cancers [203]. 1,25(OH)₂D induces tumor cell differentiation and inhibits proliferation, invasion, and metastasis [204–206]. A potential mechanism underlying the effects of 1,25(OH)₂D in cancer cells is cell cycle arrest in the G₀/G₁ phase [207], through the induction of the cyclin-dependent kinase inhibitors p21 and p27 [208]. However, the effects of 1,25(OH)₂D on increasing serum levels of calcium can lead to dangerous calcemic effects (i.e., hypercalciuria, hypercalcemia, increased bone resorption) that greatly limit the use of this multi-faceted hormone as an anti-cancer agent. Thus, a key question is how to retain the anti-cancer effects of 1,25(OH)₂D while limiting its calcemic ones.

1.4 Phytanic acid, the AMACR gene and prostate cancer

1.4.1 Phytanic acid and diet

Phytanic acid, a methyl branched-chain fatty acid, is a significant component of fat from grazing animals (including butter and other dairy products rich in fat, beef and lamb meat). The average Western diet is thought to contain between 50 and 100 mg phytanic acid per day [209].

Consumption of red meat and dairy products, major sources of phytanic acid, is associated with prostate cancer risk [210, 211]. Men with a high intake of dairy products have approximately twice the risk of developing prostate cancer and a fourfold increased risk of developing metastatic or fatal prostate cancer compared to men who consume low amounts of dairy products [212, 213]. Serum levels of phytanic acid are also increased in men with prostate cancer [214]. Interestingly, in a large population-based case-control study that included 932 cases and 1201 controls, the risk of prostate cancer from over-consumption of red meat was primarily observed in African Americans (AA), not in European Americans (EA) [215]. This effect could be due to genetic differences in fat metabolism, combined with any different patterns of dietary intake.

1.4.2 AMACR and prostate cancer

Several gene expression profiling studies have independently identified the α -Methylacyl-CoA Racemase (AMACR) gene as a marker for prostate cancer [216]. By high-throughput gene expression analyses using a cDNA microarray, the AMACR mRNA was found to be consistently over expressed in prostate cancer [217–221]. How-

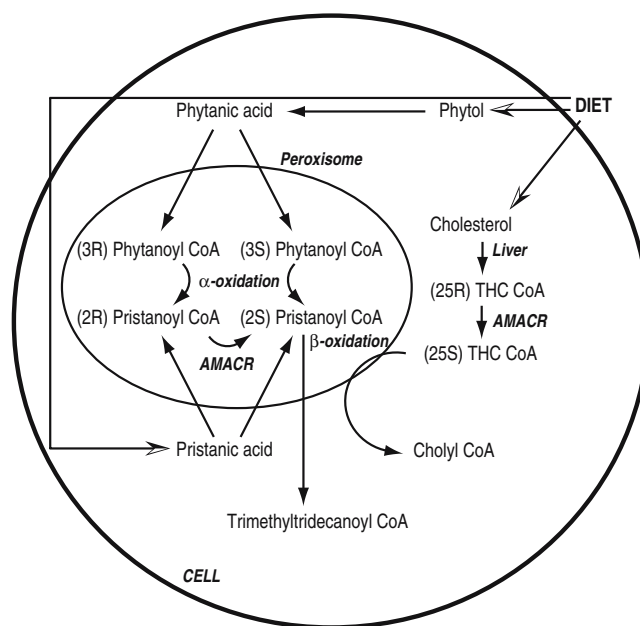


Fig. 5 Role of AMACR in phytanic, pristanic and bile acids metabolism. Dietary phytanic and pristanic acids exist in equal amount of R and S isomers. Only the S form can be metabolized through β -oxidation. AMACR is responsible for conversion of the R to S form. Similarly, during bile acid synthesis from cholesterol, AMACR catalyzes the conversion of R-THC-CoA to S-THC-CoA, which then undergoes β -oxidation to generate cholic acid. *THC* Trihydroxycholestanic acid, *AMACR* α -Methylacyl-CoA Racemase

ever, the degree of AMACR overexpression in prostate tumors varies [217, 222–224]. The up-regulation of AMACR in atrophic prostate, PIN, localized and metastatic prostate cancer was further confirmed at the protein level [220, 221]. Due to its consistent over-expression in prostate cancer, AMACR is now considered an excellent molecular marker for prostate cancer and has been widely investigated in clinical diagnosis, with both high sensitivity and specificity [220, 225–229].

1.4.3 Phytanic acid and AMACR interaction

AMACR is a well-characterized enzyme that plays a key role in peroxisomal β -oxidation of dietary branched-chain fatty acids, including phytanic acid [230] (Fig. 5). Therefore, the discovery of a link between AMACR and prostate cancer heightens the possibility of phytanic acid as a risk factor for prostate cancer. Branched-chain fatty acids derived from dairy and beef products may enhance AMACR activity: pristanic and phytanic acids were capable of increasing AMACR protein levels in LNCaP prostate tumor cells but not in normal prostate basal epithelial cells [231]. In addition, AMACR genetic variants may have different activities in metabolizing branched-chain fatty acids, which could account in part for the differential prostate cancer risk of consuming red meat in men of different ethnic backgrounds. AMACR was mapped to 5p13, a chromosomal region implicated in prostate cancer, through several genome-wide screen studies [232–234]. Multiple germline mutations in AMACR among high-risk prostate cancer patients have been identified, and the mutations segregate with prostate cancer [235].

2 Conclusions

Lipids are now widely recognized as crucial signaling molecules that affect patterns of gene expression. Therefore, it is not surprising that dietary fats can have profound influences on health in general and on cancer risk in particular. With the notable exception of n-3 PUFA and vitamin D3, consumption of high levels of fats is usually associated with increased risk of cancer. Diet is one of many environmental factors that modify cancer risk, and is constantly changing with global lifestyle changes. A significant amount of epidemiological, cell culture and animal work has been done, and yet many critical questions remain unsolved. We hypothesize that an increase in tumor-suppressing and decrease in tumor-promotive fats in our diet, together with pharmacological inhibition of FASN and HMG-CoA reductase, will have a profound cancer preventive effect. Further systematic, comprehensive, and mecha-

nistic investigations will be required to assess the relative contribution of various fats to different type of cancers, the intricate balance between exogenous dietary intake and endogenous *de novo* synthesis of fats, and the tight interconnection between fat metabolites of diverse pathways.

3 Key unanswered questions

1. Can deletion of FASN inhibit tumor cell proliferation *in vivo*?
2. Can the developmental lethality of FASN deletion be rescued by dietary palmitate?
3. Are all dietary n-3 PUFAs equally effective in tumor suppression? What are the major molecular mechanisms for tumor suppression by n-3 PUFAs?
4. Can EPA be converted to DHA in humans? Are there gender-related differences in metabolism?
5. To what extent do inhibition of *de novo* cholesterol synthesis and lowering dietary intake of cholesterol affect protein prenylation, steroid levels and bile acid synthesis? What are the consequences of these interventions on tumor progression?
6. Does AMACR play a causal role in prostate cancer development? Is it related to intake of phytanic acid?

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Expert Opinion

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Fatty acid synthase inhibitors: new directions for oncology

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Fatty acid synthase (FASN) is the enzyme that catalyzes the *de novo* synthesis of fatty acids in cells. Because of the strong expression in many cancers, FASN is an attractive and tractable target for therapeutic intervention. The discovery and development of pharmacologic agents that block FASN activity highlight the promise of these anticancer compounds. FASN inhibitors have also proven to be invaluable in developing a better understanding of the contribution of FASN and fatty acid synthesis to tumor cells. Recent advances in the development of crystal structures of FASN have provided promise towards the development of novel FASN inhibitors. This review outlines the preclinical development of FASN inhibitors, their antitumor effects and the strategies underway to develop novel inhibitors.

Keywords: C75, cerulenin, fatty acid synthesis, flavonoids, inhibitor, metabolism, orlistat, thiolactomycin

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1. Introduction

It has long been recognized that tumor cells have dramatically altered metabolism. One of the first observations was the ability of tumor cells to undergo glycolysis despite an availability of oxygen [1]. One proposal is that tumor cells have devised multiple metabolic adaptations to provide a growth advantage during exposure to stressful conditions in the microenvironment, such as hypoxia [2]. Based on the recognized difference between normal and tumor cell metabolism, recent studies have demonstrated that metabolic enzymes and pathways represent viable targets for drug intervention in many types of cancer [3].

Among the earliest and most obvious changes associated with tumor cell metabolism is the dramatic increase in fatty acid synthase (FASN) expression [4]. FASN (previously known as OA-519) is the single human enzyme that synthesizes fatty acids *de novo* [5]. Because of the strong correlation between FASN expression levels and cancer, the notion that FASN can be selectively targeted has become an attractive strategy for therapeutic intervention [6,7]. The unique expression of FASN in tumors allows for preferential targeting without harm to normal tissue. This article reviews the present understanding of the biologic role for FASN in tumor cells, as well as the clinical correlates between FASN expression and disease progression. In addition, the article highlights the early successes of several lead compounds and discusses issues related to the further development and translation of FASN inhibitors as antitumor agents into a clinical setting.

2. The fatty acid synthesis pathway

The complete synthesis of fatty acids in human cells is a coordinated process that is ultimately accomplished by multiple enzymes and enzymatic processes (Figure 1).

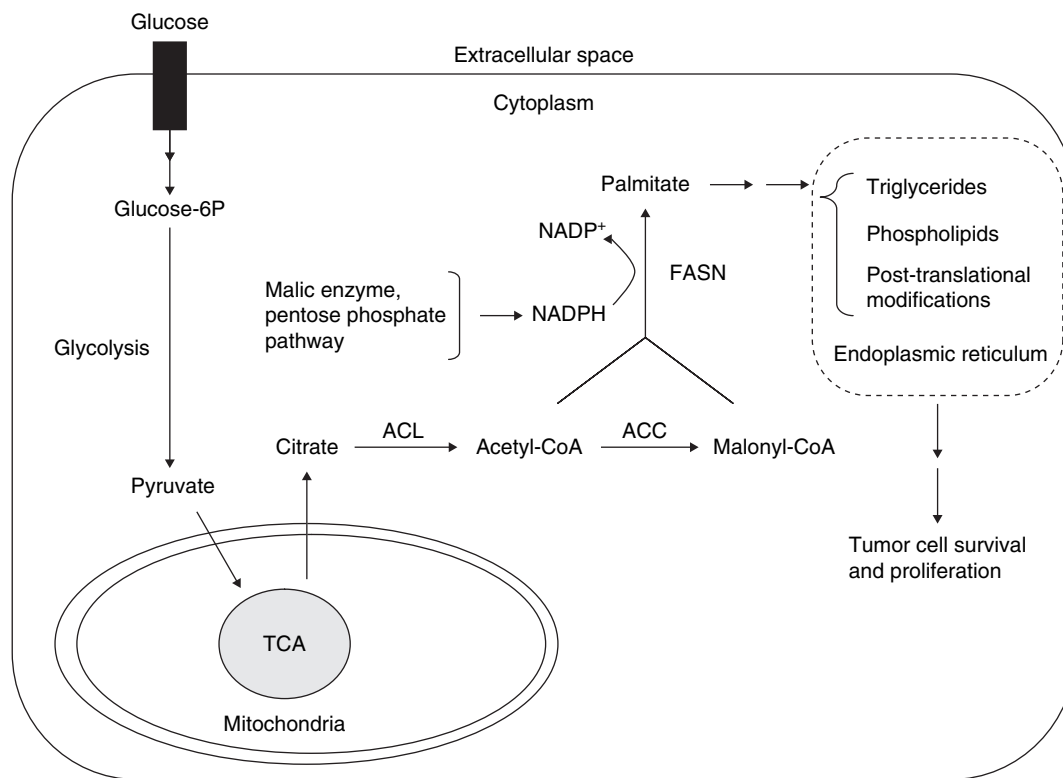


Figure 1. The fatty acid synthesis pathway. Glucose is taken up into tumor cells where it is phosphorylated to glucose-6P and shuttled into the glycolysis pathway. The resulting pyruvate enters the mitochondria and is converted to acetyl-CoA before it enters the citric acid cycle. Citrate is then shuttled from the mitochondria and converted to acetyl-CoA by ACL. ACC then converts acetyl-CoA to malonyl-CoA. FASN catalyzes the synthesis of palmitate from acetyl-CoA and malonyl-CoA using NADPH as a reducing cofactor. The NADPH can be provided by cytosolic malic enzyme or the pentose phosphate pathway. Palmitate can be desaturated or elongated and used for triglyceride synthesis, phospholipid synthesis or post-translational modification of proteins, all of which occurs in the endoplasmic reticulum.

ACC: Acetyl CoA carboxylase; ACL: ATP-citrate lyase; FASN: Fatty acid synthase; Glucose-6P: Glucose-6-phosphate.

Central to the process, FASN is the only enzyme that is able to synthesize fatty acids *de novo*. The principle product of FASN is the 16-carbon fatty acid palmitate. The other enzymes that participate either provide upstream substrates (acetyl-CoA and malonyl-CoA) and parallel cofactors such as NADPH, or are responsible for downstream modification following synthesis of the core palmitate [8]. For example, acetyl-CoA carboxylase (ACC) catalyzes the rate-limiting step of fatty acid synthesis, the carboxylation of acetyl-CoA to malonyl-CoA. Upstream of FASN and ACC, glycolysis and the citric acid cycle feed the production acetyl-CoA. The tricarboxylic acid cycle intermediate citrate is shuttled to the cytoplasm from the mitochondria where it is converted to acetyl-CoA via ATP-citrate lyase (ACL). The primary sources of NADPH to facilitate fatty acid synthesis are the pentose phosphate pathway and cytosolic malic enzyme. Ultimately, the effects of increased fatty acid synthesis in tumors are enhanced survival and proliferation. In summary, fatty acid synthesis is catalyzed by a single multifunctional enzyme, FASN, but many enzymes from several metabolic pathways

collaborate to provide the overall compliment of fatty acids in a tumor cell.

The FASN gene encodes a polypeptide that comprises seven functional domains including an acyl-carrier protein (ACP) that work together to catalyze fatty acid synthesis (Figure 2). The seven activities, in their linear order in the FASN polypeptide, are the β -ketoacyl synthase (KS), acetyl/malonyl-CoA transferase, β -hydroxyacyl dehydratase, enoyl reductase, β -ketoacyl reductase (KR), ACP and thioesterase (TE) domains. Although the primary product of FASN is the 16-carbon fatty acid palmitate, the 14-carbon myristate and the 18-carbon stearate fatty acids can also be produced, albeit at significantly lower levels. Fatty acid synthesis is an energy consuming process. For example, ATP hydrolysis is required for the conversion of acetyl-CoA to malonyl-CoA by ACC, which translates into 7 ATP per molecule of palmitate synthesized. In addition, 14 NADPH are oxidized during the FASN cycle in the following reaction scheme: $\text{acetyl-CoA} + 7\text{malonyl-CoA} + 14\text{NADPH} + 14\text{H}^+ \rightarrow \text{palmitate} + 14\text{NADP}^+ + 6\text{H}_2\text{O} + 8\text{CoA-SH} + 7\text{CO}_2$. The high energy demand that accompanies fatty acid synthesis

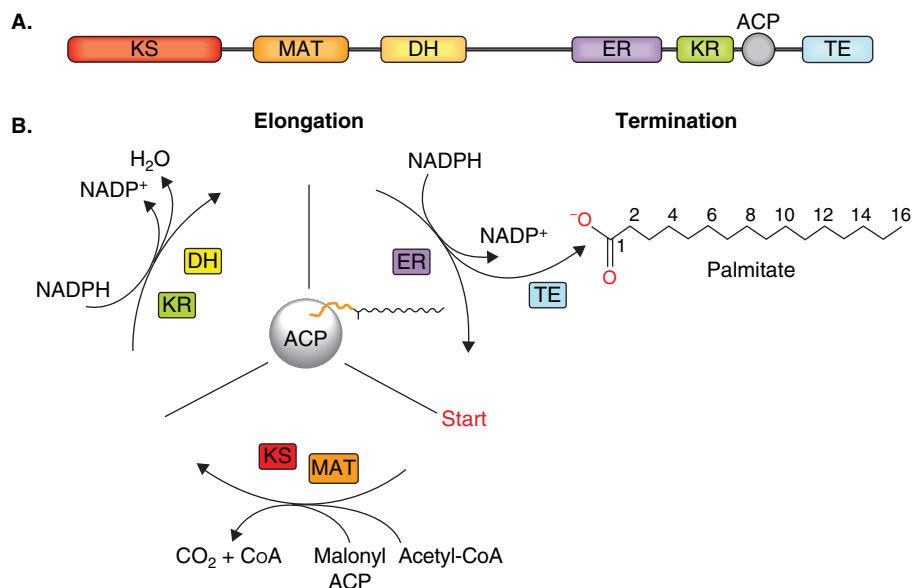


Figure 2. FASN domain organization and catalytic cycle. Initiation begins with the ACP-dependent transfer of acetyl-CoA and malonyl-CoA to the MAT domain (start). FAS performs a series (seven rounds total for palmitate) of highly coordinated chemical reactions involving condensation (KS), dehydration (β -hydroxyacyl dehydratase) and reduction (KR, enoyl reductase) of the nascent fatty-acyl chain (elongation). ACP, central to the reaction, shuttles the growing intermediates among the seven active site chambers. The mature fatty acid measuring 16 or 18 carbon units in length is selectively liberated by the resident C-terminal TE domain (termination).
 ACP: Acyl-carrier protein; FASN: Fatty acid synthase; KR: β -Ketoacyl reductase; KS: β -Ketoacyl synthase; MAT: Acetyl/malonyl-CoA transferase; TE: Thioesterase.

highlights the importance of this metabolic process to tumor cell survival and proliferation. Small molecule inhibitors of the KS and TE domains have been described as having antitumor properties. Similarly, natural product compounds that inhibit the KR domain of FASN have also been described. The expression of FASN and antitumor properties of FASN inhibitors will be discussed in the following sections.

3. FASN expression in tumors

Fatty acid synthesis is markedly increased in many tumors [6]. In fact, studies have demonstrated that the majority of fatty acids in a tumor are synthesized *de novo* [9]. Concomitant with studies that demonstrated high fatty acid synthesis in tumors, FASN protein overexpression has been documented in a wide variety of tumors. Increased FASN expression, relative to normal tissue, has been documented in tumors of the prostate [4,10-13], breast [14-17], colon [18], ovary [19,20], endometrium [21], bladder [22] and lung [23,24]. In addition, FASN overexpression has also been noted in melanoma [25], retinoblastoma [26], soft tissue sarcomas [27] and neuroblastoma (Wilms' tumors) [28,29]. FASN overexpression is primarily regulated at the transcriptional level in tumors following oncogene activation, tumor suppressor loss or growth factor stimulation (as reviewed in [6]). However, at least in prostate cancer, FASN levels can also be modulated by post-translational modification and gene duplication [30,31].

Interestingly, although FASN is an intracellular protein, it is also found in breast cancer cell culture supernatants and the blood of patients with breast cancer [32,33]. It is unclear what role extracellular FASN has or the mechanism by which FASN exits the cell. The expression levels of FASN are highest in metastatic tumors and tumor metastases, correlate with decreased survival, and are predictive of poor outcome and disease recurrence in several tumor types [10,12,14,21,23,28]. These data suggest that FASN not only provides a metabolic advantage that may drive tumor cell survival and proliferation but may also promote a more aggressive tumor phenotype.

Fatty acid synthesis is also crucial for development as mice with a homozygous deletion of *FASN* or *ACC* display embryonic lethal phenotypes [34,35]. With the exception of liver and adipose tissue, FASN is expressed at low or undetectable levels in most normal adult tissues. Therefore, fatty acid synthesis does not seem to be required for normal adult tissue maintenance. Accordingly, mice harboring liver-specific deletions of *FASN* and *ACC* display normal liver function and no obvious phenotype so long as they are maintained on a normal diet [36,37].

4. The role of FASN in mediating tumor growth

Coincident with the differences in FASN expression between normal and tumor tissues, there also seems to be

mechanistic differences in how fatty acids are used in normal and tumor cells. In liver and adipose, fatty acids are synthesized in response to excess caloric intake. These fatty acids primarily partition towards triglyceride synthesis for fat storage. In contrast, tumor FASN-derived fatty acids preferentially partition into phospholipids that segregate into detergent-insoluble membrane domains or lipid rafts [38]. Fatty acids, in general and palmitate specifically, can also be used for other functions in cells. In one example, palmitate is used for post-translational modification of proteins [39,40]. Palmitate and other fatty acids, is added by an enzymatic process to increase the hydrophobic nature of proteins to facilitate membrane-associated signaling. Among the proteins that are modified by palmitate are Ras, Wnt, Hedgehog and small protein GTPases [39,40], each of which are implicated in a variety of cancers. Although there has been no demonstration that FASN provides substrate for protein palmitoylation, it is intriguing to speculate that FASN could be important for this important process. Finally, it has been hypothesized that FASN also contributes to the redox status of tumor cells through oxidation of NADPH during the fatty acid synthesis cycle [2,41]. When all factors are taken into account, it is likely that FASN and fatty acid synthesis provide substrates to affect multiple cellular functions to provide a proliferative phenotype.

5. FASN inhibitors: *in vitro* and *in vivo*

Because fatty acid synthesis is energy expensive, a teleologic analysis suggests that fatty acid synthesis is critical for tumor cell survival and proliferation. A large body of literature supports this suggestion and has demonstrated FASN to be essential for the survival and proliferation of tumor cells *in vitro* and *in vivo*. Moreover, the pleiotropic effect of FASN inhibitors on tumor cells supports the notion FASN and fatty acid synthesis could affect multiple tumor cell pathways.

5.1 Inhibitors of FASN KS activity

The first FASN inhibitors to be described targeted the KS activity of the enzyme. These inhibitors provide the foundation and framework for the development of novel compounds that efficiently inhibit FASN in tumor cells and prevent tumor growth. Cerulenin (Figure 3) is an epoxide-containing antimetabolite discovered in *Cephalosporium caerulens* that binds covalently to and inhibits the KS activity of FASN [42]. Cerulenin inhibits FASN activity in tumor cells and induces rapid cell death and cell-cycle blockade in various tumor types [43,44]. Cerulenin also effectively inhibits the growth of ovarian tumor xenografts in mice [44]. Pharmacologic inhibition of FASN reduces fatty acid levels as well as phospholipid levels in tumor cells, which is also recapitulated when FASN is knocked-down by siRNA [45]. The antitumor effects of cerulenin have been associated with multiple phenotypic effects, including a rapid

accumulation of malonyl-CoA [46], the suppression of DNA synthesis [47], an accumulation of p53 [48] and activation of the mitochondrial cell death pathway [49].

The synthetic analog of cerulenin, C75, was developed as a chemically stable FASN inhibitor (Figure 3) [50]. As with cerulenin, C75 targets the KS activity of FASN and induces a rapid accumulation of malonyl-CoA and p53. As a result, C75 also induces apoptosis and prevents the growth of multiple tumor xenografts *in vivo* [51-53]. In addition, a recent study demonstrated that a novel FASN inhibitor C93 also inhibits FASN and limits tumor growth in an ovarian xenograft model [54]. Several relevant signaling pathways are affected by FASN inhibition in tumor cells. For example, treatment with C75 reduces Her2/*neu* expression and activation in breast cancer cells [55]. As a result, combining C75 with the anti-Her2/*neu* antibody trastuzumab synergistically stimulates cell death in breast cancer cell lines. Similar reports also demonstrate that cerulenin and C75 are able to reduce levels of phosphorylated Akt in breast and ovarian cells, respectively [53,56]. Whether the effects are directly associated with reduced PI3K activity are unknown. Considering that FASN expression in prostate cancer correlates with nuclear localization of phosphorylated Akt [57], the connection between FASN inhibition and the PI3K pathway are likely to be significant. A further illustration of the importance of the PI3K axis is provided by the demonstration that pharmacologic blockade of PI3K activity enhanced tumor cell apoptosis when FASN expression was suppressed by siRNA [58]. As mentioned in Section 4, FASN-derived fatty acids are preferentially found in phospholipid-associated detergent-soluble lipid rafts [38]. Lipid rafts are associated with signaling complexes so it should not be a surprise that FASN inhibitors affect Her2/*neu* and PI3K-mediated signaling pathways [59]. Collectively, these data provide demonstration that inhibition of FASN activity affects membrane and lipid-associated signaling pathways, thus providing a functional connection between FASN activity and the antitumor effects of FASN inhibitors.

A recent study also made the intriguing finding that C75 and the thiolactomycin-derivative C247 have chemopreventive capacity in a transgenic model of breast cancer [60]. Interestingly, Akt activation was also reduced by C75 treatment in this model. These findings combined with the lack of apoptotic phenotypes in *FASN* and *ACC*-liver specific knockouts correlate well with the lack of toxicity by FASN inhibitors in normal cells. Because the loss of FASN is acquired with time in these *Cre*-mediated models, they may not accurately reflect the acute inactivation of FASN following inhibitor administration. On the other hand, the single reported dose-limiting toxicity of C75 and other FASN KS inhibitors is weight loss [44,51] and no other toxicities have been reported. Therefore, some thiolactomycin-based FASN inhibitors, including C247 and C93, can inhibit FASN with no effects on weight, and

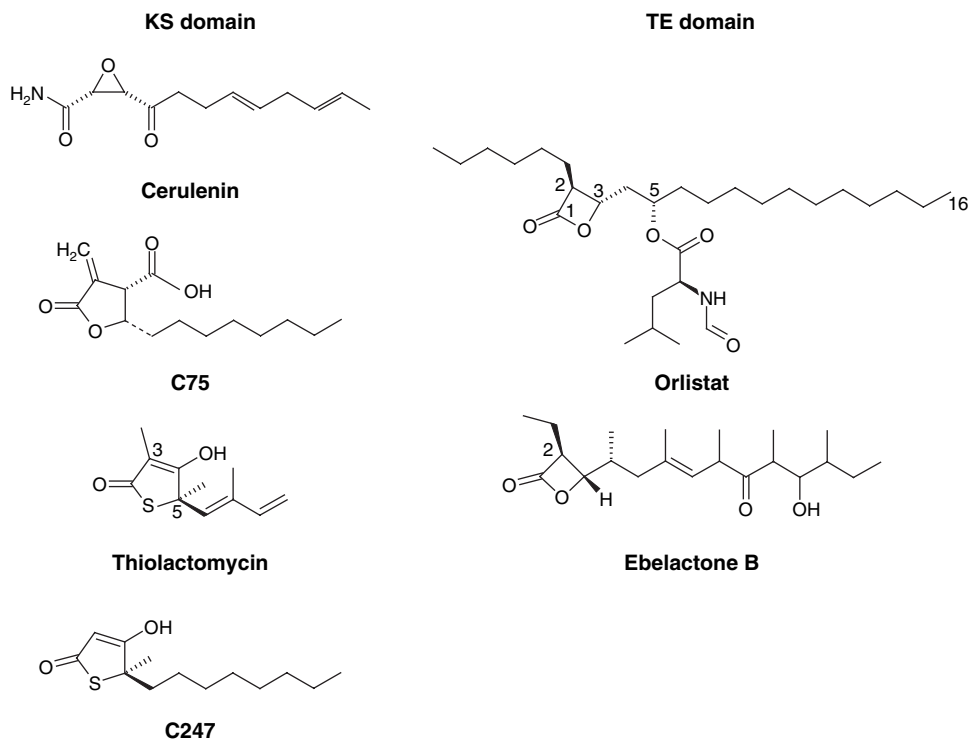


Figure 3. Pharmacologic inhibitors of the KS and the TE domains of fatty acid synthase. The dashed line in C75 represent a racemic mixture.

KS: β -Ketoacyl synthase; TE: Thioesterase.

similar compounds can induce weight loss without affecting FASN activity, suggesting that the weight loss effects may be independent of FASN [54,60-62]. Considering that FASN inhibitors have not resulted in normal tissue toxicity during chemoprevention and therapeutic intervention, the data so far indicate that FAS is a safe antitumor target.

5.2 Inhibitors of FASN TE activity

The FDA-approved drug orlistat was identified as an inhibitor of the FASN TE activity in a proteomic screen for prostate cancer-specific enzymes (Figure 3) [63]. Orlistat is presently marketed and prescribed for the management of obesity, but was not designed as a FASN inhibitor. Instead, orlistat is intended to inhibit pancreatic lipase in the digestive tract. The TE domain of FASN coordinates the terminal step of fatty acid synthesis by hydrolyzing palmitate from the 4'-phosphopantethiene arm of the ACP domain. The active moiety of orlistat is a β -lactone ring that undergoes nucleophilic attack by the active site serine of FASN TE. Orlistat is able to inhibit FASN in tumor cells and induce tumor cell-specific cell death, with little or no effect on normal cells and reduces the growth of prostate tumor xenografts in mice [63]. Orlistat also affects the cell cycle by inducing a G1/S arrest that is mimicked by FASN-specific siRNA [64]. Coincident with FASN blockade

by orlistat is a downregulation of the E3-ubiquitin ligase Skp2, which results in p27Kip1 accumulation and associates with the retinoblastoma pathway [64]. Interestingly, the cell-cycle effects induced by orlistat and FASN-specific siRNA are dissimilar from the effects of C75 and cerulenin [64-66]. However, the reasons for these differences are unclear and could potentially be attributed to cell types or secondary targets of the KS inhibitors.

The discovery of orlistat as a FASN inhibitor identified the TE domain of FASN as a potential drug target. It also provided a lead compound for the further development of inhibitors that target the TE domain. Two recent studies have reported the development of concise and scalable processes for the synthesis of orlistat and other β -lactone derivatives [67,68]. Preliminary compounds have apparent K_i values in the low micromolar range against FASN TE and provide a platform for the development of novel FASN inhibitors. In the present formulation, orlistat is unlikely to be a successful antitumor drug because of poor systemic stability and availability. On the other hand, incorporation of orlistat into liposomes, nanoparticles or some other delivery vehicle may provide a tumor specific means of efficient systemic delivery of the drug. Thus, further development and modification of orlistat-like compounds may identify compounds that could be translated into improved anticancer drugs.

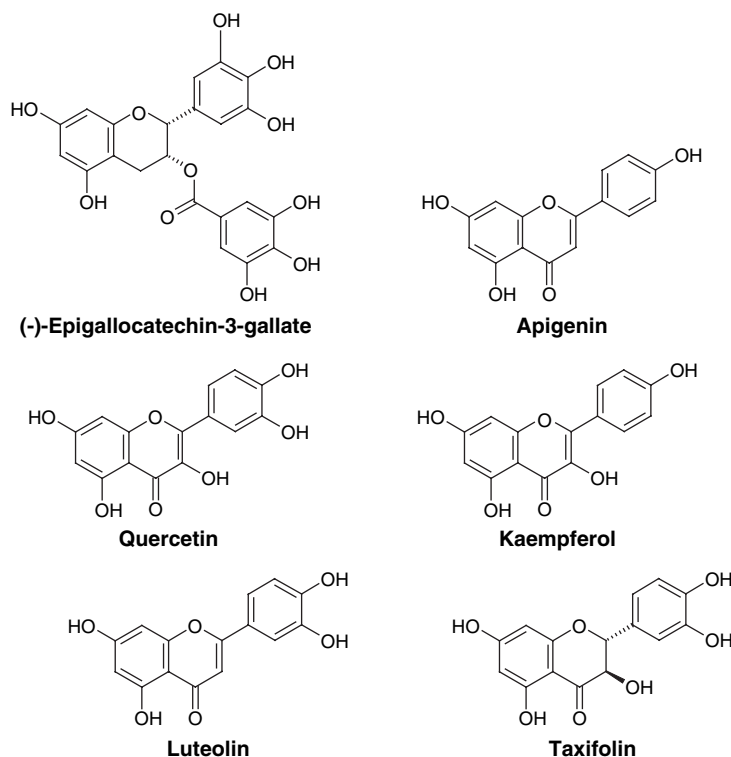


Figure 4. Natural products that inhibit fatty acid synthase.

5.3 Natural products as potential FASN inhibitors

The natural chemistry of plant-derived compounds represents another avenue that is being explored for potential FASN inhibitors and may serve to identify novel leads for pharmacologic development. Among the compounds that have been identified, the best described are several polyphenolic compounds (Figure 4) [69]. Perhaps the best characterized natural product is epigallocatechin gallate (EGCG), the principal polyphenol component of green tea. EGCG inhibits the KR activity of FASN and the galloyl moiety is indispensable for the inhibitory effects [70,71]. Interestingly, the structure of EGCG closely resembles that of NADPH and may partially explain the inhibition of NADPH-dependent FASN KR activity. The ability of EGCG to inhibit FASN coincides with the ability of EGCG to induce tumor cell-specific apoptosis [72]. Several studies have demonstrated that tea extracts are able to inhibit tumor growth in various models of prostate, ovarian and breast cancer but the effects on FASN activity were not determined [73-75]. Although EGCG is reported to have pleiotropic effects in tumor cells [76], the demonstrated ability to inhibit FASN activity and tumor growth suggest EGCG as a viable lead compound for further development or as a natural product strategy for chemoprevention or therapeutic intervention.

In addition to EGCG, other natural products have been described as potential FASN inhibitors. Specifically, independent groups have identified plant-derived flavonoids

as potential FASN inhibitors [77,78]. One study identified five flavonoids, luteolin, quercetin, kaempferol, apigenin and taxifolin, with the ability to inhibit FASN activity (Figure 4) [77]. Furthermore, the induction of apoptosis, which was reversed by the exogenous addition of palmitate, directly correlated with the reduction in *de novo* fatty acid synthesis. As with EGCG, each of these compounds is polyphenolic. Although the mechanisms by which these flavonoids inhibit FASN have not been determined precisely, based on the structural similarities between the compounds, it is tempting to speculate that they follow a similar inhibitory mechanism to EGCG. In summary, the literature suggests that several polyphenolic compounds may be excellent lead compounds for the further development of anti-FASN drugs. Moreover because many of them are safe at high doses, their chemopreventive ability may be closely linked to inhibition of FASN activity as has been demonstrated for C75 and C247 in a mouse model of breast cancer [60]. Although relatively few plant-derived compounds have been described as yet, it seems likely that other plant-derived compounds may be accessible as safe and effective FASN inhibitors.

6. Two hypotheses for FASN inhibitor-induced cell death

A large and growing body of literature supports the notion that fatty acid synthesis is a requisite process in tumor cells.

The associated studies have also identified multiple mechanisms by which FASN inhibitors negatively affect the proliferation and survival of tumor cells. However, one critical question remains. That is, why does inhibiting FASN activity in tumor cells induce cell death? There are two potential answers or hypotheses to this question. The first hypothesis is that when FASN activity is blocked the FASN substrate malonyl-CoA accumulates to supra-physiologic levels upstream to induce cell death by affecting multiple downstream pathways [51,79]. The evidence to support this hypothesis is provided by data demonstrating that pharmacologic inhibition of ACC1 and ACC2 with 5-(tetradecyloxy)-2-furoic acid does not induce cell death and is reported to block FASN inhibitor-induced cell death [51]. These studies, however, are contrasted by two reports that demonstrate that siRNA-mediated knockdown of ACC1 induces apoptosis in prostate and breast cancer cell lines [80,81]. The effects siRNA-mediated knockdown of ACC1 are associated with the generation of reactive oxygen species and can be ameliorated by reactive oxygen species scavenging and addition of exogenous palmitate [81]. Similarly, cell death resulting from inhibition of FASN can also be ameliorated by exogenous addition of palmitate, implying that cells require palmitate for survival [63]. These studies seem to support the second hypothesis that efficient fatty acid synthesis is required for tumor cell survival.

Although these two hypotheses provide potential upstream explanations to describe the antitumor effects of FASN inhibitors, it is clear that whatever the mechanism the downstream effects are actually multimodal. FASN activity clearly affects multiple pathways as discussed earlier in this review, including the cell cycle, DNA synthesis and PI3K pathways to name a few. Recent studies further highlight the pleiotropic nature of FASN inhibitors. Little *et al.* recently demonstrated that pharmacologic and genetic inhibition of FASN induces endoplasmic reticulum (ER) stress specifically in tumor cells [82]. Because the ER is the organelle in which phospholipid synthesis occurs, it is intriguing to speculate that the ER may act as a sensor of fatty acid levels and metabolic function in tumor cells and that ER dysfunction may mediate cell death when FASN is inhibited. It has also been demonstrated siRNA-mediated knockdown of FASN induces ceramide accumulation in tumor cells, and cell death is also prevented when ceramide synthesis is inhibited [83]. The mechanistic link between FASN inhibition and increased ceramide levels is unclear so far. On the other hand, the collective data related to the antitumor effects of FASN inhibition provide insight into how FASN activity acts centrally to regulate survival and proliferation of tumor cells.

Other studies also support the hypothesis that tumor cells require efficient fatty acid synthesis to maintain a growth advantage. It has been demonstrated that ACL, the enzyme that generates acetyl-CoA from citrate (Figure 1), is required for tumor cell proliferation and survival *in vitro* as well as

tumor growth *in vivo* [84,85]. Furthermore, activation of AMP-activated protein kinase (AMPK) by the AMP analog 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranosyl 5'-monophosphate has been demonstrated to inhibit the growth of prostate tumor cells *in vitro*, the effects of which can be enhanced by methotrexate treatment [86-88]. Activation of AMPK inhibits fatty acid synthesis by phosphorylating and inactivating ACC. In summary, numerous studies have demonstrated that multiple enzymes in the fatty acid synthesis pathway, including FASN, ACC and ACL, are required for tumor cells to proliferate and survive. This is consistent with the notion that tumor cells have devised alternate metabolic strategies to maintain a growth advantage. On the other hand, there is still much to be understood regarding how tumor cells use fatty acid synthesis to enable such a phenotype and how the pathway might affect global tumor cell metabolism.

7. Structure-based design of FASN inhibitors

As described briefly in Section 2, the seven functional domains of each FASN monomer (Figure 2) within the dimer coordinate to generate the fatty acid palmitate. Each functional domain of FASN represents a novel target for drug development. Efforts to understand the interrelationships of the domains have included chemical crosslinking, complementation and cryo-electron microscopy studies [89-92]. The 4.5-Å resolution crystal structure of the intact, porcine FASN has also been solved using the structures of the bacterial homologs for each domain as starting models [93]. The FASN structure clearly delineates the interface between the two monomers and gives insight into the shuttling of the growing chain between the active sites. However, the ACP and TE domains were not visible, presumably due to their high mobility. Nonetheless, it is now hopeful that a high resolution FASN structure will foster drug design efforts in the near future.

Despite the previous lack of a FASN structure, two different approaches have been used to successfully identify, design and optimize inhibitors. The first approach has combined the biochemical knowledge of the reaction mechanisms with the information from high resolution structures of individual bacterial domains in complex with ligands. In the second approach, the goal has been to express and purify each human FASN domain separately for drug screening and structural analyses. These strategies have been successful for the KS and TE domains.

7.1 Development of KS domain inhibitors

Cerulein, a compound containing a C12 acyl chain (Figure 3), has been crystallized within the active sites *Escherichia coli* KS domains [94,95]. In both instances the compound formed an irreversible, covalent adduct with the catalytic cysteine residue within the active site. These complexes are thought to mimic the condensation transition

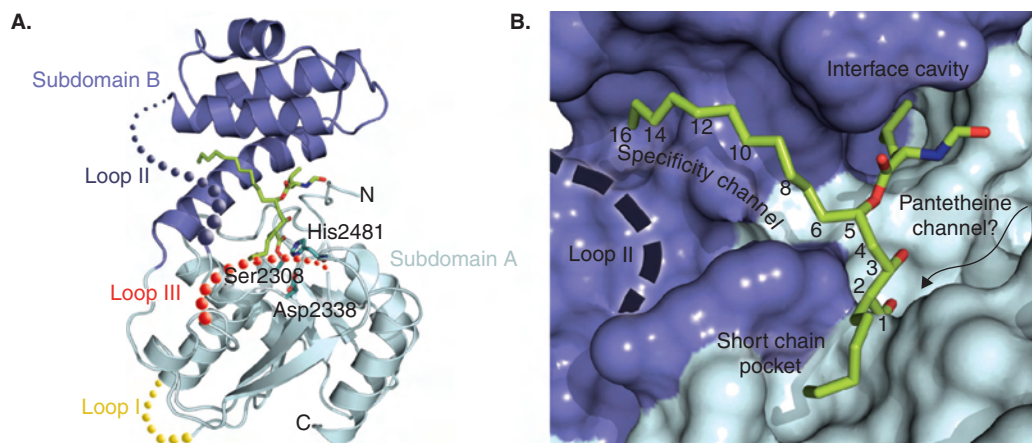


Figure 5. The covalent orlistat-TE complex. A. Overall fold of the TE domain with orlistat bound (green). Each subdomain is colored a different shade of blue. The C1 carbon atom of orlistat is linked through an ester bond to Ser2308 of the catalytic triad. Missing loop regions are indicated. **B.** Surface representation of the complex highlighting the different binding channels and pockets.

TE: Thioesterase.

state of the complex between KS and the growing acyl chain attached to ACP. Using this structural information, molecular modeling and the data from mechanistic studies, cerulenin analogs, such as C75, have been synthesized [96]. As described previously, these latter compounds can kill cancer cells but have the unfortunate side effect of weight loss.

Thiolactomycin, also known as (5*R*)-thiolactomycin (Figure 3), has been crystallized within the active site *E. coli* KS [94]. The binding of this compound is thought to represent thiomalonate attached to the ACP domain. The interactions within this putative malonyl-ACP binding pocket and the hydrophobic binding pocket for the growing acyl chain from the cerulenin complex have been used to design analogs of thiolactomycin [61]. These analogs contained modifications of the γ -thiolactone skeleton at the C3 and C5 positions and the C4 hydroxyl substituent. Remarkably, three subclasses of compounds were identified that exhibited different phenotypes. The first subclass of compounds inhibited FASN, caused effective weight loss but was not cytotoxic to cancer cells. The second subclass was cytotoxic to cancer cells and had the same remaining characteristics as subclass I. The last class of compounds inhibited FASN and killed cancer cells but did not cause weight loss. Thus, the thiolactomycin derivatives represent a unique opportunity to develop antitumor and antiobesity drugs through the inhibition of the same target.

7.2 Crystal structure of the orlistat-TE complex

The only crystal structure presently available for a domain of human FASN in complex with a ligand is the orlistat-TE complex [97]. In this study, two TE molecules were present in the repeating unit of the crystal. Each TE molecule contained a different form of orlistat: either an acyl enzyme intermediate or the hydrolyzed product. In both cases,

orlistat was bound within a cavity generated by subdomains A and B (Figure 5A). Importantly, the palmitate-like moiety of orlistat is bound within a hydrophobic channel generated by subdomain B. The authors have called this channel the specificity channel as its hydrophobicity and shape features correlate well with the selection of 16-carbon fatty acid substrates. The *N*-formyl-L-Leu moiety that extends off the C5 position of orlistat (Figure 3) binds into the interface cavity originally identified in the apoenzyme structure [98]. The C2 substituent of orlistat contains six carbons and packs against His2481 of the catalytic triad within the newly identified short chain pocket. On hydrolysis of the covalent adduct, the orlistat molecule shifts in register in the specificity channel and short chain pocket while maintaining its interaction with the interface cavity. Thus, orlistat is a substrate of FASN TE but inhibition arises from the unusual stability of the acyl enzyme intermediate, which blocks the active site.

As described in Section 5.2, orlistat targets pancreatic lipase of the gut and has minimal, if any, accessibility to the bloodstream. The orlistat-TE structures now provide an excellent primer for the design of orlistat analogs and the *in silico* search for other backbone scaffolds. Optimization of these new compounds will hopefully result in improved bioavailability, selectivity and potency. For example, the *N*-formyl-L-Leu moiety does not completely fill the interface cavity. One can easily envision extending the chain further and to install functional groups that maximize interactions with the mixed hydrophobic/hydrophilic character of this cavity. Another intriguing possibility would be to modify the C2 moiety of orlistat. Kridel *et al.* have already shown that treatment with Ebelactone B and not Ebelactone A, two orlistat congeners, leads to significant inhibition of FASN [63]. Ebelactone B contains an ethyl group at C2 while Ebelactone A contains a methyl group.

Therefore, it seems that a minimum of a 2-carbon chain is necessary at this position. The design of compounds that exploit the putative 4'-phosphopantetheine channel may also prove to be useful.

8. Conclusions

The combination of pharmacologic inhibition and genetic knockdown or knockout of FASN has demonstrated the efficacy and safety of targeting FASN in multiple tumor types. Synthetic inhibitors of the KS and TE domains of FASN have been identified. In addition, natural products that target the KR domain also have potential. Although there have been many early successes in preclinical models, FASN inhibitors have not yet entered clinical trials. However, the expression profile of FASN suggests that many tumor types could successfully be targeted by FASN inhibitors. With growing interest in the field and the target, the further development of FASN crystal structures and drug screening technologies will be likely to result in the successful translation of FASN inhibitors into the clinical setting.

9. Expert opinion

The breadth of literature in the field clearly suggests that the fatty acid synthesis pathway in general and FASN specifically, represents an attractive and tractable target for antitumor therapy for many types of cancers [6,7]. Despite the early successes outlined in this review, several questions and issues should be addressed in the coming years to more thoroughly understand the biology of fatty acid synthesis, to successfully advance FASN inhibitors past the preclinical phase and to hasten the development of novel FASN inhibitors for cancer therapy.

Although liver-specific knockout of *FASN* and *ACC* have been generated [36,37], no tissue specific knockouts have as yet been generated and crossed into spontaneous tumor models. Such models will be important in developing a complete understanding how FASN and other players in the pathway contribute to tumor development and growth and how inhibitors of the axis may be improved to impinge on the pathway. They would also provide genetic evidence to suggest that FASN can be safely inhibited in long-term chemopreventive scenarios. In addition, these models could address whether tumor cells might evolve from a FASN-dependent status to a FASN-independent status following exposure to FASN inhibitors or whether dietary fat could negatively affect the potential of FASN inhibitors as antitumor agents.

Most enzymes that are targeted for drug development have a single active site, which therefore limits the chemical diversity available in developing a therapeutic aimed at such targets. In contrast, FASN represents a unique opportunity for cancer drug discovery because it contains seven unique active sites. To take full advantage of the therapeutic potential FASN represents, it is imperative to pursue structure-based

drug design. Such a strategy would determine whether compounds can be identified to specifically inhibit each catalytic activity in FASN, not just the KS and TE domains. In parallel, high-resolution crystal structures of each human FASN domain should be determined in their native states and in complex with an inhibitor or substrate. Towards this goal, Pemble IV *et al.* have determined the structure of the FASN TE in complex with orlistat [97], which provides a blueprint for the development of novel inhibitors of the FASN TE. Similar information for other domains would be invaluable for the further development of FASN inhibitors.

As discussed in this paper, the literature clearly demonstrates that FASN inhibitors can prevent tumor growth in multiple mouse models of cancer. As it relates to target validation, however, there has been no direct demonstration that FASN inhibitors significantly block fatty acid synthesis in tumors. One modality to potentially measure fatty acid synthesis or the inhibition of FASN in tumors is positron emission tomography of [¹¹C]-acetate incorporation [99,100]. The incorporation of this promising technology into preclinical *in vivo* models and clinical studies could provide an imaging modality to correlate fatty acids synthesis with tumor growth, as well as important validation of FASN inhibitors efficacy. One study has demonstrated that C75 can affect glucose metabolism by reducing [¹⁸F]-fluorodeoxyglucose uptake in an orthotopic model of lung cancer [101]. The results correlated with reduced FASN activity *ex vivo* but do not represent a direct measure of fatty acid synthesis *in vivo*. Thus, having existing technologies to image fatty acid synthesis and subsequent inhibition by positron emission tomography represents a unique opportunity for target validation that should be further developed with FASN inhibitors for translation into a clinical setting. By addressing each of the issues outlined in this opinion section, the translation of existing FASN inhibitors as antitumor agents and the development of novel FASN inhibitors could advance more efficiently.

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Declaration of interest

The authors declare no competing financial interests.

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