Selective Inhibition of Fatty Acid Synthase for Lung Cancer Treatment

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Abstract

Purpose: Fatty acid synthase (FAS) is overexpressed in many human cancers and is considered to be a promising target for therapy. However, in vitro use of previous generations of FAS inhibitors has been limited by severe, but reversible, anorexia in treated animals, which is thought to be related to a parallel stimulation of fatty acid oxidation by these agents. This study investigated pharmacologic inhibition of FAS using C93, a rationally designed molecule that inhibits FAS activity without affecting fatty acid oxidation in preclinical models of lung cancer.

Experimental Design: Activity of C93 on FAS and fatty acid oxidation was evaluated in cultured non–small cell lung cancer (NSCLC) cells. Antineoplastic activity of the compound, given orally or by i.p. injection, was evaluated in s.c. and orthotopic NSCLC xenografts.

Results: Our experiments confirm that C93 effectively inhibits FAS without stimulating fatty acid oxidation in lung cancer cells. More importantly, C93 significantly inhibits the growth of both s.c. and orthotopic xenograft tumors from human NSCLC cell lines without causing anorexia and weight loss in the treated animals.

Conclusions: We conclude that inhibition of FAS can be achieved without parallel stimulation of fatty acid oxidation and that inhibition of tumor growth in vivo can be achieved without anorexia and weight loss. Thus, this therapeutic strategy holds promise for clinical treatment of cancers, including non–small cell lung cancer, the leading cause of cancer mortality in the United States and Europe.

Fatty acid synthase (FAS) is highly expressed in many human cancers (1–3), and previous laboratory studies have shown that cancer cell growth can be suppressed by inhibiting the activity of this enzyme with cerulenin (a natural antibiotic; ref. 4), small interfering RNA specific for the FAS gene transcript (5), orlistat, a pancreatic lipase inhibitor developed for obesity treatment (6), or C75, a stable synthetic small-molecule developed specifically for inhibiting FAS (4, 7). However, efforts to treat xenograft cancers with C75 (8–10) have been hampered by transient, but severe, anorexia and weight loss caused by drug treatment, an effect that could also limit the use of this compound in the clinical setting (11). C75 is a mimetic of malonyl-CoA, and in addition to inhibiting FAS, C75 stimulates fatty acid oxidation [most likely by activating carnitine O-palmitoyltransferase-1 (CPT1); ref. 12]. This, in turn, seems to contribute to the reduction of neuropeptide Y expression in the hypothalamus (11, 12). Based on these findings, it would seem that the limiting toxicity of C75 is due to this stimulation of fatty acid oxidation rather than the inhibition of FAS.

The present study extends the investigation of FAS as a potential target for treatment of human cancer in two important ways. First, we evaluated a new small-molecule inhibitor of FAS, C93, which was designed to specifically inhibit FAS without affecting CPT1 activity (13). This allowed us to determine whether antineoplastic activity, without anorectic effects, can be achieved by selective pharmacologic inhibition of FAS without stimulation of CPT1. Second, we examined this drug as a potential treatment of human lung cancer, the leading cause of cancer-related deaths in the United States and Europe (14). This cancer type has been previously unexplored as a target for therapy with this class of agents.

Materials and Methods

Lung cancer tissues and immunohistochemistry. We evaluated FAS expression in 181 human lung cancer tissues (94 squamous cell carcinoma, 72 adenocarcinoma, and 15 large cell carcinoma) and normal bronchi from 24 patients, selected from the pathology archives of the Johns Hopkins Hospital. Cancer samples were represented on tissue microarrays, with three core samples (0.05 mm in diameter) for...
each case. Immunohistochemistry of FAS expression was done on these tissues and on xenograft tissues using a 1:2,000 dilution of clone 6E7 antisera (FASgen) for a 1-h incubation at room temperature. Specific antibody reactivity was then visualized using biotinylated secondary antibody followed by peroxidase-labeled streptavidin (LSAB+ System-HRP, DakoCytomation) and incubation with substrate-chromogen solution according to the manufacturer’s instructions.

**Cell culture.** Human lung cancer cell lines A549, H460, and H1975 (American Type Culture Collection) were cultured in recommended medium at 37°C/5% CO₂. LX7 cells, isolated from a pleural effusion

![Fig.1. Increased FAS expression and activity in human lung cancer tumors and cell lines. A, representative immunohistochemical staining for FAS protein in pulmonary squamous cell carcinoma (left), adenocarcinoma (middle), and normal bronchus (right). The normal bronchus and adenocarcinoma shown in this figure are from the same case. B, immunohistochemical staining of xenograft tumors for human lung cell lines (H460, A549, and H1975). As shown in (C), FAS enzymatic activity is higher in the A549 and H460 lung cancer xenograft tissues than in host liver. Activity in xenograft tissues is normalized to that in liver for given experiment. Columns, means; bars, SE. Significance was determined by unpaired t test.](image)

| Table 1. FAS expression in human lung cancer tissues |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| FAS expression | Normal bronchus | Squamous cell carcinoma | Adenocarcinoma | Large cell carcinoma |
| 0 | 11 | 1 | 5 | 0 |
| 1 | 13 | 4 | 9 | 2 |
| 2 | 0 | 18 | 11 | 2 |
| 3 | 0 | 33 | 27 | 2 |
| 4 | 0 | 38 | 20 | 9 |

NOTE: Staining patterns for 181 cases of primary lung cancer and normal bronchus distant from tumors (n = 24). Tissue arrays were stained with 6E7 monoclonal antibody and scored using standard methods (3 and 4 designate strong staining in >50% or >90% of all cells, respectively, and 2 designates moderate to strong staining in at least 20% of cells). Normal bronchus stains 0 to 1+ with this antibody [comparing mean scores for each tissue group shows highly significant differences between normal bronchus and each of the cancer groups (P < 0.001 by unpaired t test), but no significant differences are noted among the various groups of lung cancer].
of a patient with pulmonary adenocarcinoma, were provided by Drs. Neil Watkins and Malcolm Brock (Johns Hopkins University, Baltimore, MD) and grown in RPMI 1640 supplemented with 10% fetal bovine serum. All cells were screened periodically for Mycoplasma contamination.

**Immunoblot analysis.** For analysis of FAS protein levels in cultured cells, samples were collected in lysis buffer [50 mmol/L Tris-Cl (pH 7.0), 1 mmol/L EDTA, 1% Triton X-100] and lysates were clarified by centrifugation at 14,000 rpm at 4°C for 15 min. Protein concentration was determined by bovine serum albumin assay (Pierce), and electrophoresis was then done using 50 μg protein from each sample on 4% to 15% gradient Tris-HCl gels. Proteins were then transferred to Trans-Blot membranes (Bio-Rad) and incubated with mouse 6E7 monoclonal anti-FAS antibody at a 1:106 dilution overnight for 4°C. Membranes were also probed with rabbit anti-actin (Sigma) at 1:2,500 dilution (for loading control) and with anti-mouse (Bio-Rad) or anti-rabbit (Sigma) secondary antibodies. Membranes were developed using SuperSignal West Femto Max Sensitivity Substrate (Pierce).

**Human lung cancer xenografts.** For s.c. xenografts, 10^6 lung cancer cells were implanted into s.c. tissue of anesthetized (xylazine/ketamine) nude mice using a 25 μL Hamilton syringe. Treatment with C93 (50 mg/kg/d, five times weekly, by i.p. injection, or 50 mg/kg, twice daily, orally) was initiated when tumors were palpable (typically 5-7 days after inoculation). Tumor nodules were measured in two dimensions using calipers on an approximately weekly basis, and tumor volumes were estimated using longitudinal and transverse measurements. Mice were euthanized and tumors were removed for examination when any animal in an experiment displayed tumor-related distress. Tumor growth was assessed at the end of the experiment by processing lungs for histology and examining whole-mount cross-sections. Tumor tissue were harvested from mice at 4, 12, 24, and 48 h after treatment (three mice per treatment group). Three samples of tissue from each mouse (300 mg each) were then transferred into individual culture wells, minced, and incubated for 2 h in medium with 1 μCi of [14C]acetate at 0.25 mCi (Sigma). Lipids were extracted from tissues, radioactivity was measured by scintillation counting, and counts were normalized to tissue weights.

Fatty acid oxidation was measured as the degradation of [14C]palmitate into acid-soluble products using methods described previously (16). In brief, H460 cells were plated at 2.5 × 10^4 per well in 24-well plates and incubated for 1 h with C75 or C93. Then, 100 μmol/L of [14C]palmitate in cyclodextran and 200 μmol/L carnitine were added to each well and incubated for an additional 30 min before extracting acid-soluble products for quantifying by scintillation counting.

**Treatments with inhibitors of FAS.** For cell culture and i.p. injection, C75 and C93 (FASgen) were dissolved in DMSO at a concentration of 50 mg/mL. For oral administration, C93 was dissolved in 80% ethanol, 10% Tween 20, and 10% polyethylene glycol at a concentration of 50 mg/mL and 1 μL/g body weight of this solution (or drug-free diluent) was given orally to alert animals by micro-pipette.

**Measuring FAS activity and fatty acid oxidation.** To measure FAS activity in cultured cells, 5 × 10^3 cells per well (24-well plates) were exposed to C93 for 2 h in serum-free medium and then pulse labeled for an additional 2 h with 1 μCi [14C]acetate (Amersham). Lipids were extracted and [14C]acetate incorporation into fatty acids was measured using a scintillation counter. To monitor effect of treatment on FAS activity in xenograft tumors, mice were given 50 mg/kg C93 (or DMSO only) by i.p. injection 21 days after implanting tumor cells. Liver and tumor tissue were harvested from mice at 4, 12, 24, and 48 h after treatment (three mice per treatment group). Three samples of tissue from each mouse (300 mg each) were then transferred into individual culture wells, minced, and incubated for 2 h in medium with 1 μCi of [14C]acetate at 0.25 mCi (Sigma). Lipids were extracted and [14C]palmitate in cyclodextran and 200 μmol/L carnitine were added to each well and incubated for an additional 30 min before extracting acid-soluble products for quantifying by scintillation counting.
Results

Increased FAS expression and activity in human lung cancer tumors and cell lines. Lung cancer is an important problem worldwide, and this study is the first to explore the possible treatment of lung cancer with pharmacologic inhibitors of FAS. Two previous publications indicate that FAS is highly expressed in some lung cancers (17, 18), and as shown in Fig. 1A and summarized in Table 1, we verify that the great majority of human non–small cell lung cancers, of various histologic types, express significantly increased levels of the FAS protein compared with normal lung bronchial epithelial tissues. For the 181 cases of lung cancer that we examined, 88.4% exhibited 2 to 4+ positivity with 71% strongly (3+ to 4+) positive. No relationship between FAS expression and survival was noted (data not shown). The high level of FAS expression is also seen in cultured human lung cancer cell lines grown as xenografts in athymic nude mice (Fig. 1B), indicating that xenografts of human lung cancer cell lines resemble primary tumors in the expression of this protein.

To confirm the enzymatic activity of FAS in lung cancer, we measured incorporation of radiolabeled acetate into free fatty acids using extracts from human lung cancer xenografts and rodent host liver tissues (Fig. 1C). Liver is the tissue that normally has the highest levels of FAS activity in the body, but our experiments showed that activity in the xenograft tissues is ~5-fold greater than those measured in liver. Thus, FAS is both highly expressed and enzymatically active in human lung cancer xenografts, and these represent suitable models to test the effects of treatments that target this pathway in lung cancer.

C93, a novel compound, inhibits FAS enzymatic activity without affecting fatty acid oxidation and without inducing weight loss. The use of C75, a first-generation synthetic compound that inhibits FAS, is hindered by severe anorexia and weight loss in treated animals. This anorexia seems to result from parallel stimulation of fatty acid oxidation, which in turn may contribute to the suppression of neuropeptide Y expression in the central nervous system (11, 12). To address this problem of anorexia induced by treatment with C75, new compounds were designed to inhibit fatty acid synthesis without parallel stimulation of fatty acid oxidation. As shown in Fig. 2, C93 inhibits FAS in human lung cancer cell cultures at dose ranges comparable with those of C75. This inhibition of enzymatic activity by C93 is not a result of decreased FAS protein levels, as shown by immunoblot analysis shown in Fig. 2B. Importantly, the specificity of C93 for FAS is supported by our finding that this compound does not cause a parallel stimulation of fatty acid oxidation, which is a function of CPT1 activity. As shown in Fig. 2C, no significant stimulation of fatty acid oxidation is seen in H460 lung cancer cells treated with C93 at any dose level, and some inhibition of fatty acid oxidation occurs at levels higher than those used to inhibit fatty acid synthesis. This contrasts to the ~80% increase in fatty acid oxidation seen after treatment with 10 μg/mL C75. Thus, C93 seems to be as potent as C75 for inhibiting FAS in cancer cells, but unlike C75, this activity is not accompanied by significant stimulation of fatty acid oxidation.

Reasoning that anorexia is due to a physiologic response to increased fatty acid oxidation, this increased specificity would be expected to result in less anorexia after treatment with C93 than after C75. Our experiments confirmed these expectations; as seen in Fig. 2D, no weight loss is seen in mice treated with C93 at levels comparable with those that cause anorexia and weight loss in mice treated with C75.

C93 inhibits tumor growth in nude mice with s.c. or orthotopic human lung cancer xenografts. To explore the potential effectiveness of C93 for lung cancer treatment in vivo, we treated athymic nude mice with human lung cancer xenografts. For our initial experiments, we gave the compound by i.p. injection, which effectively reduces FAS activity in H460 tumor

![Image of graphs and figures](https://example.com/image.png)
xenograft tissues in a time-dependent manner (Fig. 3A). S.c. xenografts were then established for four different lung cancer cell lines, and treatment was initiated after tumor nodules became palpable (5-7 days) and continued (50 mg/kg/d, five times weekly) until morbidity (e.g., tumor ulceration) was observed in untreated animals. As shown in Fig. 3, significant inhibition of tumor growth was noted for each of these types of lung cancer xenografts (Fig. 3B shows representative mice with H460 xenografts, and Fig. 3C summarizes the results for s.c. xenografts from four cell lines).

We also treated animals with orthotopic xenografts of the H460 cell line, beginning 7 days after inoculation. In this experiment, lungs were removed and processed for histologic examination at the termination of the experiment, which was determined by respiratory distress in untreated mice. Figure 4A shows histologic cross-sections of lungs removed from representative treated and control mice, showing significantly reduced growth of the H460 xenograft tumors in the treated animal. All of the animals in the treatment group did have some tumors, although four of the six treated mice had only a few-minute tumor nodules, each measuring <1 mm in diameter. Figure 4B summarizes measurements of the aggregate tumor sizes in the lungs of all animals in this experiment, showing a highly significant reduction in tumor growth by C93 treatment.

Orally given C93 effectively treats lung cancer xenografts. Because repeated i.p. injections carry a risk of infection and inflammation, we also explored the possibility of an oral administration of the compound. We used animals bearing s.c. xenografts of lung cancer cell lines for these studies, which allowed removal of tumor tissues for analysis of enzymatic activity as well as measurements of tumor growth. As shown in Fig. 5A, orally given compound effectively inhibits FAS activity in the xenograft tumor tissues with similar efficacy to that seen after i.p. administration. The half-life of this activity seems to be ~12 h, and based on this finding, we conducted experiments using 50 mg/kg C93, given orally every 12 h (and thus increasing the total daily dose to 100 mg/kg/d), to treat lung cancer xenografts established from the H460 and A549 lung cancer cell lines. As summarized in Fig. 5B, this oral administration of C93 effectively inhibited growth of xenografts for both cell
lines. No significant weight loss or other toxicity was observed in the treatment groups (data not shown).

Discussion

Distinctly high levels of FAS expression in various human cancers have attracted considerable interest as a target for therapy. Our study shows that non–small cell lung cancer, the leading cause of cancer death in the industrialized world, is among the foremost of cancers that could potentially be treated by FAS therapy. More than 70% of the lung cancers that we examined have very high levels of FAS expression, and we also confirm that FAS activity is high in xenografts of human lung cancer cells with levels of expression that are similar to those seen in primary lung cancers.

A major barrier to targeting FAS for all types of cancer has been the unavailability of highly specific pharmacologic agents. A first-generation inhibitor, C75, effectively inhibits the activity of FAS and reduces tumor growth, but dosing of this agent is limited by parallel stimulation of fatty oxidation, which leads to anorexia. Thus, a significant finding reported in this article is that C93, a second-generation inhibitor of FAS, does not stimulate fatty acid oxidation and does not cause anorexia. This improvement in toxicity profile results in the ability to dose animals at frequent intervals (twice daily in our study) and effective treatment of lung cancer xenografts without recognizable toxicity.

Remarkably, the role of increased FAS in the neoplastic phenotype and the mechanism(s) of cell killing by inhibitors of FAS are still incompletely understood. Although it might seem that cell killing due to FAS inhibition could be related to reduction of available fatty acids, variable results have been reported for experiments that inhibit acetyl-CoA carboxylase, the rate-limiting enzyme of fatty acid synthesis upstream of FAS. Some experiments, for example, suggest that acetyl-CoA carboxylase (ACC) is essential for cancer cell survival (19), whereas others find that pharmacologic inhibitors of ACC are far less toxic than FAS inhibitors for cancer cells and, in some situations, actually protect cancer cells from FAS inhibition (20, 21). Thus, accumulation of intermediate metabolite(s), such as malonyl-CoA, could be responsible for the toxicity rather than depletion of end products of the pathway.

The effect of such an intermediate metabolite of fatty acid synthesis on cancer cells is likely mediated through cell signaling pathways. For example, inhibiting FAS in ovarian cancer cells decreases the level of activated Akt (10) and suppresses HER2 overexpression (22) in breast cancer cells. In cultured prostate cancer cells, FAS inhibitors (C75, orlistat) result in increased PERK-dependent phosphorylation of the translation initiation factor eIF2α and concomitant inhibition of protein synthesis (23). Furthermore, PERK-deficient mouse embryonic ras-transformed fibroblasts and HT-29 colon cancer cells with a dominant-negative PERK were found to be more sensitive to FAS inhibitor-induced cell death than their wild-type counterparts, and increased cell killing was also observed when FAS inhibitors were combined with the endoplasmic reticulum stress inducer thapsigargin. These results were interpreted to indicate that endoplasmic reticulum stress is important in the mechanism of FAS inhibitors and that PERK function contributes to an adaptive response in tumor cells when FAS activity is inhibited. Several metabolic changes are also triggered by inhibiting FAS, and these changes seem to have involvement in the cytotoxicity. For instance, treatment with pharmacologic inhibitors of FAS resulted in activation of AMP-activated protein kinase in both neurons (24) and ovarian cancer cells (25), in parallel with an increase in the AMP/ATP ratio in these cells. Furthermore, pretreatment of ovarian cancer cells with compound C, an AMP-activated protein kinase inhibitor, substantially rescues cells from toxicity of FAS inhibition, suggesting that the toxicity is largely dependent on this AMP-activated protein kinase activation (25).

The present study addresses the complex issue of whether changes in CPT1 activity (and fatty acid oxidation) mediate, in whole or in part, the antineoplastic effects of agents intended to target FAS. Previous studies provide indecisive evidence on this issue. For example, cerulenin causes parallel inhibition of FAS and CPT1 (26), and inhibition of FAS by small interfering RNA also reportedly leads to inhibition of CPT1 (27), suggesting that these pathways are intimately linked to each other and also possibly to the antineoplastic activity of these agents. Some of this inhibition of CPT1 is expected as a result of accumulation of malonyl-CoA following inhibition of FAS (28). However, C75, an inhibitor of FAS with compelling data showing efficacy for treatment of several xenograft models of cancer, stimulates, rather than inhibits, CPT1-mediated oxidation of long chain fatty acids (12). These disparate changes in CPT1 activity observed among various agents would alone suggest that inhibition of FAS, rather than either stimulation or inhibition of CPT1, is the most important target for cancer therapy, and our experiments support that case. C93, designed to have greater specificity for FAS, is effective for treatment of human cancer xenografts at doses that inhibit FAS but have no significant effect on fatty acid oxidation. Importantly, for development of a cancer treatment, C93 does not cause anorexia or weight loss (apparently a side effect of CPT1 stimulation) at doses that are effective for antineoplastic activity. Thus, C93 and related agents represent promising new treatments for cancer.

Thus, the work reported here supports the development of FAS inhibitors for clinical use in lung cancer treatment. Human lung cancers have high levels of FAS protein and enzymatic activity, and inhibiting this enzyme can result in significant reduction in tumor burden in animals with human lung cancer xenografts. Moreover, our results indicate that antineoplastic effects of FAS inhibitors are apparently independent of effects on fatty acid oxidation, and thus, effective treatment of cancer by FAS inhibitors is possible without the side effect of anorexia.

References

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