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Fatty Acid Synthase Inhibitors Engage the Cell Death Program through the Endoplasmic Reticulum.

Fatty acid synthase (FAS), the enzyme that synthesizes the 16-carbon fatty acid palmitate, is highly expressed in prostate cancer. Because of a corresponding lack of expression in normal prostate, FAS is an attractive drug target. We have described the endoplasmic stress (ER) response as a critical mediator of the anti-tumor effects of FAS inhibitors. In this report, we also describe a novel connection between the FAS pathway and the proteasome pathway. This feedback between the two pathways can further be antagonized by co-treatment with the FDA-approved proteasome inhibitor Velcade. Velcade synergizes with FAS inhibitors to induce cell death and increase ER stress related signaling. These aspects will be followed up in vitro and in vivo. The importance of these studies is underscored by the potential relevance of FAS as a drug target in prostate cancer. Several FAS inhibitors have been developed, but none have been translated into the clinic thus far. These studies will be valuable as FAS inhibitors move toward a clinical setting.
Introduction
The basic premise of this Idea Award is to determine the mechanism by which FAS inhibitors induce endoplasmic reticulum (ER) stress-dependent cell death in prostate cancer. It is based on our initial findings that FAS inhibitors induce ER stress in tumor cell lines. Although FAS-derived fatty acids are primarily used to drive phospholipid synthesis in tumor cells and phospholipid synthesis occurs in the ER, these findings were the first to connect ER function to fatty acid synthase in any capacity. The goal of this proposal was two-fold. One was to determine the mechanism by which the ER might initiate death following FAS inhibition. The second was to determine whether FAS inhibitors can reduce prostate tumor growth in a spontaneous model of prostate cancer and whether ER stress is observed in the treated prostates. A discussion on the progress of these aims is reported in the body below. During the course of the initial funding period we have had six papers published, accepted or submitted for publication that are related to fatty acid synthase. Much of the work was supported by previous DoD/PCRP funding, but speaks to our advancement in this area or research. In addition, two abstracts were presented at national meetings and one oral presentation was given at an international meeting, all of which are listed in the Reportable Outcomes section to follow.

Body
Specific aim 1. To determine how ER stress initiates cell death when FAS activity is inhibited.
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 [1]. The status of the ER is monitored by three proximal sensors; the ER resident eIF2α 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 the application for the Idea award, inhibition of FAS does indeed induce ER stress. This finding was published in Cancer Research in February 2007 (see manuscript 1 [2] in Reportable outcomes and the appendix), and used as preliminary data for the award, so will only be summarized here. We discovered that the FAS inhibitors orlistat, C75 and cerulenin induce a PERK-dependent phosphorylation of eIF2α. Concomitant with this was the corresponding inhibition of protein synthesis. When eIF2α 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 eIF2α 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 [3,4]. Interestingly, it has also been reported that the FDA-approved proteasome inhibitor Velcade induces ER stress in tumor cells [5]. 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.

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 [6,7]. ATF4 is expressed as a function of eIF2α 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 [7].

We previously demonstrated that FAS inhibition induces the IRE1-dependent splicing of the ER stress specific XBP1 [2]. 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 [1]. IRE1 also regulates an alarm response through activation of the JNK pathway [1]. 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).

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 or 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 synergy between the two agents is currently being undertaken.

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 [8]. Therefore we hypothesized that FAS inhibitors might also be inducing autophagy. Indeed, early experiments demonstrate that FAS inhibitors induce the processing of LC3, one hallmark of autophagy (preliminary data not shown)[8]. 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.
As part of our continuing interest in developing novel inhibitors to target FAS in PCa and other cancer, a chemical library screen was performed to identify compounds that inhibit FAS thiosterase (TE) activity. A 10,000 compound library from Nanosyn, Inc. (Menlo Park, CA) was used. This pharma library is comprised of compounds that meet specific “drug-like” criteria, including solubility, reactive groups and molecular weight. Using a 96-well format, we screened compounds at 10 μM and used 40% inhibition at a single time point as our threshold for determining positive hits. This criterion identified 20 positive hits. From these 20, only 3 were able to inhibit FAS in PCa cells lines; compounds 1456, 4390 and 4393. These compounds inhibited FAS TE activity with IC50's ranging from 3.2-15 μM (Figure 8). Because of intellectual property and patent issues, the structures of these compounds are not being reported here. It is not yet known whether these compounds act as covalent inhibitors.

Figure 8. Inhibition of FAS activity in PC-3 cells. PC-3 cells in 24-well plates were incubated with the indicated concentrations of the individual compounds. 14C-acetate was the added for two hours and cells were lysed, extracted and fatty acids were quantified by liquid scintillation.

Figure 9. Novel FAS inhibitors reduce viability of tumor cell lines. PC-3, MCF-7 and U25 cells in 96-well plates were incubated with the indicated concentration of compounds. After 48 hours cell viability was measured by MTS assay

Viability assays were used to determine how effective the compounds were at retarding the growth of prostate, breast and glioblastoma tumor cell lines (Figure 9). The breast and glioblastoma lines were used to gauge how effective the compounds are in a variety of tumor types. Each of the three compounds effectively reduced the viability of the three tumor cell lines. It is interesting to note that in each case the compounds are less effective against glioblastoma. Clonogenic survival experiments are currently underway as are testing of the compounds against “normal” cell lines including PrEC’s that express little or no FAS. It is expected that these compounds will be rotated into the current repertoire of FAS inhibitors in the PTEN-/- model. We envision that a derivative of at least one of these compounds will be identified for a future translation as an anti-PCa drug that target FAS.

Specific Aim 2. To determine the effect of FAS inhibition in a spontaneous model of prostate cancer. The goal for this aim was two-fold. The first was to determine if FAS inhibitors, in general, or orlistat specifically, are able to inhibit tumor growth in a spontaneous model of prostate cancer. We chose a model in which a flowed PTEW allele is knock-out specifically in the prostate of mice. The advantages of this model are: (a) PTEN is the most mutated gene in PCa, so it is clinically relevant, (b) the model mimics the
development and progression of human PCa [9], (c) the model is spontaneous and orthotopic and (d) FAS expression is regulated via the PTEN-regulated PI3k-Akt axis[10-12]. Accordingly, tumors from Pten-/- mice have significantly elevated FAS expression relative to prostates from wild type mice (not shown). The second goal for this aim is to determine whether inhibition of FAS activity in vivo will also induce ER stress. 

Progress: After establishing a breeding colony we have mice that are currently being treated with FAS inhibitors. We have not yet collected any tumors or had any mice go long enough to determine whether there is any survival benefit. We are also working to develop optimized conditions for orlistat delivery as it has issues related to solubility.

Key Research Accomplishments:

- Fatty acid synthase inhibitors induce endoplasmic reticulum stress (manuscript 1).
- 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 (data in this report).
- FAS inhibitors when combined with the FDA-approved proteasome inhibitor Velcade induce enhanced ER stress signaling and ER stress-associated cell death.
- Determination of crystal structure of human FAS-TE domain in a covalent complex with Orlistat (manuscript 2)
- Determination of crystal structure of FAS-TE in complex with the hydrolyzed form of Orlistat (manuscript 2)
- Development of a model for β-lactone inhibition of FAS-TE
- Development a novel model of substrate binding to FAS-TE
- Identification of novel chemical inhibitors of FAS TE activity

Reportable Outcomes:

Manuscripts


Abstracts


Presentations

1. 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

None

Conclusion

As detailed in the body of this annual report, we have uncovered a novel connection between FAS, the proteasome pathway and perhaps autophagy. These results highlight the connection between FAS and prostate cancer and they add to a recently established body of literature that has established a link between ER stress and autophagy. These findings will be important not only for understanding how FAS inhibitors might induce cell death in a prostate cancer cell, but they may also provide some teleological explanation for why prostate tumor cells are addicted to FAS expression and activity.

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. Future work will provide a mechanism to explain the anti-tumor effects of FAS inhibitors as they translate toward the clinic.

References.


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 eIF2α and concomitant inhibition of protein synthesis. PERK-deficient transformed mouse embryonic fibroblasts and HT-29 colon carcinoma cells that express a dominant negative PERK (ΔC-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 coinubcation 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), 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).
Antibodies against eIF2α, phospho-eIF2α, 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). TRZol was from Invitrogen. Avian myeloblastosis virus–reverse transcriptase and Taq Polymerase were from Promega (Madison, WI). 33S-Methionine and 14C-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% CO2. 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 μg/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.

**Metabolic labeling of protein and fatty acid synthesis.** To measure new protein synthesis, cells were incubated with orlistat or thapsigargin, the phospho-eIF2α, 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). TRZol was from Invitrogen. Avian myeloblastosis virus–reverse transcriptase and Taq Polymerase were from Promega (Madison, WI). 33S-Methionine and 14C-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).

**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 μg/mL of puromycin for 48 h. The transfected cell populations were then maintained in 1 μg/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 phosphorylated Ser209 of eIF2α. Cell lysates were then transferred to Immobilon-P membrane (polyvinylidene difluoride). Immunoreactive bands were detected by enhanced chemiluminescence (Perkin Elmer Life Sciences, Inc., Boston, MA).

**Results**

**Pharmacologic inhibition of FAS induces phosphorylation of eIF2α 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 eIF2α. We first examined the phosphorylation status of eIF2α in cells treated with three different pharmacologic inhibitors of FAS (Fig. 1A). PC-3 cells were treated with orlistat (12.5–50 μmol/L, left) or cerulenin (5 or 10 μg/mL, middle) for 16 h, or C75 (10 μg/mL) for 8 to 24 h (right). Each FAS inhibitor induced robust phosphorylation of eIF2α at each concentration after 16 h of treatment, as did thapsigargin (data not shown). Similarly, all three inhibitors induced eIF2α 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 14C-acetate incorporation into total cellular lipids (Fig. 1B, left). Because the phosphorylation of eIF2α leads to the inhibition of
protein synthesis, we did a 35S-methionine labeling experiment to measure the levels of newly synthesized proteins in cells treated with orlistat. PC-3 cells were treated with orlistat (50 μM/L) for 12 and 24 h or thapsigargin (1 μM/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 eIF2α 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 eIF2α 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 eIF2α (Fig. 1D). Collectively, these data indicate that eIF2α phosphorylation induced by FAS inhibition is, indeed, specific to both FAS and tumor cells.

**PERK mediates eIF2α phosphorylation in response to orlistat treatment.** There are four known eIF2α kinases: PERK, GCN2, PKR, and HRI; however, PERK is the kinase that phosphorylates eIF2α during the ER stress response (26, 27). To determine whether PERK is the kinase responsible for the phosphorylation of eIF2α in response to FAS inhibition, wild-type and PERK−/− MEFs transformed with Ki-Ras712 were obtained and tested for their sensitivity to orlistat (27). The wild-type and PERK−/− MEFs were treated with orlistat (12.5 μM/L) for 8, 16, and 24 h (Fig. 2A) or treated with thapsigargin (1 μM/L) for 1 h (data not shown). In the wild-type MEFs, orlistat induced the phosphorylation of eIF2α within 8 h (Fig. 2A), consistent with our findings in prostate tumor cell lines (Fig. 1A). On the other hand, no significant phosphorylation of eIF2α was evident during the same time course of orlistat treatment in the PERK-deficient cells (Fig. 2A). As expected, thapsigargin only induced phosphorylation of eIF2α in the wild-type and not the PERK−/− MEFs (data not shown). These data indicate that FAS inhibition results in PERK-dependent phosphorylation of eIF2α.

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 μM/L), or thapsigargin (100 nmol/L) for 1 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.

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**Figure 1.** FAS inhibitors induce phosphorylation of eIF2α 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 μg/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-eIF2α, total eIF2α, and β-tubulin. B, PC-3 cells were treated for 2 h with C75 (10 μg/mL), orlistat (25 and 50 μM/L), or cerulenin (5 and 10 μg/mL), then incubated with 14C-acetate (1 μCi) 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 μM/L) for the indicated times or thapsigargin (Tgn; 1 μM/L) for 1 h and then pulsed with 10 μCi of 35S-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 μM/L) for 24 h or Tgn (1 μM/L) for 1 h side by side with PC3 cells and prepared for immunoblot analysis.
(ΔC-PERK) or the corresponding empty vector (23). These cells were seeded at a low density and treated with C75 (9 μg/mL), orlistat (25 μmol/L), or thapsigargin (10 nmol/L) to assess clonogenic survival. As expected, the HT-29 ΔC-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 ΔC-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 eIF2α in response to, and protects cells against, orlistat treatment. A, wild-type (WT) and PERK−/− MEFs transformed with with Ki-RasV12 were treated with orlistat (12.5 μmol/L) for the indicated times. Samples were analyzed with antibodies specific for phospho-eIF2α and total eIF2α. B, clonogenic survival assays were done in WT and PERK−/− MEFs, treated with vehicle, orlistat (25 μmol/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/ΔC-PERK cells were treated with vehicle, orlistat (25 μmol/L), C75 (9 μg/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 μmol/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 μmol/L) for 16 h or Tgn (1 μmol/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 μmol/L) for 8 to 24 h (Fig. 3A) or with cerulenin (5 or 10 μg/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 product associated with XBP-1(s) 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 μmol/L) or C75 (9 μg/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 eIF2α 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 eIF2α and subsequent indications of the ER stress response events coincide or precede cell death induced by FAS inhibition, we analyzed the temporal phosphorylation of eIF2α and markers of caspase activity and cell death. In PC-3 cells treated with orlistat, phosphorylation of eIF2α 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 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 μg/mL), orlistat (25 μmol/L), or orlistat with cycloheximide for 16 h. Samples were collected for immunoblot analysis and probed with antibodies specific for phospho-eIF2α, total eIF2α, and β-tubulin.

**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

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**Figure 4.** Inhibition of FAS activity induces mRNA expression of ER stress–regulated genes. A, DU145 cells were treated with vehicle control, orlistat (50 μmol/L, top), C75 (9 μg/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 μmol/L) for 24 h or Tgn (1 μmol/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 eIF2α is an early event in cells treated with FAS inhibitors. A, PC-3 cells were treated with 50 μmol/L of orlistat for the indicated times and samples were collected for immunoblot analysis and probed with antibodies specific for phospho-eIF2α, cleaved PARP, cleaved caspase 3, and β-actin. B, PC-3 cells were treated with DMSO, cycloheximide (CHX, 1 μg/mL), orlistat (25 μmol/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 μg/mL), orlistat (25 μmol/L), or the combination of orlistat and cycloheximide for 16 h. Samples were collected for immunoblot analysis and probed with antibodies specific for phospho-eIF2α, total eIF2α, and β-tubulin.

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stressing 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 μg/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 eIF2α separately, the level of phosphorylated eIF2α was significantly reduced in cells treated with the two agents combined, with no change in total eIF2α 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 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 μmol/L), thapsigargin (25 nmol/L), or the combination of both for 12 h (Fig. 6B, left); or (b) C75 (9 μg/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.
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 eIF2α, a concomitant attenuation of protein synthesis and the IRE1-mediated processing of XBP-1. Interestingly, phosphorylation of eIF2α 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 eIF2α, 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 eIF2α. 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 tissue. 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. These studies and RNA interference experiments have verified FAS as a key anticancer target. 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. 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 (Figs 1a, 1b). 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) domain12–14. The release of the thioesterase from FAS by limited proteolysis, however, results in the production of fatty acids containing 20 to 22 carbons. 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 FAS10,11,16,17. 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 β/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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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 adapts 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 (α4) to β-strand 5 (β5), 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 β6 to β7.

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 configuration 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

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-i-leucine moiety (or peptidyl moiety) interacts with a cavity at the interface that is also evident in the apoenzyme structure (Fig. 1f). 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 recombiant thioesterase domain and FAS in tumor cells.

The peptidyl moiety of Orlistat binds in the interface cavity, which is generated by residues from both subdomains (Fig. 2a). The i-leucine component interacts primarily with residues on the rim of the cavity, including Leu2222, Ile2250, Gln2374, Phe2370, Phe2371, Phe2375 and Phe2423. Gln2251 forms a hydrogen bond to the nitrogen atom of the N-formylamide group. The main chain nitrogen atoms of the latter two residues form the oxyanion hole and weakly hydrogen-bond with the C1 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 and Tyr2424 (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...
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 period, as there are minor structural differences 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 O1 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 inhibition7–9. 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 nucelophilic 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 domain22,23. 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
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 substrates12–14.

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 carboxyl 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 importantly, the hexanoyl tail that extends off the C2 position of Orlistat packs against His2481 (Fig. 2e). 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. 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.
(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 (C16) when attached to ACP or CoA10–14. 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 occur15. 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 FAS16,17. 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 described19. The domain was subcloned into the pET15b (Novagen) vector containing an N-terminal His6 tag with an intervening thrombin cleavage site. The thioesterase was overexpressed 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 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 complex was concentrated to 17 mg ml−1, flash-frozen in liquid nitrogen and stored at −80°C.

Table 1 Data collection and refinement statistics

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Refinement

| Resolution (Å) | 39.0–2.3 |
| No. reflections| 21,850   |
| No. atoms      | 4,016    |
| Protein        | 36.6     |
| Orlistat covalent | 60.2   |
| Orlistat hydrolyzed | 67.1   |
| DTT            | 8        |
| Water          | 53       |
| B-factors      | 30.6     |
| R.m.s. deviations | 0.01   |
| Bond lengths (Å)| 1.27    |

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

Crystallization and structure determination. Crystals of the thioesterase–Orlistat complex were grown using hanging drop/drip 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 (λ = 1.0 Å) were collected at the National Synchrotron Light Source, Brookhaven National Laboratory, on beamline X12C. Data were indexed and scaled to 2.3Å 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 Rwork and Rfree 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,
Illustrations. All structural illustrations were generated using PyMOL. 

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.

ACKNOWLEDGMENTS

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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 purification, 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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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 separated by ' - '

Cancer - Dietary fat - Saturated fatty acids - Polyunsaturated fatty acids - Fatty acid synthase - Acylation - Lipid-mediated signal transduction - Isoprenoids - Cholesterol - Bile acids - Steroids
Dietary fat–gene interactions in cancer

Yong Q. Chen · Iris J. Edwards · Steve Kridel · Todd Thornburg · Isabelle M. Berquin

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 underlying 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...
1.1 Saturated fats and cancer

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].
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 reduced 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 pathway serves 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-
there has been no direct demonstration that 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 are 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 also use for other processes in cells. Palmitate 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, ACC 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 deacylase, 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 are important to fully appreciate how membrane dynamics and membrane associated signaling are regulated.

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negative relationship with fish oil consumption [46]. For prostate cancer, review of international and intranational epidemiological evidence indicated 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 activity is increased in patients with prostate cancer, suggesting that COX-2 may play an important role in prostate cancer progression [74]. Therefore, the targeting of COX-2 in combination with other anti-cancer agents may offer a promising approach for the treatment of prostate cancer.

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
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 [Chen et al., unpublished].

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 PUFA 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 (αLNA) to eicosapentaenoic acid (EPA; Fig. 3). Both AA and EPA can be metabolized to corresponding series of eicosanoids, including prostanoids (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 PGE_2 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 PGE_2-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 pro-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-κB [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-phospholipid. 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] (Chen et al.,
unpublished). n-3 fatty acyl chains (EPA or DHA) in the
sn-2 position are poor substrates for cytosolic phospholi-
pase A_2 (cPLA_2) [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-glycerol-3-phosphoholine (PC) can be con-
verted to 1-O-alkyl-2-lyso-PC and then to the platelet
activating factor (PAF), 1-O-alkyl-2-acetyl-PC). Incorpora-
tion of n-3 PUFA at the sn-2 position may also reduce the
conversion from ether-linked PC to lyso-PC and conse-
quently 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 TNFα
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 PUFA have been proposed
to promote lipid peroxidation and thus enhance carcino-
genesis. However, studies have shown that the incorpora-
tion 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 PPARα and PPARβ/δ have been shown
to stimulate the proliferation of cancer cells [156, 157]
whereas a large body of evidence has shown that PPARγ is
growth inhibitory [158–160]. Loss-of-function mutations in
PPARγ have been associated with human colon cancer
[161]. In breast and prostate cancer cell lines, DHA induces
apoptosis through activation of PPARγ [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 PPARγ, failed to activate the
receptor to induce apoptosis.

The mechanisms of action of PUFA as 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 synthe-
sized 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 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 isoprenoids 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 is 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 to breast cancer, 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 progestosterone, 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 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., 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].

![Fig. 5 Role of AMACR in phytanic, pristanic and bile acids metabolism.](image-url)
However, 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 Phytic 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 phytic acid [230] (Fig. 5). Therefore, the discovery of a link between AMACR and prostate cancer heightens the possibility of phytic acid as a risk factor for prostate cancer. Branched-chain fatty acids derived from dairy and beef products may play a crucial role in peroxisomal β-oxidation and the inhibition of AMACR. Ample evidence suggests that dietary intake of phytic acid is associated with decreased risk of prostate cancer [231].

In addition, AMACR genetic variants may have different activities in metabolizing branched-chain fatty acids, which could account for part of the difference in 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-suppressive 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 mechanistic 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 source of 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 phytic acid?

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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 \[5\]. 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).
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: acetyl-CoA + 7malonyl-CoA + 14 NADPH + 14H⁺ → palmitate + 14 NADP⁺ + 6H₂O + 8CoA-SH + 7CO₂. The high energy demand that accompanies fatty acid synthesis

**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 shuttle 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.
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 nephroblastosoma (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 antitumor metabolite discovered in Cephalosporium caelestis 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/new 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/new 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
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 scaleable 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 3. Pharmacologic inhibitors of the KS and the TE domains of fatty acid synthase.** The dashed line in C75 represent a racemic mixture.

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 [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 [85].

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

Cerulenin, a compound containing a C12 acyl chain (Figure 3), has been crystallized within the active sites of 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
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 (5R)-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.

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.
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.
Fatty acid synthase inhibitors: new directions for oncology

Bibliography
Papers of special note have been highlighted as either of interest (*) or of considerable interest (**). to readers.


A comprehensive review that highlights many aspects of the biology of FASN and the anti-tumor actions of FASN inhibitors.


• The work in this study demonstrates that there is no deleterious phenotype when FASN is knocked out in the liver.


• This is the first work to demonstrate the ability of FASN inhibitors to induce cell death in tumor cells.


• This study is the first to demonstrate the specificity of FASIN inhibitors by using siRNA to knock down FASN in prostate tumor cells lines. In addition, it provides more demonstration that cells require fatty acid as cell death is prevented by exogenous addition of palmitate.


• This study reported the first synthesis of a pharmacologic inhibitor that targets FASN based on catalytic mechanism. Importantly, the synthetic inhibitor was also efficient at inducing cell death in tumor cells with no effect on normal cells.


• An important study that demonstrates that FASN inhibitors can be used as chemopreventive agents. Interestingly, the chemopreventive mechanism is similar to the antitumor mechanism in established tumors.
Fatty acid synthase inhibitors: new directions for oncology


*The study was the first to demonstrate that the TE domain of FASN can also be targeted with inhibitors and induce cell death in tumor cells.*


*This study not only demonstrates that FASN can be inhibited by EGCG, but also that the antitumor activity of the compounds seems to be directly linked to FASN inhibition as palmitate rescues the effects of EGCG in prostate tumor cells.*


- An important contribution to the field by providing the first crystal structure of any domain of human FASN bound to a ligand. The interaction between FASN TE and orlistat establishes a blueprint for the design of novel FASN inhibitors.


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1-11C-Acetate as a PET Radiopharmaceutical for Imaging Fatty Acid Synthase Expression in Prostate Cancer

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Although it is accepted that the metabolic fate of 1-11C-acetate is different in tumors than in myocardial tissue because of different clearance patterns, the exact pathway has not been fully elucidated. For decades, fatty acid synthesis has been quantified in vitro by the incubation of cells with 14C-acetate. Fatty acid synthase (FAS) has been found to be overexpressed in prostate carcinomas, as well as other cancers, and it is possible that imaging with 1-11C-acetate could be a marker for its expression.

Methods: In vitro and in vivo uptake experiments in prostate tumor models with 1-11C-acetate were performed both with and without blocking of fatty acid synthesis with either C75, an inhibitor of FAS, or 5-(tetradecyloxy)-2-furoic acid (TOFA), an inhibitor of acetyl-CoA carboxylase (ACC). FAS levels were measured by Western blot and immunohistochemical techniques for comparison.

Results: In vitro studies in 3 different prostate tumor models (PC-3, LNCaP, and 22Rv1) demonstrated blocking of 1-11C-acetate accumulation after treatment with both C75 and TOFA. This was further shown in vivo in PC-3 and LNCaP tumor-bearing mice after a single treatment with C75. A positive correlation between 1-11C-acetate uptake into the solid tumors and FAS expression levels was found.

Conclusion: Extensive involvement of the fatty acid synthesis pathway in 1-11C-acetate uptake in prostate tumors was confirmed, leading to a possible marker for FAS expression in vivo by noninvasive PET.

Key Words: 1-11C-acetate; fatty acid synthase; C75; TOFA

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The National Cancer Institute estimates that roughly 219,000 new cases of prostate cancer and about 27,000 deaths from this disease will occur in 2007 (1). In the early 1990s, early and widespread detection of prostate cancers was made possible by promotion of prostate-specific antigen (PSA) screening in conjunction with digital rectal examination. Elevated PSA levels are quite high in a patient with prostate cancer but, unfortunately, are also caused by benign prostatic hyperplasia and even inflammation or urinary retention (2). Transrectal ultrasound is frequently used to assist surgeons in biopsy and for local staging, CT and MRI are commonly used to determine the extent of disease, although structural changes are not always apparent with these modalities (3).

18F-FDG PET has become essential in the diagnosis of many malignancies, but it is not ideal in the detection of prostate cancer. Prostate cancer is one of a handful of tumors with low metabolism, and 18F-FDG, being a marker of glucose metabolism, is not highly effective in delineating it from surrounding tissue (4). Although 18F-FDG has been shown to be effective in the assessment of high-grade primary tumors and metastatic disease (5–9), other obstacles still leave much to be desired for its use with prostate malignancies, especially at early stages. The bladder clearance of 18F-FDG also poses an obstacle as it is in the same anatomic region as the prostate, and, therefore, the primary tumor. Studies have also shown an inability to differentiate benign hyperplasia in the prostate from malignant disease or postoperative scarring from radical prostatectomy (10,11).

Because of the problems associated with 18F-FDG imaging in prostate, alternative modalities must be used to image prostate cancer. Clinically, 1-11C-acetate has been shown to be an effective tracer for the delineation of prostate cancer and its metastases with PET in humans (12–16). Although it is accepted that the metabolic fate of 1-11C-acetate in tumors differs from that in normal tissue, the exact pathway has not been fully elucidated. Interestingly, fatty acid synthesis has been quantified in vitro by the incubation of cells with 14C-acetate.

Fatty acid synthase (FAS) is a multifunctional enzymatic protein that catalyzes fatty acid biosynthesis (17). FAS is overexpressed in prostate carcinomas as well as other cancers (18–22). On the other hand, FAS levels are low or absent in most normal tissues. FAS levels are associated with tumor aggressiveness in late-stage prostatic adenocarcinomas as well as a prognostic indicator for overall survival.
Previous studies have demonstrated that FAS inhibitors can reduce $^{14}$C-acetate incorporation in human tumor cell lines and in human lung xenografts and mouse prostate tumors ex vivo (24–27). Because of these facts, we hypothesize that FAS is involved with $^{11}$C-acetate uptake in prostate cancer. The following reports an examination of the mechanism of $^{11}$C-acetate uptake in prostate tumor models and its implications for tumor progression and patient survival. Understanding the mechanism of $^{11}$C-acetate uptake and the relation to FAS expression levels could provide a valuable tool to clinicians for the planning and monitoring of treatments because of the increased mortality with raised levels of this protein in prostate cancer. It could also be used in validating the translation of novel FAS inhibitors, as anticancer agents, into the clinical setting.

**MATERIALS AND METHODS**

**General**

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich Chemical Co., Inc. Radioactive samples were counted in a radiosotope calibrator (Capintec, Inc.) for determination of megabecquerels (millicuries) and an automated well scintillation 8000 $\gamma$-counter (Beckman Coulter) for counts per minute. Centrifugation was performed on a Sorvall Superspeed RC-6 Centrifuge (Sorvall, Inc.) refrigerated to 4°C. Male athymic nu/nu mice ($\sim$20 g; 5- to 6-wk-old) were purchased from the National Cancer Institute. Human prostate carcinoma tumor cell lines PC-3 (androgen receptor negative), LNCaP (androgen responsive), and 22Rv1 (androgen resistant) were obtained from the American Type Culture Collection and maintained by serial passage in cell culture. Both LNCaP-4 (androgen responsive) (Dr. Charles Sawyer at UCLA) and CWR22 (androgen responsive) (Bristol Myers Squibb) tumors were implanted and maintained by animal-to-animal passage. $^{11}$C-Labeled acetate was prepared by the reaction of $^{11}$C-labeled carbon dioxide with a Grignard reagent as described previously (28). Radiochemical purity was always $\geq99\%$.

**In Vitro Cell Uptake and Inhibition**

PC-3, LNCaP, and 22Rv1 prostate cells were plated in 6-well plates ($4.5 \times 10^5$, $9 \times 10^5$, and $1.2 \times 10^6$ cells per well, respectively) 24 h before the study was initiated. The cells were grown to $\sim75\%$ confluence at $37^\circ C$ and $5\%$ CO$_2$ in appropriate medium and supplemented with 10% heat-inactivated fetal bovine serum. Eighteen hours before the uptake experiment, C75 (63.5 $\mu$g), a FAS inhibitor (29), was added to the growth media (5 mL) in each well in a small amount of dimethyl sulfoxide (DMSO) (10 $\mu$L) so that the final concentration in each well was 50 $\mu$M (controls received DMSO alone). To initiate the study, the culture medium was removed, and cells were rinsed with phosphate-buffered saline (PBS). Approximately 0.37 MBq (10 $\mu$Ci) of $^{11}$C-acetate were added to the cells in 1.0 mL fresh media to initiate tracer uptake (including C75 or DMSO alone to maintain inhibitor concentration). Incubation was terminated at various times (15, 30, or 60 min) by removing the radioactive culture medium. Cell monolayers were washed with 2 mL of cold PBS 3 times to remove any excess culture medium from the extracellular spaces. Lysis of the cells was achieved by addition of 1 mL of 0.25% sodium dodecyl sulfate (SDS). Lysis extracts, as well as 1 mL of radioactive culture medium as a standard, were counted in a $\gamma$-counter and measured for protein content using a standard copper reduction/bicinchoninic acid assay (BCA; Pierce Biotechnology), with bovine serum albumin as the protein standard. Cellular uptake data for all experiments were normalized for the amount of protein present and calculated as the percentage uptake (cell-associated). A further inhibition study was performed with 5- (tetradeoxyloxy)-2-furoic acid (TOFA), a potent inhibitor of acetyl-CoA carboxylase (ACC), a key enzyme involved in fatty acid biosynthesis (30). Procedures were similar to those stated earlier, with the final concentration of TOFA being 30 $\mu$M and the pretreatment occurring 2 h before addition of $^{11}$C-acetate. The tracer was added directly to the 5 mL of growth media, rather than changing the media, to ensure continued presence of the pretreatment concentration of TOFA.

To compare the abilities of C75 and TOFA to inhibit fatty acid synthesis, PC-3 cells were seeded in 24-well plates at $1 \times 10^5$ cells per well. After 48 h the cells were treated with either C75 (0, 10, 20, 30, or 60 $\mu$M) to inhibit FAS or TOFA (0, 10, 20, or 30 $\mu$M) to inhibit ACC for 2 h, and then $2^{14}$C-acetate (0.037 MBq; 1 $\mu$Ci) was added for an additional 2 h. An additional study was performed using both PC-3 and LNCaP cells, where cells were seeded in 24-well plates at $1 \times 10^5$ cells per well. After 48 h the cells were treated with either C75 (30 $\mu$M) to inhibit FAS or TOFA (30 $\mu$M) to inhibit ACC for 2 h, and then $2^{14}$C-acetate (0.037 MBq; 1 $\mu$Ci) was added for an additional 2 h. Control cells received DMSO (0.1%) only. After the labeling period, the cells were collected and washed and lipids were extracted and quantified by scintillation counting as described previously (31,32).

To observe the contribution of the tricarboxylic acid (TCA) cycle to $^{11}$C-acetate cellular uptake, an inhibition study with 3-nitropropionic acid, a known inhibitor of succinate dehydrogenase in the TCA cycle, was performed. PC-3 cells, plated in 6-well plates 24 h before uptake, were treated with 100 $\mu$M 3-nitropropionic acid 2 h before radiotracer uptake, whereas control cells received vehicle alone. $^{11}$C-Acetate (1.11 MBq; 30 $\mu$Ci) was added to the wells; this was followed by a 25-min incubation. Cells were then washed and collected by trypsin/ethylenediaminetetraacetic acid (EDTA) for counting in the $\gamma$-counter and subsequent protein assay for normalization.

**Small-Animal PET**

All animal experiments were performed in compliance with the Guidelines for the Care and Use of Research Animals established by Washington University’s Animal Studies Committee. Single-position, whole-body imaging was performed using small-animal PET (microPET Focus 120 or Focus 220; Siemens Medical Solutions, Inc.) (33). Mice were imaged individually or in pairs in a supine position in a specially designed bed. Isoflurane (1%–2%) was used as an inhaled anesthetic to induce and maintain anesthesia during imaging. The bed was placed near the center of the field of view of the PET scanner, where the highest image resolution and sensitivity are available. Imaging was performed, 20 min after injection, with a single 10-min static scan. Images were reconstructed by Fourier rebinning, which was followed by 2-dimensional ordered-subset expectation maximization (OSEM) (34).

PET images were evaluated by analysis of the standardized uptake value (SUV) of the tumor and nontarget organ (muscle) using ASIPRO software (Siemens Medical Solutions, Inc.). The average radioactivity concentration within the tumor or tissue was obtained from the average pixel values reported in nanocuries per
milliliter within a volume of interest drawn around the entire tumor or tissue on multiple, consecutive transaxial image slices. SUVs were calculated by dividing this value, the decay-corrected activity per unit volume of tissue (nCi/mL), by the injected activity per unit of body weight (nCi/g). Necrotic tissue was excluded by analysis of the images in comparison with serial slices through the tumor postmortem. Any necrosis in a tumor was noted, and those sections (which also had no uptake) were not included in the overall SUV calculation.

An animal imaging study was performed on 22Rv1, PC-3, CWR22, and LAPC-4 tumor-bearing mice to confirm correlation of uptake of 1-11C-acetate with FAS expression in tumors. Two prostate carcinoma tumor models were prepared in culture (22Rv1 and PC-3) and then harvested for implant by trypsin/EDTA and injected in a volume of 100 μL into the right flank of intact, male nule/nu mice (15–20 g) in the appropriate media at a given concentration (5 × 10⁶ cells in Matrigel [BD Biosciences] for 22Rv1 and 3 × 10⁶ cells in Kaighn’s modification of Ham’s F12 medium for PC-3). CWR22 and LAPC-4 tumors were obtained from animal-to-animal passage. Tumors were allowed to grow until palpable, and the time varied by model. PET was performed 20 min after intravenous injection of 14.8–18.5 MBq (400–500 μCi) 1-11C-acetate (100 μL) via the tail vein. The 20-min time point after injection was chosen on the basis of the experience of other researchers (13,14,16). After imaging, the mice were euthanized, and the tumors were excised and flash frozen to −80°C for subsequent Western blot analysis to determine FAS expression. Images were analyzed for determination of SUV and compared with the Western blots.

**Western Blots**

Frozen tumors were thawed over ice and homogenized, and the cells were lysed with 1× cell lysis buffer (Cell Signaling Technology) for determination of protein concentration by BCA protein assay (Pierce). Twenty micrograms of protein of each sample were run using SDS-polyacrylamide gel electrophoresis with a 4%–20% Tris gradient gel. Standard Western blotting was performed with an anti-FAS primary antibody (rabbit; Novus Biologicals) and a goat antirabbit secondary antibody (DyLight 647; Jackson ImmunoResearch) for determination of protein concentration by BCA protein assay (Pierce) overnight in a humidified chamber at 4°C and then rinsed again with PBS. The slides were then incubated with secondary goat antirabbit antibody labeled with biotin (1:200; Immunopure GAM IgG; Pierce) for 30 min and rinsed with PBS, which was followed by incubation for 30 min with ABC Vector Elite (Vector Laboratories) solution diluted in 0.5 M NaCl. The slides were rinsed 3 times in 0.1 M Tris buffer, pH 7.4, and then incubated in 3,3’-diaminobenzidine (DAB) and chromogen (DAB + Ket; Dako) for 10 min. After repeated rinsing with tap water, the slides were counterstained with Meyer’s hematoxylin for 40 s and washed with tap water. The slides were dipped 10 times in Bluing Reagent (YWR International) before they were dehydrated and cover-slipped using Permount (Fisher Scientific). The slides were observed and compared (10x) with the H&E-stained slides under a Nikon Eclipse E600W microscope fitted with a Nikon Dxm1200F digital camera. As a result of the protocol, FAS protein should appear brown in color.

**Immunohistochemistry**

After PET, to observe inhibition of 1-11C-acetate uptake in prostate tumors by blocking of FAS, immunohistochemical techniques were used to demonstrate the extent of protein expression. Tumors from the in vivo inhibition study were formalin-fixed, paraffin-embedded, sliced, and placed on slides for immunohistochemical analysis by the Histology Core at Washington University; 1 slide per section was also stained with hematoxylin and eosin (H&E) to confirm tissue viability. Slides were baked for 30 min at 60°C and then soaked in xylene, 2 times for 3 min each, hydrated in 2 soaks of 100% ethanol for 2 min each, and soaked 2 times with 95% ethanol for 2 min each, 2 times with 70% ethanol for 2 min each, and 1 time with 50% ethanol for 2 min. Slides were then rinsed in doubly distilled water and placed in 3 washes of PBS before blocking with a protein block (Dako) in a humidified chamber for 30 min at room temperature. After aspiration of the blocking solution, the slides were incubated with the FAS primary antibody (1:1,000; anti-FASN [M] antibody 34-6E7; FASGen, Inc.) overnight in a humidified chamber at 4°C and then rinsed again with PBS. The slides were then incubated with secondary goat anti-mouse antibody labeled with biotin (1:200; Immunopure GAM IgG; Pierce) for 30 min and rinsed with PBS, which was followed by incubation for 30 min with ABC Vector Elite (Vector Laboratories) solution diluted in 0.5 M NaCl. The slides were rinsed 3 times in 0.1 M Tris buffer, pH 7.4, and then incubated in 3,3’-diaminobenzidine (DAB) and chromogen (DAB + Ket; Dako) for 10 min. After repeated rinsing with tap water, the slides were counterstained with Meyer’s hematoxylin for 40 s and washed with tap water. The slides were dipped 10 times in Bluing Reagent (YWR International) before they were dehydrated and cover-slipped using Permount (Fisher Scientific). The slides were observed and compared (10x) with the H&E-stained slides under a Nikon Eclipse E600W microscope fitted with a Nikon DXM1200F digital camera. As a result of the protocol, FAS protein should appear brown in color.

**Statistical Analysis**

Statistically significant differences between mean values were determined using ANOVA coupled to the Scheffé test or, for statistical classification, a Student t test was performed.

**RESULTS**

**Inhibition of Fatty Acid Synthesis Reduces 1-11C-Acetate In Vitro Uptake**

To confirm the hypothesis that 1-11C-acetate uptake in tumors is related FAS expression, an in vitro blocking study was performed. Cells were pretreated for 18 h with C75, a known inhibitor of FAS, before uptake of 1-11C-acetate to determine if uptake could be blocked. All cells showed a linear increase in uptake over time in both the C75-treated and the control tumors. In all cases, the uptake in the control cells was consistently higher throughout the duration of the study. By 30 min, the C75-treated cells showed inhibition of uptake by 26.4% (P = 0.0005) in the PC-3 cell line and by 16.7% (P = 0.0010) and 26.9% (P = 0.0007) for the LNCaP and 22Rv1 cell lines, respectively (Fig. 1A).

Cell viability was measured by trypan blue staining. An average of 95% viability was measured for each cell line, with no decrease in overall cell number due to the presence of C75 (data not shown).
The role of ACC in 1-11C-acetate uptake was also determined by treating cells with TOFA, a potent inhibitor of ACC. ACC is the rate-limiting enzyme in the fatty acid synthesis pathway. After pretreatment with TOFA, cell uptake of 1-11C-acetate was significantly reduced, more than with C75 (Fig. 1B). The percentage of blocking increased over time in all cases, with values of 29.8% ± 5.75%, 67.4% ± 9.22%, and 34.7% ± 9.31% for PC-3, LNCaP, and 22Rv1 cell lines, respectively, at 30 min. To demonstrate that TOFA is a more potent inhibitor of fatty acid synthesis than C75, a dose-response comparison between TOFA and C75 in PC-3 cells was performed (Fig. 2A and 2B). It is evident that even at concentrations of 10 μM, TOFA has a significantly more pronounced effect on fatty acid synthesis than C75 (10 μM: 10.7% ± 0.59% vs. 84.9% ± 2.47%). An additional one-point study comparing PC-3 cells with LNCaP cells was undertaken to show that this relationship was observed in more than 1 cell line. It was shown that 30 μM C75 inhibited about 30% of fatty acid activity in both PC-3 and LNCaP cells, whereas TOFA inhibited fatty acid synthesis about 75%–80% (both after a 2-h treatment) (Fig. 2C). These data demonstrate that 1-11C-acetate uptake is directly related to the degree of FAS inhibition in prostate tumor cell lines.

PET Demonstrates In Vivo Correlation Between 1-11C-Acetate Uptake and FAS Expression

To test the hypothesis that 1-11C-acetate may be imaging FAS expression in vivo, an imaging study was performed,
with excision of the tumors after imaging for further analysis of FAS levels by Western blot. 1-11C-Acetate PET of 4 prostate tumor models (PC-3, 22Rv1, CWR22, and LAPC-4) was performed (Figs. 3A–3C). Regions of interest were drawn on the images around the tumors, excluding any necrotic tissue. SUVs at 20 min after injection were calculated to normalize these values (nCi/mL) to the injected activity per animal (nCi) as well as body mass (g). Imaging SUVs were 0.11 ± 0.01 for LAPC-4 (n = 2), 0.26 ± 0.06 for CWR-22 (n = 2), and 0.18 ± 0.02 for 22Rv1 (n = 3). In this case, the PC-3 tumors (n = 2) could not be delineated from the surrounding tissue and, therefore, no SUVs were calculated. Visual inspection of the Western blot results revealed obvious differences in the intensity of the band near 250 kDa (FAS = 267 kDa), with PC-3 showing the lowest levels of expression, 22Rv1 and LAPC-4 with significantly more intensity than PC-3, and CWR22 being the highest of those examined (Fig. 3D). Densitometry analysis confirmed this trend quantitatively. The relative values of expression were averaged for each tumor type, resulting in 2,354.1 ± 22.8, 9,640.9 ± 2,552.3, 11,160 ± 25.0, and 15,798 ± 257.6 for PC-3, LAPC-4, 22Rv1, and CWR22, respectively. Comparison of the average SUVs from the PET data with Western blot analysis of the homogenized tumor tissue resulted in a correlation ($R^2 = 0.974$) between tumor uptake of 1-11C-acetate and FAS expression (Fig. 3E).

Small-Animal PET of FAS Inhibition (C75 Blocks 1-11C-Acetate Uptake In Vivo)

Because the in vitro results confirmed that 1-11C-acetate uptake could be diminished by inhibition of FAS, a similar study was pursued in vivo. PC-3 (as the low-expressing control) and LNCaP tumor-bearing mice were imaged with 1-11C-acetate before and after treatment with C75, so that each mouse would serve as its own control (Fig. 4A). In 6 of the 7 image sets analyzed, tumor uptake of 1-11C-acetate decreased after a single treatment with C75. LNCaP tumors showed an average decrease in uptake of 12.3%, with PC-3 SUVs reduced by an average of 49.4% (Fig. 4B). The reasoning for the significant difference in the effect of FAS inhibition on acetate uptake ($P = 0.013$) between the 2 tumor types was explored by immunohistochemical analysis of FAS expression (Fig. 4C). Visual inspection of the FAS-stained slides clearly demonstrated a much higher abundance of the protein in the LNCaP tumors compared with that of PC-3 in all cases. H&E stains of all tumor slides confirmed viability of the tissue. The brown staining also colocalized in the same areas as the hemotoxylin stain on subsequent slides, indicating protein-rich portions (data not shown).

DISCUSSION

1-11C-Acetate was first examined as a possible tracer for malignancies by Shreve et al. in 1995 (35) and has since been extensively investigated in prostate cancer and its metastases (12–16). Direct comparisons by researchers have shown greater sensitivity for detection over the standard use of 18F-FDG (4, 13). Most recent work has demonstrated 1-11C-acetate as a useful tool for detecting recurrent disease at PSA relapse in many cases (12, 14, 15, 36) and even better results when paired with CT and MRI for anatomic reference and observation of structural changes.
Despite these findings, no definitive explanation for increased uptake has been made. Acetate can be metabolized by several distinct pathways in cells. Of course, acetate can be metabolized through the TCA cycle. In tumor cells, acetate can also be used as a substrate or a substrate precursor during fatty acid synthesis. Acetate is a precursor for acetyl-CoA, which can then be converted to malonyl-CoA by ACC. Acetyl-CoA and malonyl-CoA also provide substrate for fatty acid elongation in the mitochondria and endoplasmic reticulum, respectively. In addition, acetate is a precursor for cholesterol synthesis. As a result, 1-11C-acetate incorporation could be affected by multiple pathways. Considering that FAS and the fatty acid synthesis pathway are highly expressed and active in multiple cancers—prostate cancer, in particular—this pathway could be a major determinant of 1-11C-acetate uptake in prostate tumors.

Researchers have postulated that increased acetate uptake in malignancies may be due to increased lipid biosynthesis. Accordingly, one study recently observed the uptake and metabolism of 14C-acetate into 4 nonprostate tumor cell lines (LS174T, human colon adenocarcinoma; RPMI2650, human nasal septum tumor; A2780, human ovary carcinoma; and A375, human malignant melanoma) and 1 fibroblast model (37). The authors demonstrated that all malignant lines examined had significantly higher uptake over the fibroblasts and that the acetate incorporated into the lipid-soluble fractions, and primarily phosphatidylcholine (PC). Interestingly, Swinnen et al. have demonstrated that FAS-derived palmitate primarily partitions to detergent-insoluble lipid fractions in which PC is the primary constituent (38). Similarly, uptake of 2-14C-acetate has also been measured in CWR22 and PC-3 tumors in castrated and noncastrated mice (39). The authors found that acetate uptake correlated with androgen receptor expression, suggesting that acetate uptake can be affected by androgen. FAS has been shown to be overexpressed in prostate cancer (18–22); however, specific examination of FAS levels in correlation with 1-11C-acetate uptake by PET have yet to be reported. Because high levels of FAS expression have also been found to be an indicator of a poor prognosis in patients with prostate cancer, resulting in a 4.45-fold higher risk of death (40), we hypothesized that a noninvasive imaging method such as 1-11C-acetate PET for the determination of FAS in tumors could provide clinicians with an additional tool for individualized therapy.

In this current study, pharmacologic inhibition of the FAS pathway was used to demonstrate the specificity of 1-11C-acetate for imaging FAS expression by blocking the protein. C75-treated cells showed a significant decrease in cellular accumulation of 1-11C-acetate compared with that of controls but still showed an appreciable amount of cellular uptake (Fig. 1A). It is likely that either all of the FAS was not blocked or other described pathways are also involved. Inhibition of fatty acid synthesis with TOFA, a pharmacologic inhibitor of ACC—the rate-limiting enzyme
involved in fatty acid biosynthesis—also had significant impact of the cellular accumulation of 1-11C-acetate (Fig. 1B). The demonstration that TOFA is a more efficient inhibitor of fatty acid synthesis than C75 (Fig. 2A–2C) correlates well with differences in 1-11C-acetate uptake. Furthermore, because blockade of the 2 key enzymes involved in fatty acid synthesis affected acetate uptake, our data demonstrate that a large portion of 1-11C-acetate tumor uptake and retention is related to the fatty acid synthesis pathway.

Demonstrating that in vivo uptake of 1-11C-acetate in 4 prostate tumor models correlated with FAS levels (Fig. 3), it is evident that FAS is at least involved in 1-11C-acetate uptake in vivo—to our knowledge, the first time this relationship has been demonstrated. After showing that 1-11C-acetate cellular uptake can be diminished with FAS inhibition in vitro and that uptake correlates to FAS expression levels in vivo, we performed a FAS-blocking study in vivo with C75 (Fig. 4). With each mouse serving as its own control, specific changes in overall tumor uptake were calculated and showed a small average change in the LNCaP mice (~12%) and a larger effect in the PC-3 model (~49%). LNCaP has a higher expression of FAS (Fig. 4C); therefore, the amount of C75 given is likely to show less of an effect than in PC-3 mice, where its lower expression of FAS would result in a more extreme response with the same amount of C75.

Inhibition of FAS with C75, orlistat, triclosan, and many other compounds has led to promising in vitro and in vivo results confirming FAS as a viable target for cancer therapies (41,42). Although FAS represents an important therapeutic target, there has been no in vivo demonstration that FAS inhibitors significantly block fatty acid synthesis in tumors. One study with 18F-FDG to monitor the effects of FAS inhibitors significantly block fatty acid synthesis in human A549 lung cancer was reported recently (26). A transient, reversible decrease in glucose metabolism and tumor metabolic volume was noted after C75 treatment, with the peak effect seen at 4 h. This, however, was an indirect measure of fatty acid synthesis, whereas the use 1-11C-acetate shows a direct measurement. The data presented herein provide validation for further development of 1-11C-acetate PET, as a measure of fatty acid synthesis, and incorporation of the technology into preclinical in vivo models and clinical studies. Such information could provide important validation of the efficacy of FAS inhibitors and represents a unique tool in aiding the translation of new FAS inhibitors for the treatment of cancer into the clinical setting.

As mentioned earlier, acetate may also be metabolized by other pathways. The TCA cycle is a major factor in acetate metabolism throughout the body, and its presence in the cells needs to be considered. In one experiment, 3-nitropipionic acid (a known inhibitor of the TCA cycle) was added during cell uptake and showed a 14.3% ± 3.7% reduction of 1-11C-acetate uptake in the PC-3 model (data not shown). It has been shown that small interfering RNA (siRNA)-mediated knockdown of FAS in MDA-MB-435 mammary carcinoma cells can also affect expression of genes related to the TCA cycle and glycolysis and may also influence acetate metabolism indirectly (43). This supports, in part, the idea that TCA could be a major contributor to the retention of 1-11C-acetate and is regulated by the fatty acid synthesis pathway. Given the role of cholesterol in prostate cancer (44), and that in some cell lines increased fatty acid synthesis has been shown to be accompanied by an increase in cholesterol synthesis (45), further studies on the ability of the cholesterol synthesis pathway to regulate PET of 1-11C-acetate uptake in prostate cancer may also be warranted. On the other hand, the data presented herein clearly identify FAS and the fatty acid synthesis pathway as an important determinant of 1-11C-acetate uptake in PET of prostate cancer.

CONCLUSION

These findings are promising in that they suggest a possible biomarker for more-effective treatments in prostate cancer patients, and possibly others, as FAS expression has shown links to poor prognosis in other cancers as well. Moreover, because FAS inhibitors are being developed as antitumor agents, this technology also provides a unique opportunity to monitor the effectiveness and the validation of new FAS inhibitors for translation into a clinical setting.

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Chapter 7
Fatty Acid Synthase Activity in Tumor Cells

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Abstract
While normal tissues are tightly regulated by nutrition and a carefully balanced system of glycolysis and fatty acid synthesis, tumor cells are under significant evolutionary pressure to bypass many of the checks and balances afforded normally. Cancer cells have high energy expenditure from heightened proliferation and metabolism and often show increased lipogenesis. Fatty acid synthase (FASN), the enzyme responsible for catalyzing the ultimate steps of fatty acid synthesis in cells, is expressed at high levels in tumor cells and is mostly absent in corresponding normal cells. Because of the unique expression profile of FASN, there is considerable interest not only in understanding its contribution to tumor cell growth and proliferation, but also in developing inhibitors that target FASN specifically as an anti-tumor modality. Pharmacological blockade of FASN activity has identified a pleiotropic role for FASN in mediating aspects of proliferation, growth and survival. As a result, a clearer understanding of the role of FASN in tumor cells has been developed.

Abbreviations: FASN, fatty acid synthase; ACC, acetyl-CoA-carboxylase; ACL, ATP-citrate lyase; NADPH, nicotinamide adenine dinucleotide phosphate; MAT, malonyl acetyl transferases; KS, ketoacyl synthase; KR, β-ketoacyl reductase; DH, β-hydroxyacyl dehydratase; ER, enoyl reductase; TE, thioesterase; ACP, acyl carrier protein; VLCFA, very long chain fatty acids; ELOVL, elongation of very long chain fatty acids; SCD1, stearoyl-CoA desaturase-1; AMPK, AMP-activated kinase; ME, malic enzyme; FASKOL, liver-specific deletion of FAS; PPARα, Peroxisome Proliferator-Activating Receptor alpha; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; SREBP, sterol response element binding protein; S1P, site-one protease; S2P, site-two protease; RIPCre, Cre-recombinase under the control of rat insulin 2 promoter; CPT1, carnitine palmitoyl transferase 1; MCD, malonyl-CoA desaturase; SCAP, SREBP cleavage activating protein; NF-Y, nuclear factor Y; SP1, stimulatory protein 1; PI3K, phosphatidylinositol-3 kinase; KGF, keratinocyte growth factor; EGF, epidermal growth factor; JNK, cJun N-terminal kinase; RTK, receptor tyrosine kinase; AR, androgen receptor; PR, progesterone receptor; USP2a, ubiquitin-specific protease 2a; EGCG, epigallocatechin-3-gallate; TOFA, 5-(tetradecyloxy)-2-furoic acid; FDA, food and drug administration.

Keywords: cancer, fatty acid synthase, lipogenesis

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1. FATTY ACID SYNTHESIS

1.1 The FASN Enzyme

One of the metabolic hallmarks of a tumor cell is increased lipogenesis (Kuhajda, 2006, Swinnen, et al., 2006). In fact, in many instances the vast majority of fatty acids in tumors are synthesized *de novo* (Ookhtens, et al., 1984). In mammalian cells, fatty acid synthase (FASN) is the central enzyme of long chain fatty acid synthesis. FASN is a multifunctional polypeptide that is comprised of seven separate functional domains (Figure 1A). The individual domains of FASN work in concert to catalyze thirty-two different reactions to synthesize the sixteen carbon fatty acid palmitate, using acetyl-CoA and malonyl-CoA as substrates and nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor. The fatty acid synthesis reaction mechanism can be separated into three functional groupings: to 1) bind and condense the substrates, 2) to reduce the intermediates and 3) to release the final saturated long chain fatty acid palmitate (Figure 1B). The malonyl acetyl transferase (MAT) domain binds malonyl-CoA and acetyl-CoA, while the ketoacyl synthase (KS) domain acts to condense the acyl chain (Figure 1B). This $\beta$-ketoacyl moiety is then reduced in steps by the $\beta$-ketoacyl reductase (KR), $\beta$-hydroxyacyl dehydratase (DH), and enoyl reductase (ER) domains to a saturated acyl intermediate. This derivative can then be elongated by repeating the reactions catalyzed by the five previous enzyme activities for seven cycles until the thioesterase (TE) domain cleaves the final product, the sixteen carbon fatty acid palmitate. Throughout the entire synthesis of palmitate, the acyl carrier protein (ACP) acts as a coenzyme to bind intermediates by a 4’-phosphopantetheine group (Figure 1B). In total, approximately 30 intermediates are involved in the process, but it is the high specificity of the TE domain for a 16 carbon fatty acid, as well as
the MAT specificity for malonyl-CoA that are responsible for preventing leakage of intermediates (Wakil, 1989). The overall FASN reaction is as follows:

$$\text{Acetyl-CoA} + 7 \text{Malonyl-CoA} + 14 \text{NADPH} + 14 \text{H}^+ \rightarrow \text{Palmitic acid} + 7 \text{CO}_2 + 8 \text{CoA} + 14 \text{NADP}^+ + 6 \text{H}_2\text{O}$$

The structure of FASN has yet to be definitively characterized, as there are two distinct models (Smith, 2006). Early complementation studies suggest that FASN functions as a homodimer in head-to-tail conformation with two simultaneous reactions beginning in one subunit and finishing in the other (Wakil, 1989, Smith, et al., 2003, Rangan, et al., 1998, Rangan, et al., 2001). However, a more recent crystal structure analysis of porcine FASN challenges this historical model. The 4.5 Å structure reveals FASN as an intertwined dimer in a conformation resembling an ‘X’ with one central core region with two arms and two legs (Maier, et al., 2006). However, at this lower resolution, the definitive placement of the flexible TE domain and ACP is not possible. It is also unclear whether the body of the FASN complex can be identified as two distinct monomers. In this model, the KS domain is near the bottom of the central core of the complex and two MAT domains are in the “legs” of the X shape. The DH domains are located in the top half of the central region just under the ER domains. Adjacent to the ER domains are the KR domains that comprise the “arms” of this X complex. The study equates the reaction pockets of this structure as having “double hot dog” folds but observes asymmetry of the two sides of the reaction chambers that may reveal hinge regions that allow different conformations of the FASN complex (Maier, et al., 2006, Smith, 2006).

1.2 Other Players in the Fatty Acid Synthesis Pathway

While FASN is the central enzyme of fatty acid synthesis, other enzymes and pathways upstream of FASN are required to generate and supply substrates. Glucose enters the cell and is
converted through glycolysis to pyruvate which is then shuttled into the mitochondria to enter the citric acid cycle. Citrate is shuttled out of the mitochondria, where ATP-citrate lyase (ACL) catalyzes the conversion of citrate to oxaloacetate and acetyl-CoA. Acetyl-CoA Carboxylase (ACC) catalyzes the conversion of acetyl-CoA to malonyl-CoA in the rate limiting and first committed step of lipogenesis. Unlike FASN, which is primarily regulated transcriptionally, ACC is negatively regulated by post-translational phosphorylation at serine 79 by AMP-activated kinase (AMPK). Energy deficiency stimulates AMPK to regulate energy consumption of cells, specifically by regulating ACC among other enzymes. Fatty acid synthesis requires NADPH, which is provided through the hexose monophosphate shunt and malic enzyme (ME) to donate electrons (Wakil, et al., 1983). Recent findings also suggest that glutamine metabolism in can generate sufficient NADPH in glycolytic tumor cells as well as act as a carbon source for fatty acid synthesis (Deberardinis, et al., 2007).

After fatty acid synthesis, downstream enzymes can further modify palmitate for various cellular functions. In the endoplasmic reticulum, the 16 carbon fatty acid can be modified to fatty acids with eighteen or more carbons known as very long chain fatty acids (VLCFA), such as stearate (18:0) by a family of elongase enzymes called elongation of very long chain fatty acids (ELOVL1-6) (Jakobsson, et al., 2006). Palmitate and stearate can also be desaturated by stearoyl-CoA desaturase-1 (SCD1) at the cis-9 carbon to palmitoleate (16:1) and oleate (18:1), respectively (Sampath and Ntambi, 2005).

2. FASN EXPRESSION

2.1 FASN Expression in Normal Cells

In normal tissue, FASN is expressed and active in cells that have a high lipid metabolism, such as liver and adipose tissues, to generate triglycerides in response excess caloric intake
(Jayakumar, et al., 1995, Volpe and Marasa, 1975, Wakil, et al., 1983). FASN is also expressed in a niche-specific manner in specialized tissues such as lactating mammary glands (Kusakabe, et al., 2000, Thompson and Smith, 1985) cycling endometrium (Pizer, et al., 1997, Kusakabe, et al., 2000), and various other cell types including type II alveolar cells to produce lung surfactant (Buechler and Rhoades, 1980, Kusakabe, et al., 2000), brain cells (Kusakabe, et al., 2000, Jayakumar, et al., 1995), and seminal vesicles to produce seminal fluid (Kusakabe, et al., 2000). FASN is only weakly detectable, if at all, in other rapidly dividing normal tissues such as the intestinal epithelium, stomach epithelium, and hematopoietic cells in adults and is not detectable in most other adult tissues (Kusakabe, et al., 2000).

Despite the low expression profile in adult tissues, FASN is critical for developing embryos and is highly expressed in proliferative fetal cells (Kusakabe, et al., 2000). The importance of FASN in development is underscored by the fact that mice with homozygous deletions of the FASN gene display an embryonic lethal phenotype (Chirala, et al., 2003). FASN +/- mice die before implantation around embryonic day 3.5, most likely because developing embryos are unable to acquire enough fatty acids from the mother for adequate membrane biogenesis. The importance of FASN during development is further highlighted by the fact that the majority of heterozygotes are also resorbed after implantation. Those that survive do not live long beyond birth, indicating that one FASN allele is insufficient for embryogenesis, implantation, and developing tissues (Chirala, et al., 2003). The importance of the fatty acid synthesis pathway in development is further supported by the demonstration that deletion of ACC1 in mice also results in an embryonic lethal phenotype (Abu-Elheiga, et al., 2005).

Mice harboring tissue-specific deletions of FASN have been generated to facilitate understanding the role of FASN in normal tissue. To date FASN has been deleted in liver, β-cells, and hypothalamus (Chakravarthy, et al., 2005, Chakravarthy, et al., 2007). To knock out
FASN in the liver, mice with a “floxed” FASN allele were crossed with mice harboring an allele of Cre driven by a rat albumin promoter. Although this liver-specific deletion of FASN (FASKOL) leaves animals viable without severe physiological effects, it is not without consequence. When FASKOL mice are fed a diet containing zero fat or are fasted for prolonged periods, they develop symptoms similar to those seen in mice engineered to lack Peroxisome Proliferator-Activating Receptor alpha (PPARα) (Kersten, et al., 1999). Both PPARα knockout and FASKOL mice become hypoglycemic, develop steatosis (fatty liver) that correlates with reduced serum and liver cholesterol, reduced expression of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, decreased cholesterol biosynthesis activity, and elevated SREBP-2 expression. While the hypoglycemia and fatty liver may be reversed with dietary fat, all effects including cholesterol biosynthesis, HMG-CoA reductase and SREBP2 levels, as well as cholesterol levels in the serum and liver are rescued by administration of a PPARα agonist. This reveals distinct levels of metabolic regulation between de novo and dietary fat and indicates that products downstream of FASN activity regulate cholesterol, glucose, and fatty-acid homeostasis in the liver through activation of PPARα (Chakravarthy, et al., 2005). Interestingly, mice with a liver-specific knockout of ACC1 are still able to undergo fatty acid synthesis, but this discrepancy can be attributed to the possibility that malonyl-CoA may be provided from the ACC2 isoform to compensate (Harada, et al., 2007).

To determine whether FASN plays a role in pancreatic β-cell function, a knockout of FASN was generated. Crossing floxed FASN mice with mice harboring Cre under the control of rat insulin 2 promoter (RIPCre) causes specific deletion of FASN in pancreatic β-islet cells, as well as the hypothalamus, a region of the brain known for controlling motivational states, such as feeding. The resulting FASN knockout (FASKO) mice exhibit reduced feeding behavior and are highly active, even while maintained on a high fat diet (Chakravarthy, et al., 2007).
correlates with studies showing the small molecule FASN inhibitor C75 acts in the hypothalamus to stimulate fatty acid oxidation via carnitine palmitoyl transferase 1 (CPT1) to induce a reversible anorexic phenotype (see section 4.2). Interestingly, the β-cells lacking FASN are phenotypically unaffected as loss of FASN does not alter insulin or glucose levels during glucose tolerance testing or stimulation either in vivo or in vitro. Therefore, the fasting phenotype of FASKO mice appears to be solely attributable to the effects on the hypothalamus. As a matter of fact, this observation is in agreement with a recent study showing FASN is not required for normal insulin secretion of β-cells in vitro (Joseph, et al., 2007). Intracerebroventricular injection of FASKO mice with a small molecule drug Wy14,643 to activate PPARα restores feeding and weight gain, indicating that FASN controls PPARα activation in the hypothalamus. Pharmacological activation of PPARα in these mice also restores expression of CPT-1 and malonyl-CoA desaturase (MCD) that control cellular levels of malonyl-CoA by controlling the rate of transfer of fatty acids into the mitochondria for β-oxidation and malonyl-CoA stability, respectively (Chakravarthy, et al., 2007). These studies elucidate the importance of FASN in energy homeostasis and provide a mechanism through which FASN can regulate its effects.

2.2 FASN Expression in Tumor Cells

As discussed above, FASN has historically been studied in relation to normal physiology and as a central mediator of energy balance. In the last few decades, however, it has become clear that FASN is associated with tumor development. Accordingly, high FASN expression has been identified in many tumor types (Kuhajda, 2000, Kuhajda, 2006). Haptoglobin-related protein (Hpr) was demonstrated to correlate with breast cancer stage, prognosis, as well as recurrence and patient survival (Kuhajda, et al., 1989a, Kuhajda, et al., 1989b). Shortly after this observation, Hpr, or oncogenic antigen (OA-519) protein was identified as FASN (Kuhajda, et
Since these discoveries, FASN upregulation has been demonstrated in every type of solid tumor. An initial retrospective study showed FASN expression correlated with staining of the proliferation marker MIB-1 to predict survival of breast cancer patients (Jensen, et al., 1995). Subsequent studies confirmed the association of FASN with breast cancer recurrence, as well as shorter overall and disease-free survival in early breast cancer patients (Alo, et al., 1996, Alo, et al., 1999b). Breast cancer is not the only tumor type with elevated FASN levels. FASN expression is associated with prostate cancer prognosis, progression, and stage (Shurbaji, et al., 1992, Epstein, et al., 1995, Shurbaji, et al., 1996). As a matter of fact, FASN is upregulated in androgen-independent prostate tumors and expression correlates with disease stage, as the highest levels of FASN expression are in androgen independent metastases (Pizer, et al., 2001, Rossi, et al., 2003). FASN expression correlates with poor prognosis, advanced progression, and/or decreased survival in a number of other cancers of different origins including: ovarian (Gansler, et al., 1997, Alo, et al., 2000), melanoma (Innocenzi, et al., 2003, Kapur, et al., 2005), nephroblastoma (Wilms tumor) (Camassei, et al., 2003b), retinoblastoma (Camassei, et al., 2003a), bladder (Visca, et al., 2003), pancreas (Alo, et al., 2007), soft tissue sarcoma (Takahiro, et al., 2003), non-small cell lung cancer (Visca, et al., 2004), endometrium (Sebastiani, et al., 2004), and Paget’s disease of the vulva (Alo, et al., 2005). While FASN expression correlates with decreased survival and/or poor prognosis in a large number of tumor types, there are tumor types that show elevated FASN expression, but FASN expression does not correlate with patient survival or disease stage (Rashid, et al., 1997, Nemoto, et al., 2001, Silva, et al., 2007). In addition, there are several tumor types that show increased FASN expression, but correlation with disease progression or patient survival has not been investigated or published at this time. These tumors include hyperplastic parathyroid (Alo, et al., 1999a), stomach carcinoma
(Kusakabe, et al., 2002), mesothelioma (Gabrielson, et al., 2001), glioma (Zhao, et al., 2006), and hepatocellular carcinoma (Yahagi, et al., 2005).

Increased FASN expression in tumors is an early, common event (Swinnen, et al., 2002, Myers, et al., 2001) and its correlation with reduced survival and increased recurrence rationalizes the potential for anti-FASN tumor therapeutics (Kuhajda, 2000, Kuhajda, 2006, Kridel, et al., 2007). As evidence that lipogenesis as a whole is important in cancer, many of the enzymes upstream of FASN show altered expression patterns in human tumor cells, as well. For instance, ACL is overexpressed in cancer cells of breast and bladder (Szutowicz, et al., 1979, Turyn, et al., 2003). ACC is overexpressed in breast and prostate cancer cells (Milgraum, et al., 1997, Swinnen, et al., 2000b, Heemers, et al., 2003, Swinnen, et al., 2006). Interestingly, the tumor suppressor breast cancer susceptibility gene 1 (BRCA1) can bind the phosphorylated inactive ACC to prevent re-activation (Moreau, et al., 2006). In addition, squamous cell carcinomas of the lung show lower immunohistochemical staining of phosphorylated inactive ACC than adenocarcinoma with poor prognosis (Conde, et al., 2007). The strong functional correlation between upstream mediators of fatty acid synthesis and cancer underscores the importance of this pathway in tumor biology.

3. FASN REGULATION

3.1 FASN Regulation in Normal Cells

In nonmalignant tissues, FASN expression is primarily regulated at the transcriptional level (Figure 2A) (Hillgartner, et al., 1995). There is a single FASN gene and the signals in normal cells that stimulate FASN transcription are numerous but strictly defined (Amy, et al., 1990). Transcription of FASN is stimulated by dietary carbohydrate, glucose, insulin, amino acids, sterols and cyclic-AMP through specific response elements (Paulauskis and Sul, 1988,
Rufo, et al., 2001, Foufelle, et al., 1992, Moustaid, et al., 1994, Wang and Sul, 1998, Wakil, et al., 1983, Rangan, et al., 1996, Wakil, 1989). Hormones such as the thyroid hormone triiodothyronine (T3) (Moustaid and Sul, 1991), progesterone (Lacasa, et al., 2001), androgen (Heemers, et al., 2003) and adrenal glucocorticoids (Volpe and Marasa, 1975) can also upregulate FASN in liver and adipose tissues. FASN transcription is mediated by multiple transcription factors. Upstream stimulatory factors (USFs) are required for insulin mediation of FASN expression, but other factors such as nuclear factor Y (NF-Y) and stimulatory protein 1 (SP1) can also play a role in FASN transcription (Teran-Garcia, et al., 2007, Bennett, et al., 1995). However, the vast majority of FASN-regulatory signals act through a family of transcription factors known as sterol response element binding proteins (SREBPs) that control lipid homeostasis and bind to various elements in the FASN promoter. There are three SREBP family members: SREBP-1a, SREBP-1c, and SREBP-2. SREBP-1a and SREBP-1c have been most widely linked to regulation of lipogenic gene transcription, while SREBP-2 is most linked to cholesterol metabolism. The SREBPs exist as endoplasmic reticulum membrane bound precursors that are activated after proteolytic processing by site-one and site-two proteases (S1P, S2P). When sterol levels are low, S1P cleaves the SREBP molecule to release the N terminal portion from the endoplasmic reticulum (Sakai, et al., 1998). SREBP then binds to the SREBP cleavage activating protein (SCAP) and is translocated to the Golgi where S2P further processes the molecule so that the transcription factor is activated. The processed SREBP then translocates to the nucleus to bind specific E box motifs and sterol response elements (SREs) (Magana and Osborne, 1996). There is evidence that dietary factors stimulate the expression of FASN in a manner mediated through signaling pathways such as the PI3K pathway. For instance, nonmalignant 3T3-L1 adipocytes regulate insulin-mediated FASN expression through Akt in a
manner independent of both mitogen activated protein kinase (MAPK) and P70 S6 kinase, but dependent on SREBP1 (Wang and Sul, 1998, Porstmann, et al., 2005).

Expression of FASN is tightly controlled so that transcription does not continue unabated under typical circumstances. Polyunsaturated fatty acids (PUFAs) (Xu, et al., 1999, Moon, et al., 2002, Jump, et al., 1994), sterols (Adams, et al., 2004, Bennett, et al., 1995), and leptin (Fukuda, et al., 1999) all act to repress FASN transcription and do so by specifically down-regulating SREBP-1 in hepatocytes (Worgall, et al., 1998, Teran-Garcia, et al., 2007). This highly complex organization of checks and balances for FASN expression is necessary to supply the cell with essential de novo fatty acids for cellular function and growth (Figure 2A). Just as importantly, controls keep the cell from continuing unnecessary lipogenesis.

3.2 FASN Regulation in Tumor Cells

While FASN expression is tightly controlled through dietary and hormonal stimuli in nonmalignant cells, tumor cells ignore these restrictions and increase FASN beyond typical levels (Figure 2B). In fact, an early study of orthotopic hepatomas revealed that while low-fat, high-fat, and high-cholesterol diets all affected rates of fatty acid synthesis in the normal liver, the rates of hepatoma fatty acid synthesis were unchanged (Sabine, et al., 1967). It has since been discovered that deregulation of upstream signals drive FASN expression in a manner that is largely transcriptional in tumors (Figure 2B) (Swinnen, et al., 2006).

Overexpression of FASN in tumor cells is induced at the transcriptional level by receptor tyrosine kinase (RTK)-stimulation of Ras and Akt (Figure 2B). Keratinocyte growth factor (KGF) can induce the Akt- and cJun N-terminal kinase (JNK)-dependent expression of FASN in pulmonary cancer cells (Chang, et al., 2005). Epidermal growth factor (EGF) has also been shown to increase FASN in prostate cancer cells (Swinnen, et al., 2000a).
In addition to growth factor signaling, activation of the RTK HER2/Neu is linked with FASN expression in tumor cells. HER2/Neu upregulates PI3K-dependent FASN transcription in breast cancer cells (Kumar-Sinha, et al., 2003, Yoon, et al., 2007). Interestingly, blocking HER2/Neu with Herceptin decreases FASN expression (Kumar-Sinha, et al., 2003). In fact, there appears to be a crosstalk between these pathways, as inhibition of FASN activity leads to the downregulation of HER2/NEU (Menendez, et al., 2004). While HER2/Neu is primarily associated with breast cancer progression, HER2/Neu and FASN expression correlate in squamous cell carcinomas of the tongue, as well (Silva, et al., 2007). Surprisingly, HER2/Neu can also regulate FASN expression in prostate cancer cells (Yeh, et al., 1999). These data suggest there is a coordinate regulation of activated HER2/Neu and FASN upregulation in tumor cells.

Downstream of RTK signaling, the PI3K/Akt pathway has been shown to upregulate FASN. Loss of PTEN is a frequent transformation event in cancer, that leads to a gain of function in Akt signaling (Mulholland, et al., 2006, Blanco-Aparicio, et al., 2007). In prostate cancer cells, this signaling cascade drives androgen receptor (AR)-mediated oncogenic transcription and progression to metastatic disease (Wang, et al., 2003, Mulholland, et al., 2006). The PTEN-null LNCaP tumor cell line has high levels of FASN. Reintroducing PTEN or using the PI3K inhibitor LY294002 can decrease FASN expression, whereas introducing constitutively active Akt can restore FASN expression (Van de Sande, et al., 2002). The connection between FASN expression and PI3 kinase activity is further observed in prostate carcinoma samples with high Gleason scores, where high FASN expression correlates with phosphorylated Akt that is localized to the nucleus (Van de Sande, et al., 2005). Moreover, a crosstalk between these pathways has been identified. In ovarian cancer cell lines, phosphorylated Akt correlates with and drives FASN expression. Conversely, inhibiting FASN results in decreased Akt
phosphorylation (Wang, et al., 2005). These data suggest that PI3K signaling through Akt is an important mediator of FASN transcription in tumor cells.

In addition to RTK-driven stimulation of Akt, there is evidence that the small GTP-ase protein Ras can influence FASN expression in tumors. Constitutively active H-ras induces increased PI3K and MAPK-dependent FASN expression in MCF-10A cells (Yang, et al., 2002). Consistent with this notion, the expression of activated K-ras correlates with FASN expression in human colorectal cancer samples (Ogino, et al., 2006, Ogino, et al., 2007). Altogether, these data suggest that RTK signaling, the Ras and PI3K-Akt pathways can drive transcriptional up-regulation of FASN expression in tumor cells (Figure 2B).

Not surprisingly, hormones are another common factor driving FAS expression in tumor cells (Figure 2B). Progestins stimulate FASN expression in breast cancer cells (Chalbos, et al., 1987, Lacasa, et al., 2001, Menendez, et al., 2005a). Consistent with this finding, increased FASN expression in endometrial carcinoma correlates with expression of both estrogen and progesterone receptors (PR) (Pizer, et al., 1998b). In prostate cancer, FASN expression can be regulated by androgens in prostate cancer through upregulation of transcription factors such as S14 and SREBPs (Swinnen, et al., 1997a, Swinnen, et al., 1997b, Heemers, et al., 2000, Heemers, et al., 2001). In addition, HER2/Neu can drive activation of AR in prostate cancer cells to increase MAPK-dependent induction of FASN in the absence of androgen (Yeh, et al., 1999).

While the main mechanism of FASN overexpression in tumors is through transcriptional upregulation, there is also evidence that FASN is regulated by post-transcriptional mechanisms (Figure 2B). For instance, HER2/Neu driven expression of both FASN and ACC can be regulated at the translational level through Akt, PI3K, and mTOR-dependent mechanisms (Yoon, et al., 2007). FASN stabilization is tightly linked with the de-ubiquitinating enzyme ubiquitin-specific protease 2a (USP2A) in prostate cancer cells. USP2A is androgen regulated and is not
only upregulated similarly to FASN, but actually interacts with FASN to enhance FASN stability (Graner, et al., 2004). Treating prostate tumor cells with the proteasome inhibitor MG-132, also increases FASN expression, further supporting evidence that FASN is regulated by the proteasome (Graner, et al., 2004). Interestingly, yeast studies provided early evidence of FASN regulation by proteasomal degradation (Egner, et al., 1993). It is also worth mentioning that FASN can also be upregulated in cancer cells by FASN gene amplification (Shah, et al., 2006). The fact that numerous mechanisms act to increase FASN expression in tumor cells highlights the importance of FASN in tumor progression.

3.3 Palmitate Utilization in Normal and Tumor Cells

Upregulation of FASN activity causes the increased production of fatty acids, particularly palmitate. While the mechanisms that drive FASN expression are different in tumors as compared to normal cells, the utilization of its products differs, as well. Fatty acids are used for a variety of cellular functions. In nonmalignant adipose and hepatic tissue, palmitate is incorporated into triglycerides for secretion and storage to be ultimately used as an energy source through β-oxidation (Thupari, et al., 2002). Fatty acids such as palmitate can also comprise a regulatory pool that activates energy mediators such as PPARα in the liver and hypothalamus (Chakravarthy, et al., 2005, Chakravarthy, et al., 2007). In addition, key signaling molecules, such as Ras and Hedgehog, can be palmitoylated to target these proteins to cellular membranes (Resh, 2006). So far, a link between protein palmitoylation and FASN activity has not been established though. In development, fatty acids can segregate into phospholipids to create cellular membranes (Chirala, et al., 2003). Similarly, tumor FASN-derived palmitate segregates into phospholipid microdomains known as lipid rafts (Figure 2B) (Swinnen, et al., 2003). Lipid rafts are involved in a number of key biological functions including signal transduction,
polarization, trafficking, and migration (Freeman, et al., 2005, Freeman, et al., 2007). Considering that palmitate can ultimately be used for a number of cellular processes, including being elongated and desaturated for subsequent events, it is apparent that FASN occupies an important niche in tumor cells.

4. INHIBITING FASN ACTIVITY

4.1 Small Molecule Inhibitors of FASN

Because of the unique expression of FASN in tumors, much emphasis has been put toward the development of pharmacological agents that inhibit FASN activity and, therefore, inhibit tumor growth and progression. Historically, a Cephalosporium caerulens mycotoxin metabolite known as cerulenin [(2S, 3R)-2,3-epoxy-4-oxo-7,10-dodecadienoylamide] has been the primary FASN inhibitor used in biological studies. Cerulenin covalently binds the β-ketoacyl synthase domain in FASN that is responsible for binding and condensing the substrates (Funabashi, et al., 1989). More recently, C75 was formulated as a synthetic analog of cerulenin due to instability and poor systemic availability of cerulenin (Kuhajda, et al., 2000). C93 is the newest generation of C75 analogues (Zhou, et al., 2007). Both C75 and C93 target the β-ketoacyl synthase activity of FASN (Kuhajda, et al., 2000, Zhou, et al., 2007). Recently, orlistat (Xenical©), a FDA-approved drug for obesity that targets gastrointestinal lipases, was described as a novel inhibitor of FASN thioesterase activity (Kridel, et al., 2004). There also exists a growing body of literature showing that various natural products such as the green tea polyphenolic component epigallocatechin-3-gallate (EGCG) can inhibit FASN activity (Tian, 2006).

4.2 Effects In vivo

### 4.3 Cell Cycle Effects *In vitro*

To determine the cellular consequences of FASN inhibition, numerous studies have focused on the *in vitro* anti-tumor effects of these inhibitors. Many studies have linked FASN inhibitors with cell cycle and growth arrest (Figure 3). Cerulenin acts *in vitro* to inhibit fatty acid
synthesis-mediated growth of breast carcinoma cells that can be rescued with palmitate (Kuhajda, et al., 1994b). Cerulenin induces a block at the G2/M cell cycle checkpoint in an androgen-independent prostate cancer cell line that correlates with an induction of cyclin-dependent kinase inhibitors p21 and p27 (Furuya, et al., 1997). However, glioma cells accumulate in S phase after cerulenin treatment (Zhao, et al., 2006). Different hepatocellular carcinoma cell lines treated with C75 undergo either G1 or G2 cell cycle arrest independent of p53 status (Gao, et al., 2006). In melanoma A-375 cells, cerulenin induces accumulation of cells in S phase, while C75 induces accumulation of G2/M phase cells (Ho, et al., 2007). RKO colorectal cancer cells treated with either cerulenin or C75 show a transient accumulation of cells in S and G2/M phases, but accumulation in G1 and G2/M phases later (Li, et al., 2001). Both cerulenin and C75 induce S phase arrest and inhibit DNA replication in breast, colorectal, and promyelocytic leukemia cancer cells (Pizer, et al., 1998a). Orlistat induces cell cycle arrest by downregulating Skp2, a deubiquinating enzyme, leading to decreased turnover of p27/kip1, therefore blocking prostate tumor cells from entering S phase (Knowles, et al., 2004). Orlistat has also been shown to induce an accumulation of breast cancer cells in S phase (Menendez, et al., 2005b). Use of RNAi to mediate knockdown of both the FASN and ACCα genes induces a decrease in S phase cells, further supporting the role of fatty acid synthesis in progression to or in S phase (Brusselmans, et al., 2005). The data show there is little consensus on the phase that tumor cells arrest growth after inhibition of FASN in various tumor cells, which may be attributed to different tumor cell types. It is likely that a lack of de novo fatty acid synthesis in tumor cells impacts on phospholipid synthesis required for proper DNA synthesis and cell cycle progression (Jackowski, 1994).

4.4 Cell Signaling Effects
The effects of FASN inhibitors are also mediated through key tumor signaling pathways. For example, it has been demonstrated that pharmacological inhibition of FASN activity results in reduced Akt activation in multiple tumor cell lines (Figure 3) (Wang, et al., 2005, Liu, et al., 2006). As mentioned previously, it has been demonstrated that PI3 kinase and Akt can drive FASN expression in tumor cells (Figure 2B) (Van de Sande, et al., 2002, Wang, et al., 2005). The demonstration that reduced FASN activity negatively effects Akt activation identifies a feedback between the two pathways. Not surprisingly, inhibiting the PI3K pathway synergizes with cell death induced by genetic and pharmacological inhibition of FASN (Bandyopadhyay, et al., 2005, Wang, et al., 2005, Liu, et al., 2006).

In addition to the PI3 kinase pathway, HER2/Neu has also been linked with FASN expression in breast and prostate cancer cells (Kumar-Sinha, et al., 2003, Yoon, et al., 2007, Yeh, et al., 1999). Inhibiting FASN with cerulenin and C75 reduces expression of Her2/neu expression in breast cancer cell lines (Figure 3) (Menendez, et al., 2004, Kumar-Sinha, et al., 2003). Additionally, inhibiting Her2/Neu with Herceptin synergizes with FASN inhibitors to induce cell death (Menendez, et al., 2004). Altogether, these data indicate that the very pathways that drive FASN expression in malignant cells are also affected when FASN activity is blocked. Moreover, tumor cell killing can be potentiated when FASN inhibitors are combined with inhibitors of these signaling pathways. The reason for this crosstalk has not been clearly defined, but it is tempting to speculate that inhibition of FASN activity directly impacts on lipid raft function, which results in reduced kinase signaling.

4.5 In vitro Tumor Cell Death

breast and prostate cancer cell death that correlates with DNA fragmentation and morphology characteristic of apoptosis (Pizer, et al., 1996a, Furuya, et al., 1997, Pizer, et al., 2000b). The mitochondria have also been linked to facilitation of cell death induced by cerulenin. For instance, the anti-apoptotic mitochondrial factor Bax rescues cerulenin-induced cell death when overexpressed (Heiligtag, et al., 2002). This correlation between cerulenin and the mitochondrial pathway of apoptosis is further supported by the induction of cytochrome c release (Figure 3) (Heiligtag, et al., 2002). FASN inhibition has been linked to p53 status of tumor cells, but whether p53 plays any role in FASN-expressing cells is unclear, as FASN is expressed in tumors independent of p53 status. FASN is strongly and significantly associated with p53 expression in hyperplastic parathyroids (Alo, et al., 1999a). In various cancer cells, blocking p53 activity with a dominant negative construct potentiates FASN inhibitor-induced cell death (Li, et al., 2001). Conversely, others have reported that FASN inhibitors work equally well in tumors independent of p53 status (Heiligtag, et al., 2002).

Cell death induced by FASN inhibitors could be a result of the cell lacking fatty acid for membrane biogenesis. Inhibiting FASN and ACC reduce incorporation of fatty acid into membrane phospholipids, which occurs in the endoplasmic reticulum (Zhou, et al., 2003). Inhibiting FASN incorporation into phospholipids corresponds to a decrease in cell volume and other morphological changes ultimately leading to apoptosis (De Schrijver, et al., 2003). Inhibiting FASN with small molecules cerulenin, C75, orlistat, or with siRNA induces endoplasmic reticulum stress and activation of the unfolded protein response (UPR) (Little, et al., 2007). The UPR is able to induce cell death if homeostasis is not restored and, therefore, FASN inhibitors may be inducing cell death that is mediated by the UPR (Figure 3) (Little, et al., 2007).
When FASN is inhibited malonyl-CoA accumulates (Pizer, et al., 2000a). One hypothesis for the mechanism of FASN inhibitor-induced cell death is attributed to this accumulation of malonyl-CoA and, potentially, its interaction with CPT-1, the enzyme responsible for transferring fatty acids into the mitochondria for oxidation. Malonyl-CoA acts as a natural inhibitor of CPT-1 activity so that fatty acids are not simultaneously synthesized and then oxidized (McGarry, et al., 1983). Driving this hypothesis is a study showing that co-treating breast or ovarian cancer cells with the ACC inhibitor 5-(tetradecyloxy)-2-furoic acid (TOFA) partially rescues cell death induced by FASN inhibitors C75 and cerulenin (Pizer, et al., 2000a, Zhou, et al., 2003). However, C75 alone can increase CPT-1 activity and directly compete with malonyl-CoA (Thupari, et al., 2002, Yang, et al., 2005). Therefore, it is important to note that MCF-7 cells co-treated with C75 and the CPT-1 inhibitor etomoxir show no effect on C75-induced cell death (Zhou, et al., 2003). Hence, malonyl-CoA accumulation, not CPT-1 activation, is mediating death induced by FASN inhibitors (Figure 3). In addition, siRNA-mediated knockdown of FASN induces accumulation of ceramide and malonyl-CoA that leads to inhibition of CPT-1 and induction of apoptotic genes BNIP3, TRAIL, and DAPK2 (Bandyopadhyay, et al., 2006).

Upstream lipogenesis mediators ACL and ACC are also important in maintaining tumor cell survival. RNAi-mediated knockdown or chemical inhibition of ACL in human tumor cells decreases proliferation and induces cell death in vitro and limits tumor growth by stimulating differentiation of tumor cells in vivo (Hatzivassiliou, et al., 2005). ACL inhibition can also can impair Akt-mediated tumorigenesis of tumor cells and induce cell death (Bauer, et al., 2005). In addition, silencing ACC using RNA interference, breast and prostate cancer cells undergo apoptosis (Brusselmans, et al., 2005, Chajes, et al., 2006). Chemical inhibition of ACC can also induce tumor cell death (Beckers, et al., 2007). While the effects of FASN inhibitors on tumor
cells are clearly pleiotropic, and in some cases maybe even specific to the tumor type, it is evident that many of the effects can ultimately be tied to decreases in de novo synthesized fatty acids which can be extended to phospholipid synthesis. Whatever the mechanisms may be, the data clearly suggest that FASN occupies an important regulatory position in tumor cells to facilitate the processes that lead to tumor cell proliferation and survival.

5. CONCLUDING REMARKS

In summary, FASN is upregulated in multiple tumor types and correlates with poor patient prognosis and reduced survival. Correspondingly, a body of literature has demonstrated a requirement of FASN activity for tumor cell viability. Phospholipids synthesized from FASN-derived palmitate are important for cell cycle progression, lipid raft signaling, and endoplasmic reticulum homeostasis, all of which contribute to tumor cell survival, thereby, underscoring the importance of FASN. These findings signify a central role for fatty acid synthesis in critical cellular processes. In addition, tumor cells have developed feedback mechanisms to mediate crosstalk between FAS and signaling pathways like PI3-kinase and Her2/Neu. The discovery and development of pharmacological agents that block FASN activity suggest that FASN can be targeted for anti-tumor therapy. So far, anti-FASN drugs have successfully inhibited tumor growth in several tumor models with minimal side effects. Therefore, FASN represents a highly tractable anti-tumor target with significant clinical potential.

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Figure Legends

Figure 1: The FASN Enzyme
A. The FASN polypeptide comprises seven functional domains: the ketoacyl synthase (KS), malonyl acetyl transferase (MAT), β-hydroxyacyl dehydratase (DH), enoyl reductase (ER), β-ketoacyl reductase (KR), the acyl carrier protein (ACP), and thioesterase (TE) domains. B. The FASN reaction mechanism. The MAT domain of the enzyme binds malonyl-CoA and acetyl-CoA, while the KS domain acts to condense the growing acyl chain. The resulting β-ketoacyl moiety is then reduced in steps by the KR, DH, and ER to a saturated acyl intermediate. This process is repeated in seven cycles when the TE domain releases the sixteen carbon fatty acid palmitate.

Figure 2: Regulation of FASN Expression in Normal and Tumor Cells.
A. In normal cells (hepatocytes and adipocytes) FASN expression through multiple stimuli is primarily regulated through transcriptional mechanisms. B. In tumor cells, FASN expression is regulated by transcriptional and non-transcriptional mechanisms via multiple pathways.

Figure 3: Inhibiting FASN in Tumor Cells.
Several small molecule drugs can inhibit FASN activity. Blockade of FASN activity leads to a reduction in lipogenesis and phospholipid content in tumor cells. Inhibiting FASN also induces cycle arrest, cytochrome c release, and endoplasmic reticulum stress. In addition, FASN inhibitors can reduce the activation and expression of Akt and HER2/Neu.
A.

N → KS → MAT → DH → ER → KR → ACP → TE → C

B.

- acetyl-CoA + 7 · CO₂ → 8 · CoA + 7 · CO₂
- 7 · malonyl-CoA → 7 · NADPH
- palmitate → 7 · NADPH
- 6 · H₂O

START

TERMINATION

ELONGATION
A. transcriptional

- glucose
- PUFAs
- insulin

SREBP

\( \rightarrow \)

FASN

\( \rightarrow \)

palmitate

\( \downarrow \)

triglycerides

PPAR\( \alpha \)

\( \rightarrow \)

phospholipid

Normal cell

B. transcriptional post-transcriptional

- growth factors
- dietary stimuli

(EGFR, HER2/Neu, KGF2)

PTEN

\( \rightarrow \)

p-Akt

\( \rightarrow \)

Akt, mTOR

LY294002

H-Ras

\( \rightarrow \)

FASN

\( \rightarrow \)

palmitate

\( \downarrow \)

phospholipid

\( \downarrow \)

lipid rafts

Normal cell

Normal cell

MG-132
FASN

⇒ palmitate

⇒ phospholipid

- cell cycle arrest
- cytochrome c release
- endoplasmic reticulum stress
- p-Akt
- HER2/Neu

⇒ cell death