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Novel Therapy for Breast Cancer

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13. ABSTRACT (Maximum 200 Words)

This grant proposed to study the effect of fatty acid synthesis inhibition in human breast cancer xenografts using C75, a novel inhibitor of fatty acid synthesis. We also proposed to study the mechanism of cell death by C75 and the effect of dietary fatty acids on this model. The purpose of this study is to demonstrate that the fatty acid synthesis is a novel pathway for breast cancer therapy development. We found that C75 inhibited fatty acid synthase (FAS) and had significant anti-tumor activity in both human breast cancer and human mesothelioma xenografts. In addition, we determined that malonyl-CoA is likely the trigger of apoptosis during FAS inhibition in cancer cells. During treatment of animals with C75, we noted significant reversible weight loss. Mechanistic studies of the weight loss showed that FAS inhibition leads to reduced neuropeptide-Y production in the hypothalamus. Thus, we have found that inhibition of FAS in mammals has a significant anti-tumor effect. In addition, FAS inhibition can cause weight reduction and correction of Type II diabetes in obese (ob/ob) mice.

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Table of Contents

INTRODUCTION: This grant proposed to study the effect of fatty acid synthesis inhibition in human breast cancer xenografts using C75, a novel inhibitor of fatty acid synthesis. We also proposed to study the mechanism of cell death by C75 and the effect of dietary fatty acids on this model. The purpose of this study is to demonstrate that the fatty acid synthesis is a novel pathway for breast cancer therapy development. Since no effective fatty acid synthesis inhibitors exist, we are utilizing C75 which we have developed as the first synthetic, chemically stable inhibitor of mammalian fatty acid synthase. In addition, to testing the effects of C75 against breast cancer xenografts, we are also studying the mechanism of action of C75 in human cancer cells. As we will show during the last four years we have made significant progress toward these goals and have made fundamental observations concerning the mechanism of cancer cell death from fatty acid synthesis inhibition. We now understand that inhibition of fatty acid synthase kills cancer cells through the generation of superphysiological levels of malonyl-CoA which selectively toxic to cancer cells.

BODY:
1. Fatty acid synthesis inhibition kills human breast cancer cells through the generation of toxic levels of malonyl-CoA (See reprint in Appendix).
As I reported one year ago, recent data from Dr. Ellen Pizer showed that pharmacological inhibition of mammalian fatty acid synthase activity lead to inhibition of DNA replication within about 90 minutes of drug application. While generating a great deal of interest, the question of how inhibition of fatty acid synthase triggers this phenomenon remained unknown.

We have made a significant breakthrough linking fatty acid synthase inhibition with breast cancer cell apoptosis. In the enclosed reprint of our paper published in *Cancer Research*, we demonstrate that inhibition of FAS leads to high levels of malonyl-CoA which occurs within 30 minutes of C75 treatment. These superphysiological levels of malonyl-CoA, *not low levels of endogenously synthesized fatty acids,* are responsible for breast cancer cell apoptosis. This finding furthers our understanding of the mechanism of fatty acid synthesis inhibition and cell death. In addition, this is a novel pathway which leads to selective apoptosis of cancer cells. In addition to its role as a substrate for FAS, malonyl-CoA acts at the outer mitochondrial membrane to regulate fatty acid oxidation by inhibition of carnitine palmitoyltransferase 1 (CPT-1). Inhibition of CPT-1 has been shown to sensitize cells to fatty acid induced apoptosis (1); CPT-1 may also interact directly with BCL-2, the anti-apoptosis protein, at the mitochondria (2). We hypothesize that FAS inhibition leads to high levels of malonyl-CoA inhibiting CPT-1 which induces cancer cell apoptosis. Without high levels of FAS, normal cells will not be affected by this therapeutic strategy.

This paper also has the latest xenograft experiment with C75 demonstrating a significant inhibition of cancer cell growth *in vivo.* It provides the necessary validation of the preliminary xenograft study documented in the 1998 report.

In summary, this paper identifies a novel means to trigger apoptosis in cancer cells through inhibition of fatty acid synthase and the demonstration of its effectiveness *in vivo.*

Relationship of Studies to the Statement of Work: These studies complete the i.p. model as outlined in Task 5. In addition, it completes analysis of the fatty acid synthesis pathway activity in breast cancer cells *in vitro* and *in vivo* as outlined in Tasks 6 and 7. The discovery of a novel

4

apoptotic pathway was unanticipated in the original application, but it provides important details of the mechanism of action of C75 and other future FAS inhibitors.

2. *Human breast cancer cells have a radically altered distribution of Coenzyme A derivatives in vivo*. Since the acceptance of the paper to *Cancer Research* we have continued to make progress as we can now measure malonyl-CoA levels in xenograft tissues and liver. Figure 1 demonstrates that the MCF-7 human breast cancer xenograft has markedly elevated levels of malonyl-CoA compared to mouse liver. In addition, the distribution of other CoA derivatives are markedly altered. For example, while liver has about 5-6 fold less malonyl-CoA compared to the xenograft, it has about 4 fold higher levels of acetyl-CoA. These data indicate significant energy metabolism alteration in human cancer cells which merits further investigation. Figure 1B illustrates the quantitative differences in malonyl-CoA levels between the tumor tissue and mouse liver. The methods used for extraction and quantitation of CoA derivatives was the same as in the reprint (See Appendix).
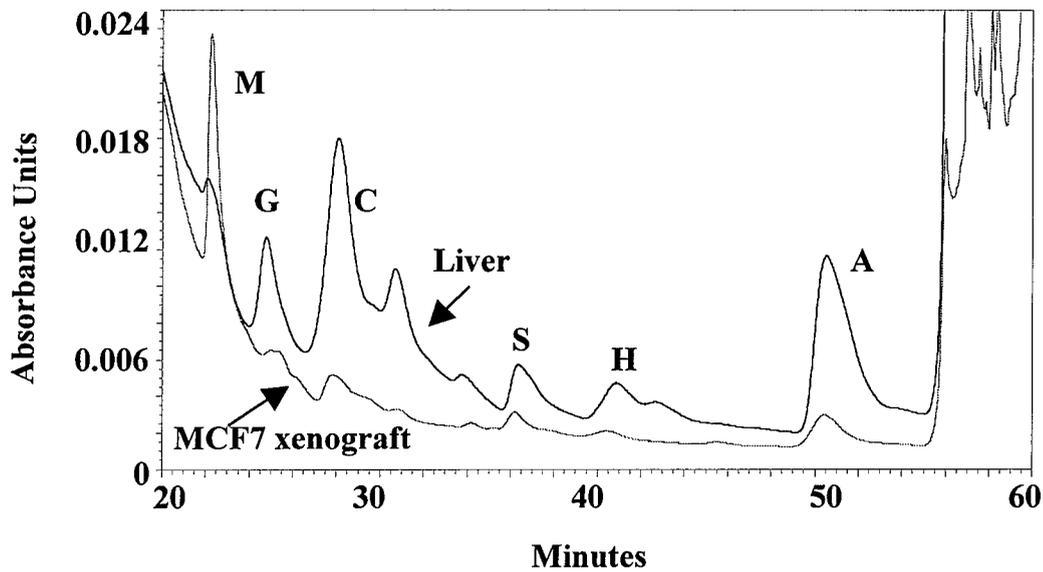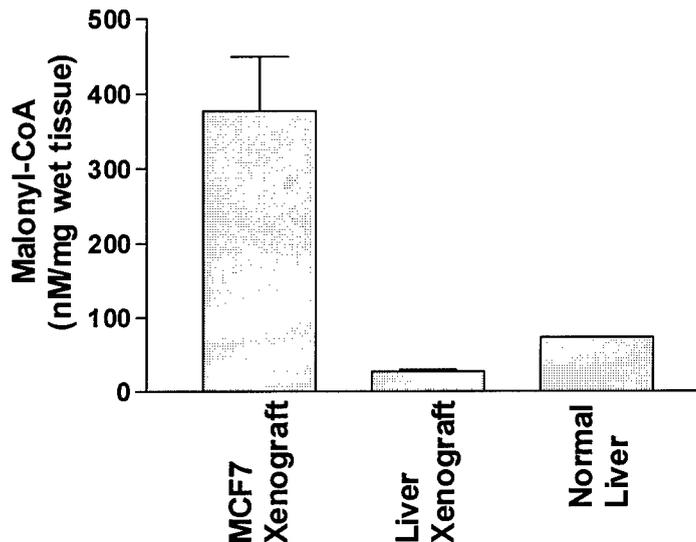
Figure 1A

Figure 1B

Figure 1A. *MCF7 xenograft has high steady-state levels of malonyl-CoA.*
HPLC separation of coenzyme-A derivatives from 10% TCA extracts of tumor
and liver tissue from the same athymic nude mouse xenografted with MCF7
cells. **M**=malonyl-CoA, **G**=glutaryl-CoA, **C**=free CoA, **S**=succinyl-CoA, **H**=HMG-
CoA, **A**=acetyl-CoA. Note the high levels of malonyl-CoA in tumor compared to
liver reflecting elevated levels of fatty acid synthesis. All other CoA derivatives
are at higher levels in the liver compared to the tumor tissue. **1B.** *Quantitation
of malonyl-CoA in MCF7 xenograft, xenograft liver, and normal liver.* The
MCF7 xenograft has a 10 fold higher level of malonyl-CoA than control liver from
the same animal. Normal liver from a female mouse with the estrogen implant
without tumor had a higher level of malonyl-CoA than liver from tumor bearing
mice. Error bars represent standard error of the mean. Xenograft
measurements represent duplicate measurements from the same animal. Only
one normal liver sample was tested.

2. Synthesis of [³H-C75].

Dr. Townsend's group has recently been able to synthesize [³H-C75] with a specific activity of
14mCi/mM which is sufficient for *in vitro* and *in vivo* studies. Briefly, the strategy employed to
introduce the tritium label involved reduction of a 9 carbon aldehyde with sodium borotritiide to
an alcohol with subsequent treatment with PCC (pyridoxine chlorochromate). This labeled
aldehyde was subsequently used to synthesize C75 as follows.

Two equivalents of lithiumhexamethyldisilyl amide (LiHMDS) were added to a solution of *p*-
methoxybenzyl itaconate dissolved in dry tetrahydrofuran (THF) at -78 °C. After 1 h, an
equivalent of [³H-aldehyde] in THF was added at low temperature and stirred for 3-4 h. The
reaction was quenched by the addition of cold 6*N* sulfuric acid, and the products were extracted
into ether. The organic solution was dried over anhydrous magnesium sulfate and evaporated to
a gummy solid, which was dissolved in methylene chloride and treated with trifluoroacetic acid
at room temperature for 10-12 h. The products were partitioned into aqueous sodium
bicarbonate, reacidified and extracted again into ether. Drying and removal of the solvent as
before gave the lactones as a mixture of *trans-* and *cis*-diastereomers as a crystalline solid.
These were separated by flash column chromatography on silica gel using ethyl
acetate:hexanes:acetic acid 30:70:1 as eluent, and individually crystallized from boiling hexanes.

3. Distribution of [³H-C75] in MCF7 xenograft bearing nude mice.

C75 is widely distributed in tumor and normal tissues, except for the brain. In addition, a large
amount of drug remains in the blood which may represent binding to albumin. Figure 2 shows
the quantitation of [³H-C75] in the MCF7 xenograft and normal tissues. C75 targets rapidly to
liver as expected from i.p. administration. Over 24 hours, tumor C75 levels approximate those
of liver. All levels fall dramatically after 48 hours which may represent renal excretion or
peripheral metabolism. Importantly, C75 does not reach the brain in significant quantities
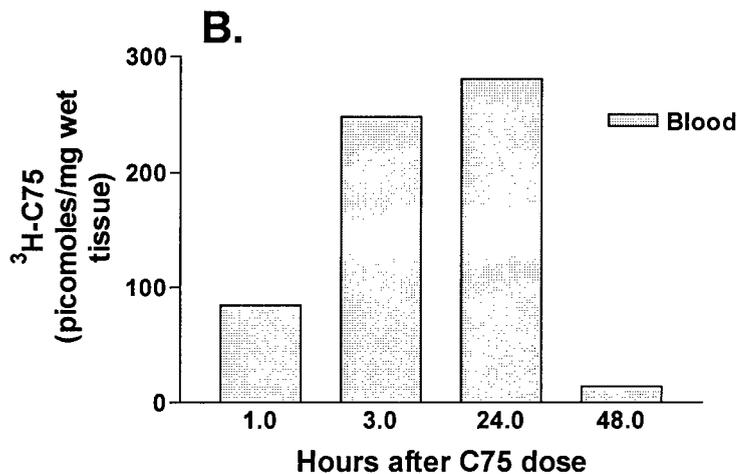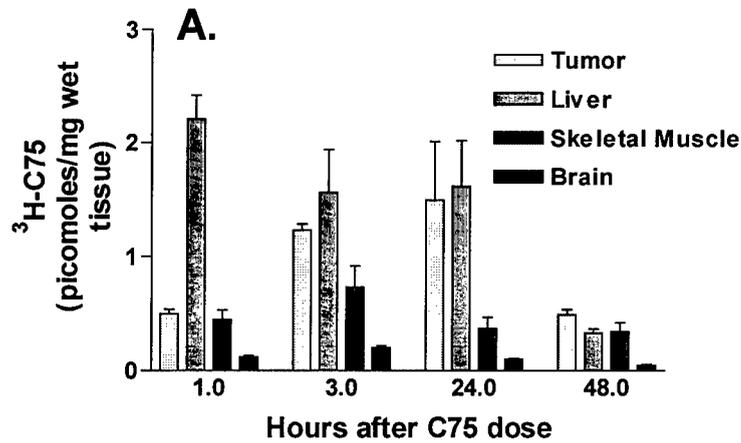indicating that it does not easily pass the "blood-brain barrier".

Figure 2A and B. *Tissue distribution of [³H-C75] in the MCF7 xenograft and normal tissues.* Athymic nude mice with subcutaneous MCF7 xenografts were treated with 0.6 mg of [³H-C75] i.p. in 200 ul of RPMI vehicle. At the indicated times, animals were sacrificed and 1-2 mg of tissues and 1 μl of blood were removed and counted for ³H. Note the rapid rise in blood and liver levels except for brain. Tumor levels remain elevated above liver after 48 h. Error bars represent standard error of the mean.

Relationship of Studies to the Statement of Work: The synthesis of [³H-C75] and its tissue distribution fulfill tasks 1-3. These data provide substantial support to the C75 xenograft treatment studies demonstrating that labeled drug targets tumor nearly as well as liver despite i.p. administration of drug.

4. [³H-C75] binds to FAS in human cancer cells in vitro.

1×10^5 MCF7 cells were incubated with 10 μg/ml [³H-C75] for 1 hr in the presence and absence of 10 μg/ml cerulenin. Cells were washed thrice with PBS, and analyzed by SDS-PAGE and

fluorography. [^{3}H-C75] labeled a band at approximately 260 kDa consistent with fatty acid synthase. Pretreatment with cerulenin reduced the labeling suggesting competition for the target. Immunoprecipitation of [^{3}H-C75] treated cells with anti-FAS antibodies also shows a 260 kDa band demonstrating that C75 indeed binds to FAS. Furthermore, the interaction is likely to be covalent since it survives SDS-PAGE treatment. The reduction of binding with cerulenin pretreatment suggests that C75 may bind at or near the site of cerulenin binding on FAS. This would suggest that C75 binds and inhibits the β-ketoacyl condensing site on FAS, similar to cerulenin. These studies have now been reported in the *Proceedings of the National Academy of Science* contained in the appendix.

5. C-75 induced weight loss is due to inanition and may represent appetite suppression.

In the last report we detailed a series of studies analyzing the C75 associated weight loss, the only significant C75 toxicity thus far identified. A formal collaboration has been established with Dr M. Daniel Lane, a member of the National Academy of Science, and Professor of Biological Chemistry, and Dr. Gabriele Ronnett in Neuroscience to further investigate this phenomenon. Further studies have clearly shown that C75 alters feeding habits of mice by reduction of appetite.

During the past 12 months, an extensive collaborative effort was made to identify the mechanism responsible for the profound weight loss. Surprisingly, these studies showed that C75 reduces Neuropeptide-Y (NPY) production in the hypothalamus. NPY is one of the most potent appetite stimulating substances produced in the brain. The reduction in NPY is blocked by inhibition of acetyl-CoA carboxylase by TOFA, indicating that malonyl-CoA production is likely responsible for this effect. These data were recently reported in *Science* which is included in the appendix. Further studies have now shown that fatty acid synthase is present in both the rodent and human hypothalamus and may function as an energy sensing pathway in the CNS.

6. C-75 has significant anti-tumor activity against a human mesothelioma xenograft.

Due to the high prevalence of malignant mesothelioma in our patient population and its resistance to all types of chemotherapy, we decided to study FAS expression in human mesothelioma. In our study in press in *Cancer Research* we found that at least 50% of human mesotheliomas express FAS and expression is present in both epithelial and sarcomatous varieties. Using the H-meso established human mesothelioma cell line, we also detected fatty acid synthesis activity. Finally, C75 had a significant anti-tumor effect in H-meso Xenografts. These studies are detailed in the preprint of our paper in the appendix.

KEY RESEARCH ACCOMPLISHMENTS:

1) Identification of malonyl-CoA as a novel trigger to induce apoptosis in breast cancer cells.
2) Successful treatment of the MCF7 xenograft with C75.
3) Identification of radically altered CoA derivatives in human breast cancer cells *in vivo*.
4) Synthesis of [^{3}H-C75].
5) C75 is widely distributed in xenograft tissues including MCF7 tumor.
6) C75 binds covalently to FAS.

7) C75 induces reversible altered feeding behavior in mice leading to weight loss.
8) Inhibition of FAS causes reduction in neruopeptide-Y (NPY).
9) FAS is present at high levels in at least 50% of human mesotheliomas.
10) C75 has anti-tumor activity against the H-meso human mesothelioma xenograft.

REPORTABLE OUTCOMES:

Manuscripts:

1. Kuhajda FP, Pizer ES, Mani NS, Pinn ML, Han WF, Chrest FJ, Townsend CA. Synthesis and anti-tumor activity of a novel inhibitor of fatty acid synthase. Proceedings of the American Association of Cancer Research 40: 121, 1999.

2. Parrish NM, Kuhajda FP, Heine HS, Bishai WR, Dick JD. Antimycobacterial activity of cerulenin and its effects on lipid biosynthesis. J. Antimicrobial Chemotherapy 43: 219-226,1999.

3. Pizer ES, Thupari J, Han WF, Pinn ML, Chrest FJ, Frehywot GJ, Townsend CA, Kuhajda FP. Malonyl-Coenzyme-A is a Potential Mediator of Cytotoxicity Induced by Fatty Acid Synthase Inhibition in Human Breast Cancer Cells and Xenografts. Cancer Research 60:213-218, 2000.

4. Kuhajda FP. Fatty acid synthase and human cancer: new perspectives on its role in tumor biology (Invited Review). Nutrition 16:202-208,2000.

5. Kuhajda FP, Pizer ES, Li JN, Mani NS, Freywot GL, Townsend CA. Synthesis and antitumor activity of an inhibitor of fatty acid synthase. PNAS 97: 3450-3454, 2000.

6. Loftus TM, Townsend CA, Lane MD, Kuhajda FP. Weight loss mediated by fatty acid synthase inhibition. A link between feeding behavior and fatty acid metabolism. Science 288:2379-2381, 2000.

7. Gabrielson E., Pinn M, Testa JR, and Kuhajda FP. Increased Fatty Acid Synthase is a Therapeutic Target in Mesothelioma. Cancer Research, in press.

Presentations:

Kuhajda FP, Pizer ES, Mani NS, Pinn ML, Han WF, Chrest FJ, Townsend CA. Synthesis and anti-tumor activity of a novel inhibitor of fatty acid synthase. 1999 Annual Meeting of the American Association of Cancer Research, 1999.

Patents:

Inhibition of Fatty Acid Synthase as a Means to Reduce Weight and/or Adipocyte Mass in Humans, Mammals and other Species Capable of Fat Storage"
Granted U.S.application filed November 28, 1996; formal issuance occurred in 1999.

Inhibition of Carnitine Palmitoyltransferase-1 (CPT-1) as a Means to Selectively Kill Cancer Cells U.S. patent filed November 2000

Depletion Of Cellular Coenzyme-A Levels As A Means To Selectively Kill Cancer Cells U.S. patent filed November 2000

There is a significant patent estate owned by the Johns Hopkins University surrounding diagnostic and therapeutic applications of this technology.

Other grant applications:

We have received NIH RO1 funding (RO11CA087850-01) as of July,1 2000 for further studies on the mechanism of action of FAS inhibition and apoptosis in cancer cells.

In the Spring of 2000, we plan to submit a program project grant with the Departments of Chemistry and Neuroscience to study the biology of feeding behavior and metabolism of FAS inhibition as it applies to weight loss and reduction of adipocyte mass.

CONCLUSIONS

Thus far we have demonstrated that FAS inhibition using C75 has a significant anti-tumor effect on the MCF7 human breast cancer xenograft and the H-meso human mesothelioma xenograft. Radiolabeled C75 has enabled us to directly study the interaction of C75 with FAS and begin pharmacokinetic analysis of C75 in the xenograft. Through studies of C75 mechanism of action we have discovered a novel biochemical pathway leading to apoptosis involving malonyl-CoA. There is now a potential link between FAS inhibition, high levels of malonyl-CoA, CPT-1 inhibition, and the mitochondria to target apoptosis to cancer cells. Further analysis of coenzyme-A derivatives in the MCF7 xenograft and liver has found striking differences indicating profound metabolic abnormalities in cancer which could lead to new target pathways.

Thus, we believe that the medical application of this technology will be significant. C75, the first chemically stable FAS inhibitor has significant anti-tumor activity in breast cancer xenografts without toxicity to proliferating cells. Since many human solid tumors express high levels of FAS and undergo high levels of fatty acid synthesis, this strategy may lead to treatments affecting many common tumors. The high levels of FAS found in *in situ* breast cancer raises the possibility of treatment of precancerous lesions in the future. Since this technology targets a novel pathway, FAS inhibitors may enhance the cytotoxicity of conventional chemotherapy without increasing toxicity to proliferating cells. Finally, these

studies seek to expand our fundamental knowledge of metabolism in human breast cancer which may yet provide new insights for therapeutic strategies.

In addition, we now know that C-75 inhibits NPY production in the CNS. This could lead to FAS therapeutics for obesity and Type II diabetes. Further studies on the weight loss mechanism of action could enable us to separate the weight-loss effects from the anti-tumor effects of FAS. This would also further the anti-tumor therapy effort.

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1) Paumen MB *et al.* Inhibition of carnitine palmitoyltransferase 1 augments sphingolipid synthesis and palmitate-induced apoptosis. J. Biol. Chem. 272, 3324-3329, (1997).

2) Paumen MB *et al.* Direct interaction of the mitochondrial membrane protein carnitine palmitoyltransferase 1 with BCL-2. Biochem. Biophys. Res Com. 231, 523-525 (1997).

Advances in Brief

Malonyl-Coenzyme-A Is a Potential Mediator of Cytotoxicity Induced by Fatty-Acid Synthase Inhibition in Human Breast Cancer Cells and Xenografts[1]

Ellen S. Pizer, Jagan Thupari, Wan Fang Han, Michael L. Pinn, Francis J. Chrest, Gojeb L. Frehywot, Craig A. Townsend, and Francis P. Kuhajda[2]

Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21224 [E. S. P., J. T., W. F. H., M. L. P., F. P. K.]; Research Resources Branch/Flow Cytometry Unit, Gerontology Research Center, National Institute on Aging, Baltimore, Maryland 21224 [F. J. C.]; and Department of Chemistry, The Johns Hopkins University, Baltimore, Maryland 21218 [G. L. F., C. A. T.]

Abstract

A biologically aggressive subset of human breast cancers and other malignancies is characterized by elevated fatty-acid synthase (FAS) enzyme expression, elevated fatty acid (FA) synthesis, and selective sensitivity to pharmacological inhibition of FAS activity by cerulenin or the novel compound C75. In this study, inhibition of FA synthesis at the physiologically regulated step of carboxylation of acetyl-CoA to malonyl-CoA by 5-(tetradecyloxy)-2-furoic acid (TOFA) was not cytotoxic to breast cancer cells in clonogenic assays. FAS inhibitors induced a rapid increase in intracellular malonyl-CoA to several fold above control levels, whereas TOFA reduced intracellular malonyl-CoA by 60%. Simultaneous exposure of breast cancer cells to TOFA and an FAS inhibitor resulted in significantly reduced cytotoxicity and apoptosis. Subcutaneous xenografts of MCF7 breast cancer cells in nude mice treated with C75 showed FA synthesis inhibition, apoptosis, and inhibition of tumor growth to less than 1/8 of control volumes, without comparable toxicity in normal tissues. The data suggest that differences in intermediary metabolism render tumor cells susceptible to toxic fluxes in malonyl-CoA, both *in vitro* and *in vivo*.

Introduction

A number of studies have demonstrated surprisingly high levels of FAS[3] expression (EC 2.3.1.85) in virulent human breast cancer (1, 2), as well as other cancers (3, 4). FAS expression has also been identified in intraductal and lobular *in situ* breast carcinoma, lesions associated with increased risk for the development of infiltrating breast cancer (5). FAS is the principal synthetic enzyme of FA synthesis, which catalyzes the NADPH-dependent condensation of malonyl-CoA and acetyl-CoA to produce predominantly the 16-carbon saturated free FA palmitate (6). *Ex vivo* measurements in tumor tissue have revealed high levels of both FAS and FA synthesis, indicating that the entire genetic program is highly active consisting of some 25 enzymes from hexokinase to FAS (3). Cultured human cancer cells treated with inhibitors of FAS, including the fungal product cerulenin and the novel compound C75, demonstrated a rapid decline in FA synthesis, with subsequent reduction of DNA synthesis and cell cycle arrest, culminating in apoptosis (7, 8). These findings suggested a vital biochemical link between FA synthesis and cancer cell growth. Importantly, these effects occurred despite the presence of exogenous FAs in the culture medium derived from fetal bovine serum. Although

it has been possible to rescue the cytotoxic effect of cerulenin on certain cells in FA-free culture conditions by the addition of exogenous palmitate, most cancer cells were not rescued from FA synthesis inhibition by the pathway end product (data not shown; Ref. 9). Thus, it has been unresolved whether the cytotoxic effect of FA synthesis inhibition on most cancer cells resulted from end product starvation or from some other biochemical mechanism. If FA starvation mediated the cytotoxic effects of cerulenin and C75, then any other FA synthesis inhibitor of similar potency should produce similar effects. To test this idea, we compared the effects on cancer cells of inhibition of ACC (EC 6.4.1.2), the rate-limiting enzyme of FA synthesis, with the effects of FAS inhibitors.

Fig. 1A outlines the portion of the FA synthesis pathway containing the target enzymes of the inhibitors used in this study. TOFA is an allosteric inhibitor of ACC, blocking the carboxylation of acetyl-CoA to malonyl-CoA. Once esterified to CoA, TOFA-CoA allosterically inhibits ACC with a mechanism similar to long chain acyl-CoAs, the physiological end-product inhibitors of ACC (10). Both cerulenin (11) and C75 (8) are inhibitors of FAS, preventing the condensation of malonyl-CoA and acetyl-CoA into FAs. Cerulenin is a suicide inhibitor, forming a covalent adduct with FAS (12), whereas C75 is likely a slow-binding inhibitor (13). We now report that using TOFA, we achieved FA synthesis inhibition in human breast cancer cell lines comparable to inhibition by cerulenin or C75. Surprisingly, however, TOFA was essentially nontoxic to human breast cancer cells. These data suggest that FA starvation is not a major source of cytotoxicity to cancer cells in serum supplemented culture. Rather, high levels of the substrate, malonyl-CoA, resulting specifically from inhibition of FAS, may mediate cytotoxicity of cerulenin and C75.

Materials and Methods

FA Synthesis Inhibitors. Cerulenin was obtained from Sigma. C75 and TOFA were synthesized in the laboratory of C. A. Townsend in the Department of Chemistry, Johns Hopkins University.

Cell Lines, Culture Conditions, Metabolic Labeling, and Clonogenic Assays. The human breast cancer cell lines, SKBR3 and MCF7 were maintained in RPMI with 10% fetal bovine serum. Cells were screened periodically for *Mycoplasma* contamination (Gen-probe). All inhibitors were added as stock 5 mg/ml solutions in DMSO. For FA synthesis activity determinations, 5×10^4 cells/well in 24-well plates were pulse labeled with [U^{14}C]acetate after exposure to drug, and lipids were extracted and quantified as described previously (8). For MCF7 cells, pathway activity was determined after 2 h of inhibitor exposure. SKBR3 cells demonstrated slower response to FAS inhibitors, possibly because of their extremely high FAS content, so pathway activity was determined after 6 h of inhibitor exposure. For clonogenic assays, 4×10^5 cells were plated in 25-cm^2 flasks with inhibitors added for 6 h in concentrations listed. To rescue MCF7 cells with TOFA (see Fig. 3C), the TOFA was added 1 h prior to the FAS inhibitors. Equal numbers of treated cells and controls were plated in 60-mm dishes. Clones were stained and counted after 7–10 days.
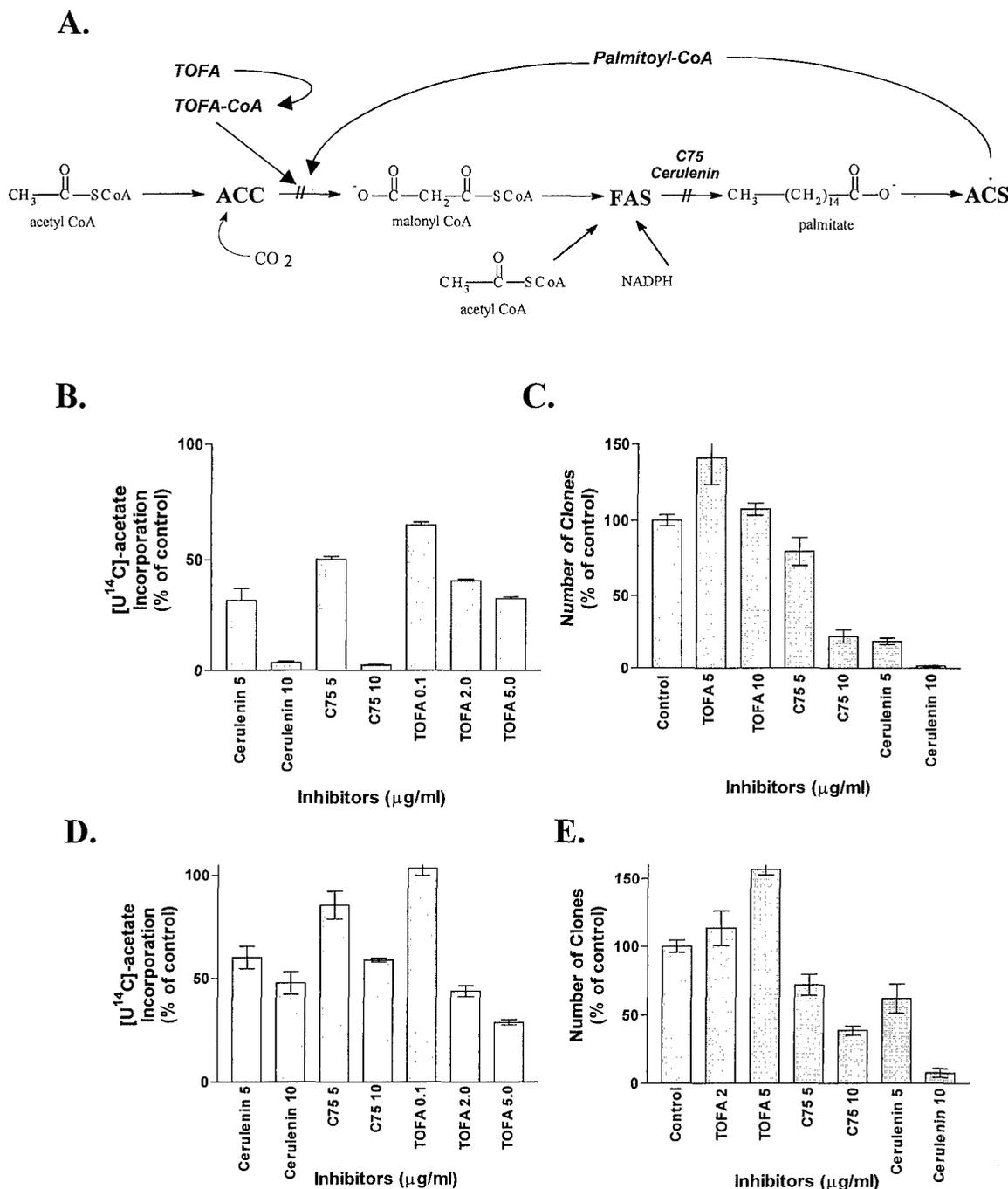
Fig. 1. Inhibitors of the FA synthesis pathway. *A*, schematic representation of the FA synthesis pathway showing the specificity of cerulenin and C75 for FAS and of TOFA for ACC. The three FA synthesis inhibitors reduced FA synthesis activity (incorporation of [$U^{14}C$]acetate into extractable lipids) by comparable amounts in SKBR3 breast carcinoma cells (*B*) and in MCF7 breast carcinoma cells (*D*). The cytotoxic activity of the three FA synthesis inhibitors was determined by clonogenic assay in the dose range for FA synthesis inhibition. A 6-h exposure to cerulenin or C75 reduced the clonogenic fraction of SKBR3 breast carcinoma cells (Student's t test, $P = 0.0002$ for C75, $P < 0.0001$ for cerulenin; *C*) and MCF7 breast carcinoma cells ($P = 0.0004$ for C75, $P < 0.0001$ for cerulenin; *E*), whereas TOFA did not.

Flow-cytometric Quantitation of Apoptosis. Apoptosis was measured by multiparameter flow cytometry using a FACStar^{Plus} flow cytometer equipped with argon and krypton lasers (Becton Dickinson). Apoptosis was quantified using merocyanine 540 staining (Sigma), which detects altered plasma membrane phospholipid packing that occurs early in apoptosis, added directly to cells from culture (8, 14). In some experiments, chromatin conformational changes of apoptosis were simultaneously measured as decreased staining with LDS-751 (Exciton; Ref. 15). Merocyanine 540 (10 μg/ml) was added as a 1 mg/ml stock in water. Cells were stained with LDS-751 at a final concentration of 100 nM from a 1 mM stock in DMSO. The merocyanine 540-positive cells were marked by an increase in red fluorescence, collected at 575 ± 20 nm,

0.5–2 logs over merocyanine 540-negative cells. Similarly, the LDS-751 dim cells demonstrated a reduction in fluorescence of 0.5–1.5 logs relative to normal cells, collected at 660 nm with a DF20 band pass filter. Data were collected and analyzed using CellQuest software (Becton Dickinson). In these experiments, all LDS-751 dim cells were merocyanine 540 bright; however, a population of merocyanine 540 bright cells were detected that were not yet LDS-751 dim. All merocyanine 540 bright cells were classified as apoptotic.

Measurement of Malonyl-CoA. Malonyl-CoA levels were measured in MCF-7 cells using the HPLC method of Corkey (16). Briefly, 2.5 × 10^5 cells/well in 24-well plates were subjected to 1.2 ml of 10% trichloroacetic acid at 4°C after various drug treatments. The pellet mass was recorded, and

the supernatant was washed six times with 1.2 ml of ether and reduced to dryness using vacuum centrifugation at 25°C. CoA esters were separated and quantitated using reversed-phase HPLC on a 5-μm Supelco C18 column with a Waters HPLC system running Millenium[32] software, monitoring 254 nm as the maximum absorbance for CoA. The following gradients and buffers were used: buffer A, 0.1 M potassium phosphate, pH 5.0; buffer B, 0.1 M potassium phosphate, pH 5.0, with 40% acetonitrile. Following a 20-min isocratic run with 92% buffer A, 8% buffer B at 0.4 ml/min, flow was increased to 0.8 ml/min over 1 min, whereupon a linear gradient to 10% buffer B was run until 24 min and then held at 10% buffer B until 50 min, at which point a linear gradient was run to 100% buffer B at 55 min, completing at 60 min. The following CoA esters (Sigma) were run as standards: malonyl-CoA, acetyl-CoA, glutathione-CoA, succinyl-CoA, HMG-CoA, and free CoA. Samples and standards were dissolved in 50 μl of buffer A. CoA esters eluted sequentially as follows: malonyl-CoA, glutathione-CoA, free CoA, succinyl-CoA, HMG-CoA, and acetyl-CoA. Quantitation of CoA esters was performed by the Millenium[32] software.

Xenograft Studies. s.c. flank xenografts of the human breast cancer cell line, MCF-7 in nu/nu female mice (Harlan) were used to study the antitumor effects of C75 *in vivo*. All animal experiments complied with institutional animal care guidelines. All mice received a 90-day slow-release s.c. estrogen pellet (Innovative Research) in the anterior flank 7 days before tumor inoculation. MCF7 cells (10^7 cells) were xenografted from culture in DMEM supplemented with 10% FBS and 10 μg/ml insulin. Treatment began when measurable tumors developed about 10 days after inoculation. Eleven mice (divided between two separate experiments of five and six mice) were treated i.p. with weekly doses of C75 at 30 mg/kg in 0.1 ml of RPMI. Dosing was based on a single dose LD_{10} determination of 40 mg/kg in BALB/c mice; 30 mg/kg has been well tolerated in outbred nude mice. Eleven control mice (divided in the same way as the treatment groups) received RPMI alone. Tumor volume was measured with calipers in three dimensions. Experiment was terminated when controls reached the surrogate end point. In a parallel experiment to determine FA synthesis activity in treated and control tumors, a group of MCF-7 xenografted mice were treated with C75 or vehicle at above doses and sacrificed after 3 h. Tumor and liver tissue were *ex vivo* labeled with [$U^{14}C$], lipids were extracted and counted as described (3). In an additional parallel experiment to histologically examine treated and control tumors, six C75-treated and six vehicle control mice were sacrificed 6 h after treatment. Tumor and normal tissues were fixed in neutral-buffered formalin and processed for routine histology, and immunohistochemistry for FAS was performed.

FAS Immunohistochemistry. Immunohistochemistry for FAS was performed on the MCF-7 xenografts using a mouse monoclonal anti-FAS antibody (1) at 1:2000 on the DAKO Immunostainer using the LSAB2 detection kit.

Results and Discussion

TOFA, Cerulenin, and C75 All Inhibited FA Synthesis in Human Breast Cancer Cells but Showed Differential Cytotoxicity. In standard pulse labeling experiments in which breast cancer cell lines SKBR3 and MCF7 were labeled for 2 h after exposure to FA synthesis inhibitors, TOFA, C75, and cerulenin all produced dose-dependent inhibition of [$U^{14}C$]acetate incorporation into lipids (Fig. 1, *B* and *D*). The maximal pathway inhibition achieved with each drug was somewhat variable among cell lines. In numerous similar experiments (not shown), TOFA maximally inhibited FA synthesis in the 1–5 μg/ml dose range in all cell lines tested, and cerulenin and C75 maximally inhibited FA synthesis at about 10 μg/ml Although all inhibitors reduced FA synthesis to comparable degrees, TOFA was nontoxic or stimulatory to the cancer cell growth in the dose range for ACC inhibition, as measured by clonogenic assays, whereas cerulenin and C75 were significantly cytotoxic in the dose range for FAS inhibition (Fig. 1, *C* and *E*). The profound difference between the cytotoxic effects of ACC and FAS inhibition demonstrated that the acute reduction of FA production *per se* was not the major source of cell injury after FAS inhibition. Alternatively, these data suggested that cytotoxicity resulted from a biochemical effect of FAS inhibition that was not shared by ACC inhibition.
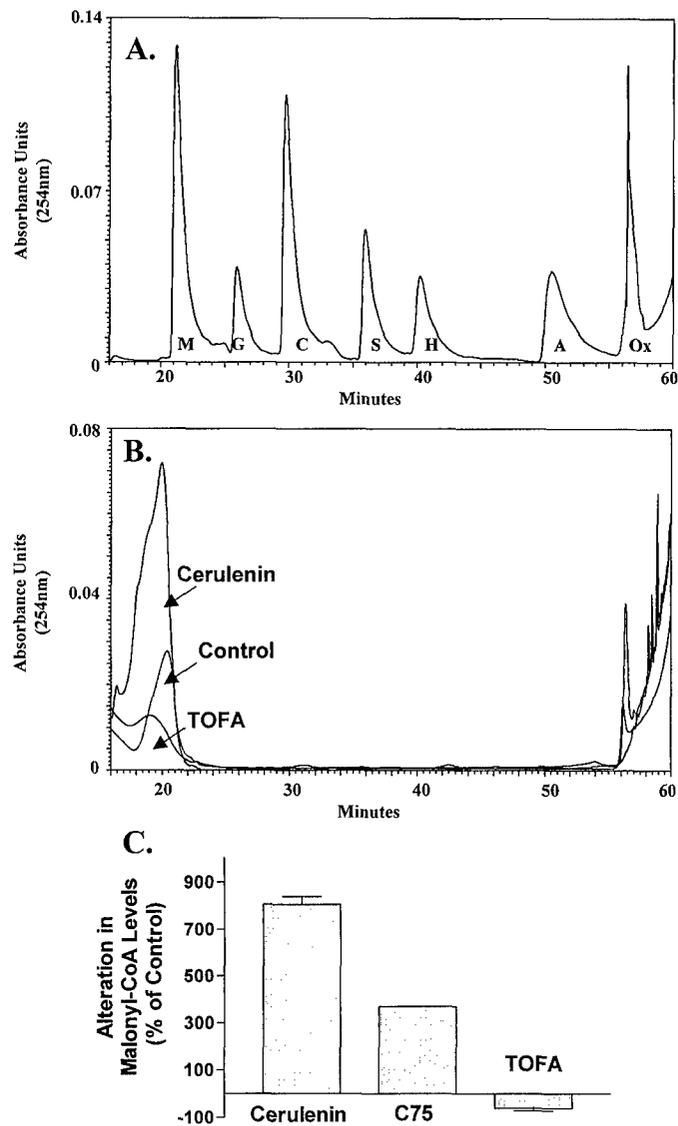

Fig. 2. Effects of the FA synthesis inhibitors on malonyl-CoA levels. Chemical standards (*A*) and acid soluble extracts of MCF7 breast carcinoma cells (*B*) were resolved by reversed-phase HPLC for quantitation of malonyl-CoA. Changes in intracellular malonyl-CoA content after exposure to the three FA synthesis inhibitors are shown in C. Assays were performed in triplicate.

Malonyl-CoA Levels Were Markedly Increased with FAS Inhibition and Reduced by TOFA. The most obvious difference in the expected results of inhibiting these two enzymes was that malonyl-CoA levels should fall after ACC inhibition but increase after FAS inhibition. Although not previously investigated in eukaryotes, recent data in *Escherichia coli* have demonstrated elevated levels of malonyl-CoA resulting from exposure to cerulenin (17).

Direct measurement of CoA derivatives in MCF-7 cells by reversed-phase HPLC of acid soluble extracts from drug-treated cells confirmed that both cerulenin and C75 caused a rapid increase in malonyl-CoA levels, whereas TOFA reduced malonyl-CoA levels. Fig. 2A is a representative chromatograph demonstrating the separation and identification of CoA derivatives important in cellular metabolism. Malonyl-CoA is the first of these to elute, with a column retention time of 19–22 min. The overlay of chromatographs in Fig. 2B shows that cerulenin treatment led to a marked increase in malonyl-CoA over the control, whereas TOFA caused a significant reduction. The chemical identity of the malonyl-CoA was independently confirmed by spiking samples with standards (not shown). The anal-
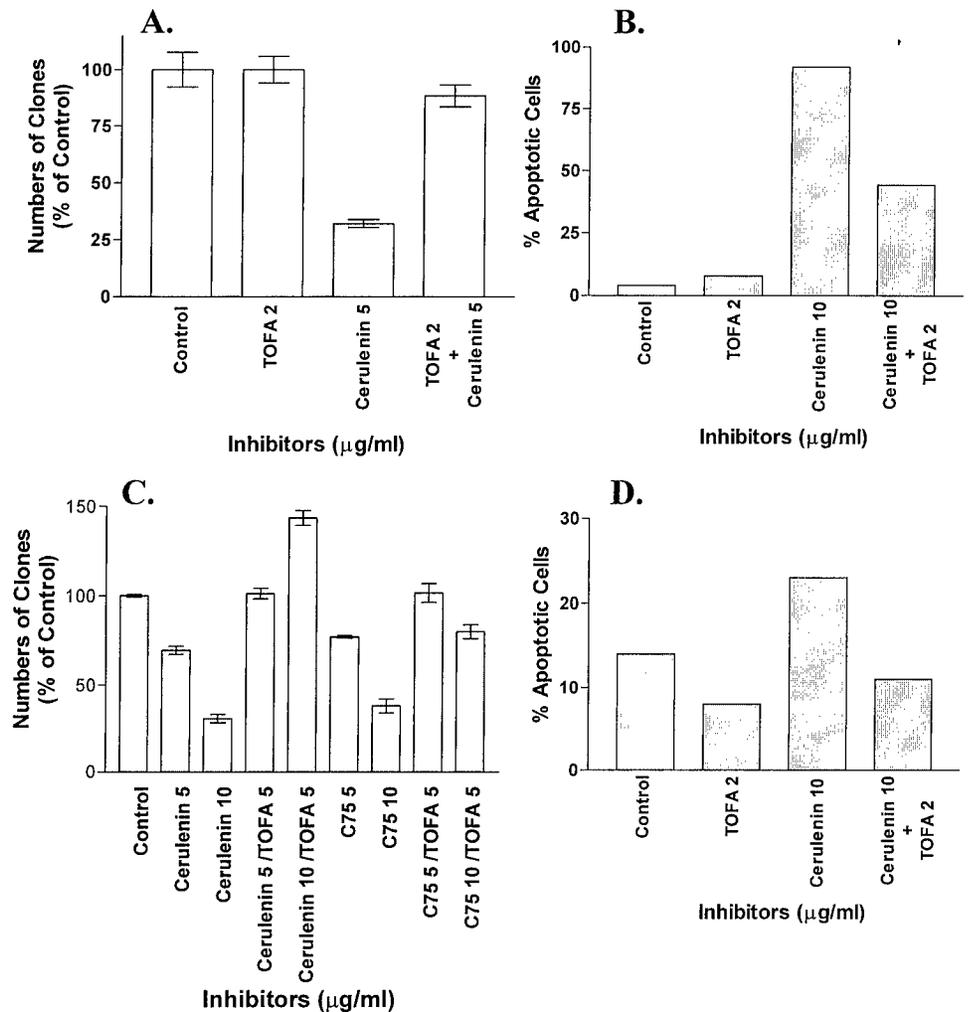
Fig. 3. ACC inhibition rescued the cytotoxic effects of FAS inhibition in breast cancer cells. *A*, pretreatment with TOFA rescued SKBR3 cells from cerulenin cytotoxicity as determined by clonogenic assays (Student's *t* test, *P* = 0.001). *C*, similarly, TOFA reduced both cerulenin and C75 cytotoxicity in MCF7 cells (*P* = 0.0016 for C75, *P* < 0.0001 for cerulenin). Using merocyanine 540 staining as an indicator of apoptosis, TOFA rescued both SKBR3 cells (*B*) and MCF7 cells (*D*) from cerulenin cytotoxicity.

ysis of multiple experiments shown in Fig. 2C demonstrated that following a 1-h exposure to cerulenin or C75 at 10 μg/ml, malonyl-CoA levels increased by 930 and 370%, respectively, over controls, whereas TOFA treatment (20 μg/ml) led to a 60% reduction of malonyl-CoA levels. The concentration of TOFA required for maximal reduction of malonyl-CoA levels was 4-fold higher than the dose for pathway inhibition shown in Fig. 1, *B* and *D*. However, optimal cultures for extraction of CoA derivatives had 5-fold higher cell density than the cultures used in the other biochemical and viability assays presented. The remarkable increase in malonyl-CoA after FAS inhibition can be attributed in part to the release of long-chain fatty acyl-CoA inhibition of ACC, leading to an increase in ACC activity (Fig. 1A). Moreover, the cerulenin-induced increase in malonyl-CoA levels occurred within 30 min of treatment (930 ± 15% increase over control, data not shown), within the time frame of FA synthesis inhibition and well before the onset of DNA synthesis inhibition or early apoptotic events (8). Thus, high levels of malonyl-CoA were a characteristic effect of FAS inhibitors and temporally preceded the other cellular responses, including apoptosis.

Inhibition of ACC Rescued Breast Cancer Cells from FAS Inhibition. If the elevated levels of malonyl-CoA resulting from FAS inhibition were responsible for cytotoxicity, then it should be possible to rescue cells from FAS inhibition by reducing malonyl-CoA accumulation with TOFA. Co-administration of TOFA and cerulenin to SKBR3 cells (Fig. 3A) abrogated the cytotoxic effect of cerulenin alone in clonogenic assays. In MCF7 cells (Fig. 3C), TOFA rescued

both cerulenin and C75 when cells were exposed to TOFA for 1 h prior to the FAS inhibitors. Representative flow cytometric analyses of SKBR3 cells (Fig. 3B) and MCF7 (Fig. 3D) substantiated these findings, because TOFA rescued cells from cerulenin induced apoptosis. These experiments also confirmed the differential cytotoxicity between TOFA (<5% increase in apoptosis; no reduction in clonogenicity) compared to cerulenin (>85% apoptosis; 70% reduction in clonogenicity). Taken together, these studies suggest that high malonyl-CoA levels may play a role in the cytotoxic effect of FAS inhibitors on cancer cells.

***In Vivo* Inhibition of FAS Led to Reduced Tumor Growth.** Previous studies have demonstrated local efficacy of cerulenin against a human cancer xenograft (18) but were limited by the failure of cerulenin to act systemically. The similar responses of breast cancer cells to cerulenin and C75 *in vitro* suggested that C75 might be effective *in vivo* against xenografted breast cancer cells. To determine whether the effects of FAS inhibition seen *in vitro* would translate to an *in vivo* setting requiring systemic activity, we tested C75 against s.c. MCF-7 xenografts in athymic nude mice, to quantitate effects on FA synthesis and the growth of established solid tumor.

FA synthesis pathway activity in tissues of xenografted mice was determined by *ex vivo* pulse labeling with $[U^{14}C]$acetate. The tumor xenografts had 10-fold higher FA synthesis activity than liver, highlighting the difference in pathway activity between benign and malignant tissues (Fig. 4A). FAS expression in the MCF-7 xenograft paralleled the high level of FA synthesis activity (Fig. 4B). i.p.
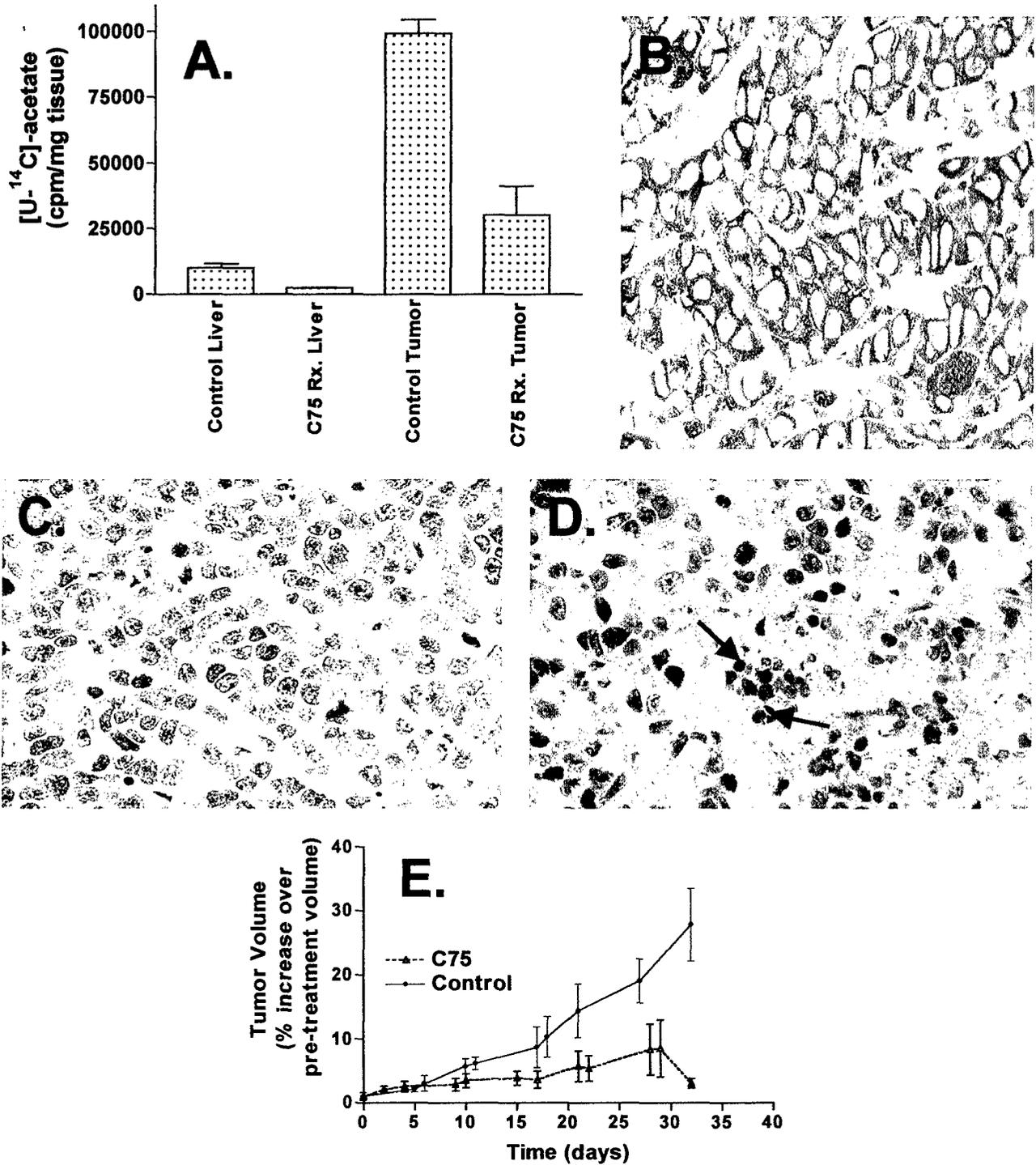
Fig. 4. Selective cytotoxicity of the FAS inhibitor, C75, to MCF7 breast carcinoma flank xenografts in nude mice. *A*, xenografted tumor had high FA synthesis activity relative to liver, determined by *ex vivo* metabolic labeling. A standard i.p. dose of C75 (30 mg/kg) inhibited FA synthesis in both liver and tumor by 76 and 70%, respectively, at 3 h (Student's *t* test, $P = 0.04$ for liver, $P = 0.03$ for tumor). *B*, FAS expression was elevated in the xenografted tumor in parallel with FA synthesis, determined by immunohistochemistry. *C* and *D*, a standard i.p. dose of C75 (30 mg/kg) produced histological evidence of widespread apoptosis in the xenografted tumor at 6 h (*D*), which was not evident in vehicle-treated animals (*C*). *E*, weekly treatment with i.p. C75 (30 mg/kg) inhibited the growth of established MCF7 xenografts, resulting in a greater than 8-fold difference in mean tumor growth between vehicle and drug-treated tumors after 32 days (Student's *t* test, $P = 0.0003$). *Arrows*, apoptotic bodies.

injections of C75 at 30 mg/kg reduced FA synthesis in *ex vivo* labeled liver by 76% and in the MCF-7 xenografts by 70% within 3 h (Fig. 4*A*). These changes in FA synthesis preceded histological evidence of cytotoxicity in the xenograft, which became evident 6 h after treatment (Fig. 4, *C* and *D*). The C75-treated xenografts showed numerous apoptotic bodies throughout the tumor tissue, which were not seen in vehicle-treated tumors. Histological analysis of liver and other host tissues following C75 treatment showed no evidence of any short or long term toxicity (not shown).

Weekly i.p. C75 treatment retarded the growth of established s.c. MCF-7 tumors compared to vehicle controls, demonstrating a systemic antitumor effect (Fig. 4*E*). After 32 days of weekly treatments, there was a greater than 8-fold difference in tumor growth in the treatment group compared to vehicle controls. Sim-

ilar to cerulenin, transient reversible weight loss was the only toxicity noted (18).

The systemic pharmacological activity of C75 provided the first analysis of the outcome of systemic FAS inhibitor treatment. The significant antitumor effect of C75 on a human breast cancer xenograft in the setting of physiological levels of ambient FAs was similar to the *in vitro* result in serum supplemented culture and was consistent with a cytotoxic mechanism independent of FA starvation. Furthermore, the result suggested that malonyl-CoA accumulation may not be a significant problem in normal tissues, possibly because FA synthesis pathway activity is normally low, even in lipogenic organs, such as the liver. It is of further interest that whereas malonyl-CoA was the predominant low molecular weight CoA conjugate detected in breast cancer cells in these experiments, other studies have reported predominantly succinyl-CoA and acetyl-CoA in cultured hepatocytes (16). Differences in CoA derivative profiles may be indicative of larger differences in energy metabolism between cancer cells and hepatocytes.

The identification of malonyl-CoA as a potential mediator of cytotoxicity, possibly via induction of apoptosis in cancer cells, although unanticipated, was not surprising given its pivotal role in cellular metabolism. In addition to its function as a substrate for FA synthesis, malonyl-CoA regulates FA oxidation by inhibiting carnitine palmitoyltransferase I at the outer mitochondrial membrane (19). Physiologically, the elevated levels of malonyl-CoA occurring during FA synthesis reduce FA oxidation to prevent a futile cycle of simultaneous FA synthesis and degradation. During starvation or feeding with high-fat diets, fat synthesis ceases, malonyl-CoA levels fall, and FAs enter the mitochondrion for energy production. Malonyl-CoA is thus a crucial regulatory metabolic intermediate in cellular energy metabolism. How superphysiological levels of malonyl-CoA may lead to apoptosis is not yet known; however, carnitine palmitoyltransferase I, which is regulated by malonyl-CoA, has been shown to interact directly with Bcl-2 at the mitochondrial membrane (20). This convergence suggests that high levels of malonyl-CoA may either induce apoptosis directly or alter mitochondrial metabolism to increase susceptibility to apoptosis from other signals. Thus, further investigation of malonyl-CoA and CoA metabolism in cancer cells may yield new insights into cancer cell metabolism and selective susceptibility to antimetabolite therapy.

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Synthesis and antitumor activity of an inhibitor of fatty acid synthase

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Compared to normal human tissues, many common human cancers, including carcinoma of the colon, prostate, ovary, breast, and endometrium, express high levels of fatty acid synthase (FAS, EC 2.3.1.85), the primary enzyme responsible for the synthesis of fatty acids. This differential expression of FAS between normal tissues and cancer has led to the notion that FAS is a target for anticancer drug development. Recent studies with C75, an inhibitor of fatty acid synthesis, have shown significant antitumor activity with concomitant inhibition of fatty acid synthesis in tumor tissue and normal liver. Importantly, histopathological analysis of normal tissues after C75 treatment showed no adverse effects on proliferating cellular compartments, such as bone marrow, gastrointestinal tract, skin, or lymphoid tissues. In this study, we describe the *de novo* synthesis of C75 based on the known mechanism of action of cerulenin and the theoretical reaction intermediates of the β-ketoacyl synthase moiety of FAS. In addition, we demonstrate that C75 is a synthetic, chemically stable inhibitor of FAS. C75 inhibits purified mammalian FAS with characteristics of a slow-binding inhibitor and also inhibits fatty acid synthesis in human cancer cells. Treatment of human breast cancer cells with [5-³H]C75 demonstrates that C75 reacts preferentially with FAS in whole cells. Therefore, we have shown that the primary mechanism of the antitumor activity of C75 is likely mediated through its interaction with, and inhibition of, FAS. This development will enable the *in vivo* study of FAS inhibition in human cancer and other metabolic diseases.

Fatty acid synthesis is common to most organisms. In well-nourished humans, however, the fatty acid synthetic pathway is down-regulated because of sufficiently high levels of dietary fat (1). Although normal tissues have low levels of fatty acid synthesis, a number of recent studies have demonstrated surprisingly high levels of fatty acid synthase expression (FAS, EC 2.3.1.85) in a wide variety of human malignancies and their precursor lesions, including carcinoma of the colon (2), prostate (3, 4), ovary (5), endometrium (6), and breast (7–9). *Ex vivo* measurements in tumor tissue have revealed high levels of both FAS and fatty acid synthesis, indicating that the entire genetic program is highly active, consisting of some 25 enzymes from hexokinase to FAS (2, 10). This differential expression of FAS between normal tissues and cancer has led to the notion that FAS is a target for anticancer drug development.

The widespread expression of FAS in human cancer and its association with aggressive disease in breast (5, 9, 11), prostate (3, 4), and ovarian cancer (5) suggests that fatty acid synthesis provides an advantage for tumor growth. This is in marked contrast to its role as an anabolic energy storage pathway in liver and adipose tissue. Treatment of cancer cells *in vitro* with cerulenin, a covalent inactivator of the β-ketoacyl synthase reaction on FAS, led to cell death by means of apoptosis, demonstrating that cancer cells with highly active fatty acid synthesis require a functional pathway (12). Because of its chemical instability, however, cerulenin had limited activity *in vivo*.

To study systemic anticancer effects of FAS inhibition *in vivo*, a chemically stable type I FAS inhibitor was required. In this

study, we describe the *de novo* synthesis of a synthetic, chemically stable inhibitor of mammalian FAS, C75, based on the known mechanism of action of cerulenin and the theoretical reaction intermediates of the β-ketoacyl synthase moiety of FAS. We further show that C75 binds to and inhibits mammalian FAS and inhibits fatty acid synthesis in human cancer cells. Recent studies have shown C75 to have significant *in vivo* antitumor activity against human breast cancer xenografts (13). Thus, the development of C75 should enable extensive *in vivo* study of FAS inhibition in human cancer and other diseases associated with dysfunctional fatty acid synthesis activity.

Materials and Methods

Synthesis of C75 and Related α-Methylene-γ-Butyrolactones. Lithiumhexamethyldisilyl amide (LiHMDS 1 M in THF; 40 ml, 40 mmol) was added to a solution of *p*-methoxybenzyl itaconate (**1**, 20 mmol) dissolved in dry tetrahydrofuran (THF, 200 ml) at −78°C. After 1 h, the aldehyde (20 mmol in 10 ml THF) was added at low temperature and stirred for 3–4 h. The reaction was quenched by the addition of cold 6N sulfuric acid (20 ml), and the products were extracted into ether. The organic solution was dried over anhydrous magnesium sulfate and evaporated to a gummy solid, which was dissolved in methylene chloride (100–125 ml) and treated with trifluoroacetic acid (1.5 ml) at room temperature for 10–12 h. The products were partitioned into aqueous sodium bicarbonate, reacidified, and extracted again into ether. Drying and removal of the solvent as before gave the lactones a mixture of *trans-* and *cis*-diastereomers (**3** and **4**) as a crystalline solid. These were separated by flash column chromatography on silica gel by using ethyl acetate:hexanes:acetic acid 30:70:1 as eluent, and individually crystallized from boiling hexanes. The following analytical data were collected for C75 (**3**, $r = n - C_8H_{17}$) and are representative: *trans*-isomer (**1**, see Fig. 1 **3**, $r = n - C_8H_{17}$): 25–30% isolated yield, mp 76–77°C; IR (film) 3000–3400, 2924, 2852, 1743, 1717, 1660, 1621, 1460 cm⁻¹; ¹H NMR (CDCl₃) δ 0.84 (t, 3H, $J = 6.8$ Hz), 1.2–1.8 (m, 14H), 3.59 (dt, 1H, $J = 2.8, 5.6, 12.8$ Hz), 4.77 (q_{app}, 1H, $J = 6, 12.8$ Hz), 6.0 (d, 1H, $J = 2.8$ Hz), 6.4 (d, 1H, $J = 3.2$ Hz); ¹³C NMR (CDCl₃) δ 14.0, 22.6, 24.7, 29.12, 29.14, 29.3, 31.7, 35.1, 49.4, 78.7, 125.9, 132.2, 168.1, 174.5; exact mass calculated for 254.1518, found 254.1514.

Synthesis of [5-³H]C75. [1-³H]Nonanal was obtained by treatment of unlabeled aldehyde (1.42 g, 10.0 mmol) with sodium borohydride (0.18 g, 0.5 mmol) in absolute ethanol followed by sodium [³H]borohydride (Amersham Pharmacia; 500 mCi, 15 mCi/mmol). After 18 h at room temperature, the reduction was completed by the addition of excess sodium borohydride (0.4 g,
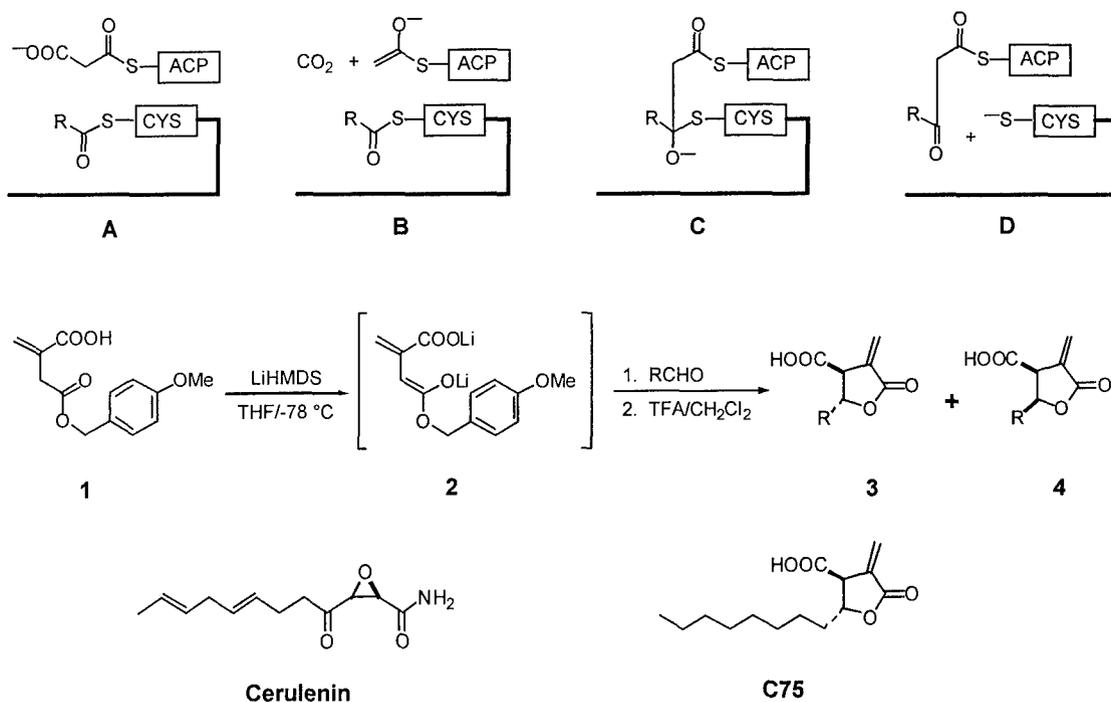
Fig. 1. Proposed mechanism of chain elongation in fatty acid biosynthesis and the synthesis of 3-carboxy-4-alkyl-2-methylenebutyrolactones. (*A*) Malonate, bound as its thioester to ACP, enters the active site where the elongating fatty acid chain is covalently linked to a reactive cysteine residue. (*B*) Decarboxylation of the malonyl-ACP results in a reactive enolate anion. (*C*) Enolate anion attacks the acyl-ACP in a thioester Claisen condensation to form the transiently generated tetrahedral oxyanion. (*D*) This rapidly decomposes to generate the resting state of KAS and the homologated β-ketoacyl intermediate bound to ACP. **1**, Deprotonation of *p*-methoxybenzyl itaconate at low temperature produces the dianion **2**, which underwent aldol reaction with a series of aldehydes, RCHO, to give on strongly acidic workup a mixture of γ-lactones **3** and **4**. Separation on silica gel and crystallization afforded the pure diastereomers. Note the structural differences and similarities between cerulenin [(2S, 3R) 2,3-epoxy-4-oxo-7E, 10E-dodecadienamide] and C75. Although C75 lacks the highly reactive epoxide moiety, it retains the 8-carbon lipid tail without the two double bonds.

11 mmol). After filtration through a short column of silica gel, the tritiated alcohol was oxidized to [1-³H]nonanal with pyridinium chlorochromate (1.5 equiv.) in methylene chloride, and the product was purified by silica gel chromatography in 72% overall yield. [5-³H]C75 was obtained by reaction of the [1-³H]nonanal as above.

Growth Inhibition of α-Methylene-γ-Butyrolactones and Cerulenin in SKBR3 Breast Cancer Cells and Normal Human Skin Fibroblasts. Both SKBR3 cells and fibroblasts were maintained in RPMI medium 1640 with 10% FBS under standard culture conditions. Cells were plated in 96-well plates at a density of 5,000 cells per well. After overnight growth, compounds solubilized in DMSO were added at concentrations ranging from 0.08 to 40 μg/ml in a volume of 100 μl with a maximum DMSO concentration of 1.25%. Vehicle controls were included for each plate. In addition, *cis* and *trans* isomers of C75 were tested against SKBR3 cells solubilized in DMSO or RPMI. Each concentration was plated in quadruplicate. After 72 h of incubation with drug at 37°C, plates were stained with crystal violet (0.2% in 12% ethanol), and absorbance was measured at 490 nm. ID$_{50}$ (dose resulting in 50% growth inhibition) was computed by using linear regression (EXCEL). Error determinations were computed from 95% confidence intervals of the regression model.

Purification of FAS and FAS Enzyme Assay. FAS was purified from rat liver without enzyme or protease inhibitors by using stepwise polyethylene glycol and ammonium sulfate precipitations, gel filtration, and anion exchange chromatography as previously described (14). FAS was 95% pure as estimated from SDS/PAGE with Coomassie blue staining. FAS activity was measured by spectrophotometrically monitoring oxidation of NADPH at

340 nm as described (15). Briefly, 6.25 μg FAS, 50 μl of 1 M K$_2$PO$_4$ (pH 7.6) at 25°C, 25 nm of acetyl-CoA and 75 nm of NADPH in 0.5 ml reaction volume were monitored at 340 nm in a heated chamber spectrophotometer at 37°C for 3 min to measure background NADPH oxidation. After the addition of 27 nm of malonyl-CoA, the reaction was assayed for an additional 3 min to determine FAS-dependent oxidation of NADPH. One A$_{340}$ unit is equivalent to 80.5 nm of NADPH oxidized. To detect slow-binding inhibition, FAS, C75, water, and K$_2$PO$_4$ buffer were incubated at 37°C for increasing incubation times from 0 to 30 min before commencing the standard FAS assay. Controls consisted of DMSO vehicle without drug.

[5-³H]C75 Binds to FAS in Human Cancer Cells. To demonstrate [5-³H]C75 binding to FAS in a whole cell lysate, 1 × 10⁶ SKBR3 cells plated in 60-mm dishes and cultured as above were incubated with [5-³H]C75 at 10 μg/ml for 4 h. After washing in PBS, cells were solubilized in Laemmli's buffer, resolved with 5% SDS/PAGE, and visualized by using fluorography with En³Hance (New England Nuclear), per manufacturer's instructions.

To confirm that [5-³H]C75 bound to FAS, FAS was immunoprecipitated from SKBR3 cells. A total of 5 × 10⁶ SKBR3 cells were treated with [5-³H]C75 at 10 μg/ml for 4 h. Cells were then lysed and immunoprecipitated by using 20 μg polyclonal rabbit anti-human FAS with protein-A agarose (Roche Molecular Biochemicals) according to the manufacturer's protocol. Immunoprecipitated proteins were resolved with 5% SDS/PAGE and fluorography as above.

Measurement of Fatty Acid Synthesis in HL60 Cells. HL60 human promyelocytic leukemia cells were grown in serum- and fatty

acid-free medium as previously described (16). C75 (10 μg/ml in DMSO) or vehicle alone was added to cultures of 5×10^5 cells. After 2 h, cultures were labeled in triplicate with 1.0 μCi of [U-^{14}C]acetate for 2 h, and total lipids were extracted with chloroform/methanol as previously described (16, 17). Labeled lipids were subjected to TLC (Analtech) in hexane/diethyl ether/acetic acid 90:10:1, which separates the following lipid classes: cholesterol, cholesterol ester, triglycerides, phospholipids, and free fatty acids; ^{14}C-labeled standards were run for each of the lipid classes. After chromatography, labeled lipid classes were identified on a Molecular Dynamics Storm PhosphorImager and quantitated with IMAGEQUANT software.

Results

Design and Synthesis of C75 and Preparation of [5-^{3}H]C75. The iterative two-carbon chain extension characteristic of fatty acid biosynthesis takes place at the β-ketoacyl synthase (KAS) domain of FAS (18, 19). Malonate, bound as its thioester to acyl carrier protein (ACP), enters the active site where the elongating fatty acid chain is covalently linked to a reactive cysteine residue (Fig. 1A). Decarboxylation of the malonyl–ACP takes place to give a reactive enolate anion (Fig. 1B), which attacks the acyl-ACP in a thioester Claisen condensation. The transiently generated tetrahedral oxyanion (Fig. 1C) rapidly decomposes to generate the resting state of KAS and the homologated β-ketoacyl intermediate bound to ACP (Fig. 1D).

The design of inhibitors of FAS was predicated on both an understanding of the mechanism of fatty acid biosynthesis, in particular that of the critical β-ketoacyl synthase step (18) and the well-studied inactivation of FAS by cerulenin (20). We have conducted simple molecular modeling exercises and structure-based searches based on the mechanism outlined in Fig. 1 A–D. A number of potential inhibitors of the reaction were identified. Among these, α-methylene-γ-butyrolactones having hydrocarbon side chains were chosen for further study. Several natural products of this general type are known, notably methylenolactocystin (3, $r = n - C_5H_{11}$), which was shown earlier to have activity against Gram-positive bacteria and prolong the survival of mice inoculated with Ehrlich carcinoma (21). Although the mechanisms of action for either of these activities were unknown, these observations prompted us to survey several side chain lengths to test their antitumor and FAS inhibitory behavior.

Although total syntheses of methylenolactocystin have been reported, we sought a procedurally simpler approach to rapidly assemble alkyl variants of this structural family. A modification of the reaction developed by Carlson and Oyler (22) achieved this goal and is set out in Fig. 1. Deprotonation of p-methoxybenzyl itaconate (1) at low temperature gave the dianion 2, which underwent aldol reaction with a series of aldehydes, RCHO, to give on strongly acidic workup a mixture of γ-lactones 3 and 4. Separation on silica gel and crystallization afforded the pure diastereomers. The results obtained for a representative selection of these compounds are shown in Table 1. One of these, C75 (Table 1, $r = n - C_8H_{17}$), was prepared in tritium-labeled form. Nonanal was reduced by the "sandwich" procedure with sodium [^{3}H]borohydride (23) and oxidized to [1-^{3}H]nonanal, which was elaborated as above to give [5-^{3}H]C75.

α-Methylene-γ-Butyrolactones Selectively Inhibit Growth of Human Cancer Cells in Vitro. The family of α-methylene-γ-butyrolactones was screened for selective growth inhibition based on differential ID_{50} between SKBR3 cells, a human breast cancer cell line with high levels of fatty acid synthesis (24, 25), and normal skin fibroblasts. Table 1 summarizes the structures and growth inhibition obtained for a representative selection of the α-methylene-γ-butyrolactones with varying alkyl side chains. An alkyl

Table 1. In vitro growth inhibition of compounds against SKBR3 human breast cancer cells

Compound	Alkyl side chain ($r =$)	SKBR3 cells ID_{50}, μg/ml	JW fibroblasts ID_{50}, μg/ml
Cerulenin	—C_8H_{13} (1–4 trans diene)	3.3 ± 0.2	7.2 ± 3.1
C83	—$C_{13}H_{27}$	3.9 ± 0.1	10.6 ± 0.3
C81	—$C_{11}H_{23}$	4.8 ± 0.2	29.0 ± 5.0
C77	—C_9H_{19}	5.2 ± 0.3	12.8 ± 1.2
C75*	—C_8H_{17}	5.0 ± 0.1	21.6 ± 1.4
C49	—C_7H_{15}	4.8 ± 0.5	21.7 ± 0.5
C73	—C_6H_{13}	8.4 ± 0.2	12.4 ± 0.8
C271	—C_8H_{13} (1–4 trans diene)	26.3 ± 2.4	N.T.
DMSO control		N.T.	N.T.

N.T., no toxicity identified; C75, racemic mixture of cis and trans isomers.

side chain length of seven or eight carbon atoms gave optimum selective growth inhibition and aqueous solubility.

Initially, a racemic mixture of cis and trans C75 isomers dissolved in DMSO and diluted into buffer displayed selective toxicity against SKBR3 cells (Table 1). Although the cis isomer was less soluble in aqueous solutions, the trans isomer of C75 was soluble in RPMI culture medium and showed activity against SKBR3 cells (ID_{50} 3.2 +/− 0.2) similar to cerulenin (Table 1). The reason for the increased activity of trans-C75 in aqueous vehicle has not been determined. Interestingly, adding the double bonds to C75 in the same location of the alkyl side chain as cerulenin (Table 1, C271) markedly reduced growth inhibitory activity.

C75 Inhibits Purified Mammalian FAS. C75 exhibits characteristics of a slow-binding inhibitor of FAS. Slow-binding inhibitors have been defined as compounds in which equilibrium among enzyme, inhibitor, and enzyme–inhibitor complex occurs on a scale of seconds to minutes and include a number of commonly prescribed drugs (26). Under conditions of the standard assay for FAS activity, enzyme and inhibitor are preincubated for approximately 3 min at 37°C, before initiation of the enzyme reaction by the addition of malonyl-CoA (16). C75 is inactive in this standard assay. When 37°C preincubation times were increased, there was a directly proportional inactivation of FAS activity, consistent with slow-binding inhibition, up to 83% after 30 min (Fig. 2A). Interestingly, C83 (Table 1, $r = n - C_{13}H_{17}$), a compound with an alkyl side chain five carbons longer than C75, is active in the standard assay, suggesting that alkyl side chain length influences enzyme-inhibitor binding (data not shown).

C75 Inhibits Fatty Acid Synthesis in Vitro and in Vivo. HL60 human promyelocytic leukemia cells proliferate in serum and fatty acid-free culture media and as such provide a convenient in vitro model for the study of fatty acid synthesis in cancer cells (16). In this environment, the cells are solely dependent on endogenous fatty acid synthesis for the production of storage, structural, and signaling lipids. As shown in Fig. 2B, after 4 h exposure to drug at 5 μg/ml, [U-^{14}C]acetate incorporation into phospholipids and triglycerides was inhibited by 87% and 89%, respectively. The transfer of label into the free fatty acid pool was also reduced by 44%. In HL60 cells grown in 10% FBS, fatty acid synthesis was down-regulated, but the fractional inhibition by C75 was similar to serum-free conditions (27). C75 treatment of HCT116 human colon cancer cells (27) and MCF7 and SKBR3 human breast carcinoma cells grown with 10% FBS showed similar magnitudes of pathway inhibition (data not shown).

C75 Binds to Human FAS. Although C75 inhibits purified mammalian FAS and inhibits fatty acid synthesis, we used [^{3}H]C75 to
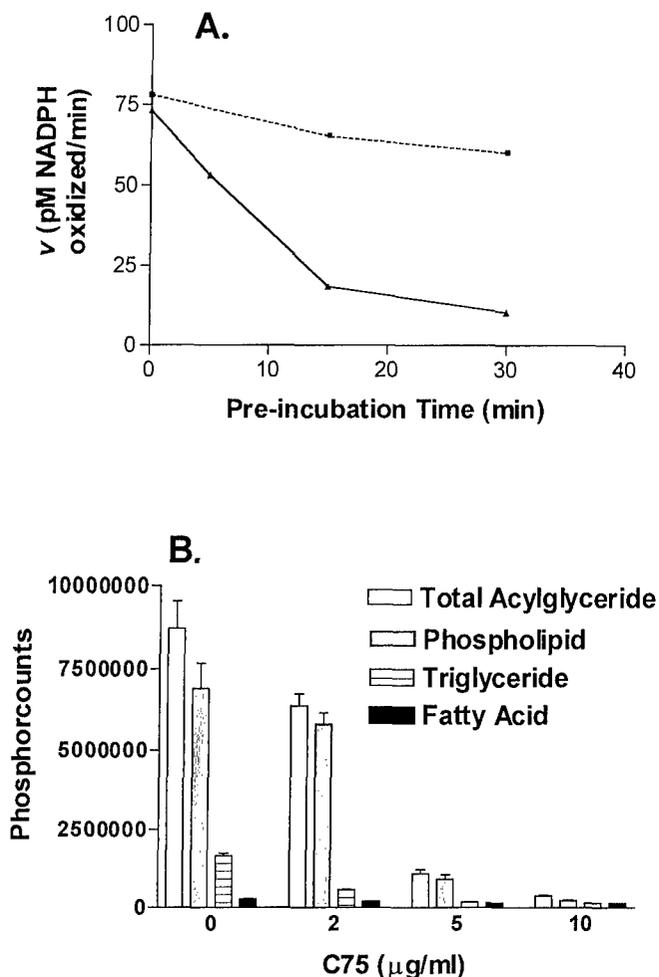
Fig. 2. C75 inhibits FAS and fatty acid synthesis. (A) C75 has characteristics of a slow-binding inhibitor of type I FAS. With increasing preincubation time of FAS and C75, there is greater inhibition of FAS activity (solid line) compared with control (dotted line). (B) C75 inhibits fatty acid synthesis in HL60 cells. With increasing doses of C75, [U-^{14}C]acetate incorporation into acylglycerides is inhibited by 80%. Incorporation into the free fatty acid pool is reduced by about 50%.

Fig. 3. C75 labels FAS. SDS/PAGE and fluorography of SKBr3 cell lysate after 4 h exposure of cells to ^{3}H-C75 [5 μg/ml]. Lane 1, whole cell lysate. Lane 2, FAS immunoprecipitation. Note the labeling of the approximately 250-kDa band, which represents human FAS.

examine its interaction with FAS in whole cells. SKBr3 cells are ideal for these studies as they express the highest per-cell amounts of FAS yet described in human established cancer cell lines. After a 4-h exposure of SKBr3 cells to [^{3}H]C75, a band of approximately 250 kDa is labeled from a whole cell lysate (Fig. 3, lane 1). Immunoprecipitation with anti-human FAS from similarly labeled cells, identifies the 250-kDa protein as FAS (Fig. 3, lane 2). In a parallel experiment, the 250-kDa band was confirmed as FAS by an immunoblot with polyclonal rabbit anti-human FAS antibodies (data not shown). The additional 60-kDa band labeled in lane 1may represent [^{3}H]C75 binding to (*i*) albumin from the culture medium, (*ii*) an FAS fragment, or (*iii*) binding to an unknown cellular target.

Discussion

C75 treatment of MCF-7 human breast cancer xenografts has shown significant anti-tumor activity with concomitant inhibition of fatty acid synthesis in tumor tissue and normal liver (13). Importantly, histopathological analysis of normal tissues after C75 treatment showed no adverse effects on proliferating cellular compartments, such as bone marrow, gastrointestinal tract,
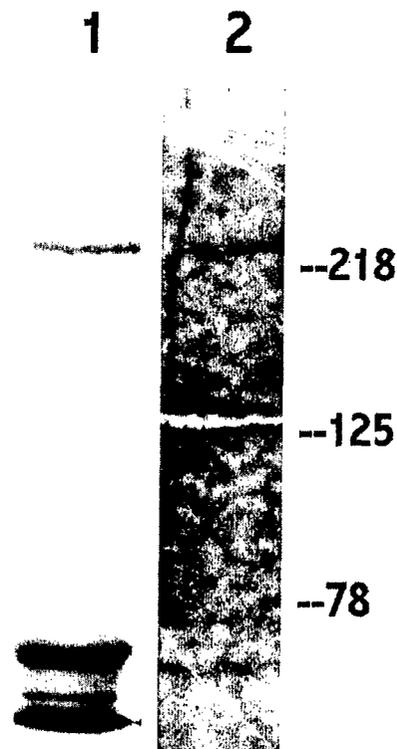
skin, or lymphoid tissues. Transient reversible weight loss was the only toxicity noted.

C75 treatment of human cancer cells *in vitro* led to rapid inhibition of fatty acid synthesis, followed by inhibition of DNA replication culminating in apoptosis (27). Further *in vitro* and *in vivo* mechanistic studies have shown that high levels of malonyl-CoA resulting from C75 treatment is likely the mediator of apoptosis in cancer cells (13). Although these biological data strongly suggested that C75 action occurred by means of inhibition of FAS, this study provides direct evidence of C75 interaction with human FAS.

The synthetic strategy used for FAS inhibitor design led to a family of α-methylene-γ-butyrolactones with anti-tumor activity. Structurally, they all lack the reactive epoxide present on cerulenin, enhancing chemical stability and specificity (Fig. 1). Moreover, C75 synthesis was accomplished in two steps resulting in high yields of a pure crystalline stereoisomer with aqueous solubility.

C75 demonstrated kinetics consistent with slow binding inhibition of purified mammalian FAS by C75 (Fig. 2*A*) in which 50% inhibition occurred within 10 min. Slow-binding inhibition is a desirable pharmacological characteristic because, in contrast to classical inhibitors, buildup of substrates following enzyme inhibition is less likely to reverse the inhibition because of isomerization of the enzyme–inhibitor complex (26). Slow-binding inhibitors include many important drugs, such as methotrexate, allopurinol, and acyclovir (26).

Reflecting its inhibition of purified FAS, C75 is an effective inhibitor of fatty acid synthesis both *in vitro* and *in vivo*. C75 significantly inhibited radiolabeled acetate incorporation into phospholipids and triglycerides in HL60 cells (Fig. 2*B*). Because these biochemical measurements took place within 4 h of drug exposure, they represented fatty acid synthesis pathway inhibi-

tion in viable cells before the onset of early apoptotic events as measured by flow cytometry (27).

In whole cell lysates of SKBr3 human breast cancer cells treated with [³H]C75, labeled C75 bound to FAS with a high degree of specificity. In addition to FAS, an additional 60-kDa protein was labeled, which represents binding to serum proteins from the culture medium, a FAS fragment, or an additional unknown cellular target. In addition to specificity, these data demonstrate that C75 binds tightly to FAS surviving highly denaturing conditions. This tight-binding may represent a covalent interaction with FAS, which is often characteristic of slow-binding inhibitors.

In summary, we have described the design and synthesis of a chemically stable inhibitor of mammalian FAS, C75. This compound afforded the first demonstration of systemic inhibition of fatty acid synthesis resulting in specific anti-tumor cytotoxicity. The synthetic strategy, which led to these compounds, will likely yield other inhibitors, other type I and type II fatty acid synthases. Further development of C75 should enable extensive study of fatty acid synthesis inhibition in human cancer and other diseases associated with dysfunctional fatty acid synthesis activity.

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Science

Reduced Food Intake and Body
Weight in Mice Treated with
Fatty Acid Synthase Inhibitors

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With the escalation of obesity-related disease, there is great interest in defining the mechanisms that control appetite and body weight. We have identified a link between anabolic energy metabolism and appetite control. Both systemic and intracerebroventricular treatment of mice with fatty acid synthase (FAS) inhibitors (cerulenin and a synthetic compound C75) led to inhibition of feeding and dramatic weight loss. C75 inhibited expression of the prophagic signal neuropeptide Y in the hypothalamus and acted in a leptin-independent manner that appears to be mediated by malonyl–coenzyme A. Thus, FAS may represent an important link in feeding regulation and may be a potential therapeutic target.

Excess body weight is a major health problem in developed nations, affecting over 50% of the U.S. population (1), and is increasing both in prevalence and severity. This condition is associated with increased risk of type II diabetes, cardiovascular and cerebrovascular diseases, and increased mortality (1). The magnitude of this health problem and the recent difficulties with several weight-loss therapies emphasize the need for different approaches to weight-loss therapy.

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FAS catalyzes the reductive synthesis of long-chain fatty acids from acetyl–coenzyme A (acetyl-CoA) and malonyl-CoA (2). The mechanism through which two carbon units from malonyl-CoA are sequentially added to the growing fatty acid chain is unique among vertebrates, making FAS an attractive target for the design of therapeutic agents. Cerulenin, a natural FAS inhibitor, forms a well-characterized complex with the enzyme (3); however, its epoxide structure is thought to limit its utility as a drug. We synthesized a FAS inhibitor, C75. Intraperitoneal (ip) injection of mice with C75 leads to a 95% reduction in ^{14}C-acetate incorporation into fatty acids and to a 110% increase in the level of hepatic malonyl-CoA, the principal substrate of FAS (Web fig. 1) (4).

To investigate the physiological conse-quences of in vivo inhibition of fatty acid synthesis on global lipid metabolism, we administered cerulenin [60 mg/kg body weight per day (mg/kg/day) for 7 days] or C75 (single dose of 7.5 to 30 mg/kg) to mice by ip injection. We observed profound weight loss following treatment (Fig. 1). Weight loss occurred in a dose-dependent manner and persisted for a duration that increased with dose. In all cases, treated mice recovered lost body weight after the effect of the drug dissipated, arguing against induction of a persistent state of wasting. The treatment was well tolerated by the mice with no obvious toxicity. Necropsy and histological analysis of all major organs in treated mice revealed no adverse pathology and plasma alanine aminotransferase activity was unchanged (Web fig. 2A). In addition, C75-induced weight loss was observed in mice lacking interleukin-1r and tumor necrosis factor–α r1a receptors, suggesting that the weight loss is not mediated by an inflammatory response (Web fig. 2B) (4).

C75-induced weight loss was due primarily to inhibition of feeding. Intraperitoneal administration of C75 (15 mg/kg) reduced food intake by more than 90% over the first 24 hours (Fig. 1C). Feeding then returned to normal over the next 48 to 72 hours as the drug effect dissipated. Inhibition of feeding was observed within 20 min of treatment (Web fig. 3) (4). Forced feeding largely reversed the observed weight loss (5).

There was a 40% reduction in both water intake and urinary output in C75-treated mice (6). This is consistent with a change in osmotic balance resulting from decreased intake of salts and other solutes in the diet. However, we cannot exclude the possibility that some of the observed weight loss is due to water loss. The loss of adipose mass was

accompanied by a reduction of lean body mass typical of that observed in fasting (6).

To determine whether C75-induced weight loss is attributable entirely to suppression of feeding, we compared ip C75 treatment with fasting. C75-treated mice lost 45% more weight than did the fasted animals (Fig. 2A). Because the normal response to fasting is to reduce energy utilization (7), this result suggests that C75 treatment may allow maintenance of a normal energy utilization as well as inhibition of feeding. No gross changes in the animals' activity were observed (6).

Neuropeptide Y (NPY) acts in the hypothalamus as a central member of a coordinated group of neuropeptides, regulated by adiposity and feeding status, that control feeding and energy utilization. NPY promotes feeding (8) and its expression increases in the fasted state (9). To ascertain whether C75 affects NPY expression in the hypothalamus, we performed Northern blot analysis of hypothalamic tissue from fed, fasted, and C75-treated mice (Fig. 2). As expected, fasting markedly up-regulated NPY mRNA. However, hypothalamic NPY mRNA levels in C75-treated mice were even lower than those in fed control mice, even though the C75-treated mice had not eaten. This indicates that C75 inhibits feeding, at least in part, by blocking NPY-induced feeding.

We next examined whether NPY could reverse C75-induced anorexia. Intracerebroventricular (ICV) injection of NPY (2.5 μg) into mice pretreated with ip injection of either vehicle or C75 (C75-NPY) rapidly led to voracious feeding, whereas ICV injection of vehicle had no effect on feeding. Total food intake within 1 hour by C75-NPY–treated mice was similar to that by mice treated with NPY alone, and intake was nine times greater than that by C75-treated mice (Fig. 2C). This result indicates that the pathways downstream of NPY are functional in C75-treated mice and that C75 acts upstream of NPY. C75 also suppresses voracious feeding in fasted animals that have already up-regulated their endogenous NPY (Web fig. 3) (4), suggesting that C75 must have additional feeding regulatory effects.

Leptin is elevated in the fed state and inhibits NPY production and feeding (10) in a manner similar to that observed with C75 treatment. Because leptin is synthesized primarily in white adipose tissue (11), a primary site of fatty acid synthesis, we tested whether it mediates the effects of C75. Serum leptin levels were reduced rather than elevated in C75-treated mice (Fig. 3A), indicating that leptin does not mediate the C75 signal. Northern blot analysis of leptin mRNA levels in white adipose tissue supported this observation (6).

If C75 acts by a leptin-independent mech-anism, it should reduce the obesity of leptin-deficient ob/ob mice (11). A 2-week course of ip C75 treatment was found to reduce body weight by 10 g (Fig. 3, B and C). Histological examination of the liver from C75-treated mice revealed a dramatic normalization of the fatty liver that is characteristic of ob/ob mice (Fig. 3D). There was no evidence of histological abnormality. In addition, C75 treatment corrected the hyperglycemia observed in control ob/ob mice, leading to a nearly threefold reduction in serum glucose levels. A 24-hour ip treatment of wild-type mice had no effect on serum glucose beyond that attributable to fasting (Web fig. 4) (4).

The role of metabolism in controlling feeding is well established. The infusion of physiological fuels such as glucose (12) or fatty acids [reviewed in (13)] has long been known to inhibit feeding. Furthermore, inhibitors of glucose or fatty acid oxidation (e.g., 2-deoxyglucose or mercaptoacetate) stimu-late feeding. However, the FAS inhibitors described here operate by a distinct mechanism because they induce a feeding-inhibitory signal in the absence of an added physiological fuel.

A physiological link between feeding inhibition and fatty acid synthesis is consistent with the fact that synthesis occurs only during energy surplus, when excess physiological fuels are channeled into energy storage. However, the observation that FAS inhibition produces the same response predicted for active fatty acid synthesis argues against an effect of the end product of the pathway. Rather it suggests that an intermediate, preceding FAS in the pathway, mediates the metabolic signal. We postulate that elevation in the level of the FAS substrate, malonyl-CoA, during fatty acid synthesis may be linked to feeding control (Fig. 4A). It is unlikely that inhibition of fatty acid synthesis per se causes feeding inhibition. In previous studies (14), treatment of mice with inhibitors of acetyl-
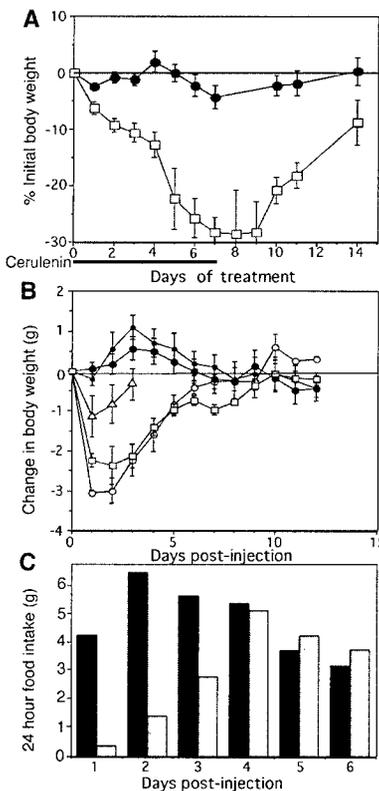

Fig. 1. Effect of FAS inhibitors on body weight and food intake. (A) Female BALB/c mice were treated by ip injection of RPMI medium vehicle (●) or cerulenin at 60 mg/kg/day (□) for 7 days. (B) Female BALB/c mice were given a single ip injection of C75 at doses of 7.5 (△), 15 (○), or 30 (□) mg/kg or (●) RPMI vehicle. Mice were housed in metabolic cages and monitored for body weight, food and water intake, and urine and feces output. Mean change from initial body weight is expressed ± SEM. (C) Total food intake per day by mice treated with vehicle (black bars) or C75 (15 mg/kg) (gray bars) from (B). Experiments consisted of three animals per group and were repeated four times.
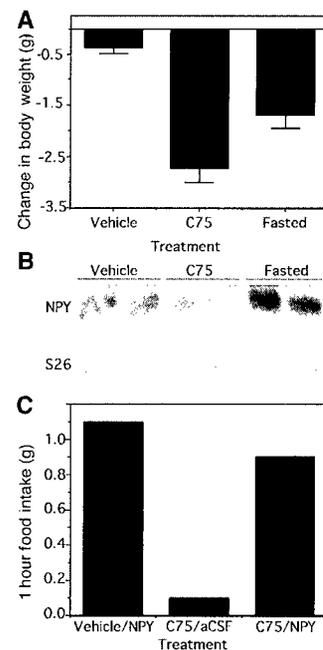

Fig. 2. Role of NPY in regulation of feeding by C75. (A) Male BALB/c mice were preweighed and fasted or were treated with vehicle or C75 (30 mg/kg) and fed ad libitum. After 24 hours, mice were weighed (n = 6 mice, repeated seven times). Change from initial body weight is expressed as mean ± SEM. (B) Hypothalamic RNA from mice in (A) was analyzed by Northern blot using random primed probes for transcripts encoding NPY and the ribosomal protein S26 (which was used as a loading control) (n = 6 mice). (C) Mice were pretreated for 4 hours with C75 (30 mg/kg) or with RPMI by ip injection, anaesthetized by inhaled metofane, and given an ICV injection of 2.5 μg of NPY (2.5 μl) or artificial cerebrospinal fluid vehicle. Mice were observed for feeding behavior and were monitored for food intake for 1 hour (three repetitions of three mice per group, nine mice total).

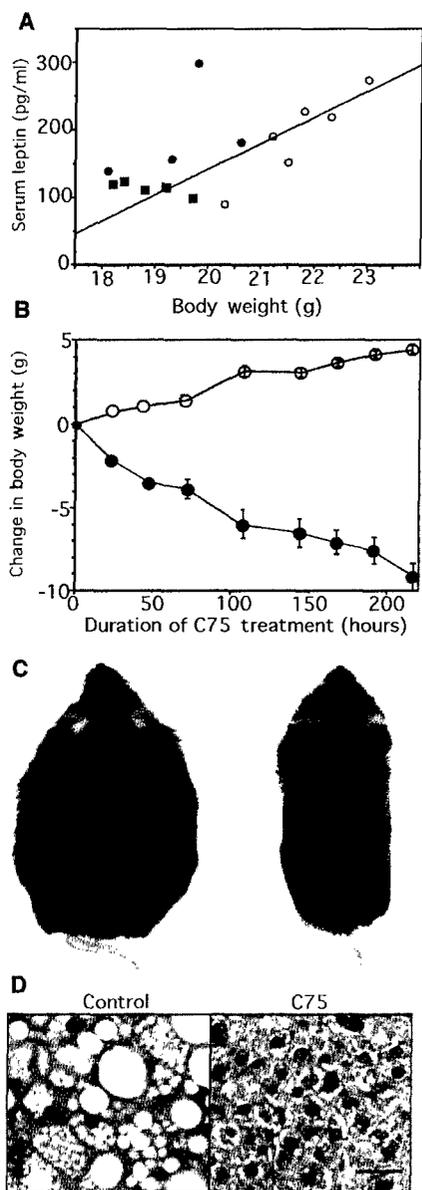CoA carboxylase (ACC), the enzyme preceding FAS in the pathway, inhibited fatty acid synthesis but not feeding. Administration of ACC inhibitors would block malonyl-CoA production and, thus, would not be expected to inhibit feeding. In contrast, inhibition of FAS by C75 results in elevation of malonyl-CoA levels in vivo that may mimic fatty acid synthesis and, thus, the fed state.

This model predicts that feeding inhibition by FAS inhibitors should be attenuated by inhibitors of ACCs. To test this, we pretreated mice with either the ACC inhibitor TOFA or vehicle by ICV injection and examined the ability of an ip injection of C75 to inhibit feeding. TOFA largely restored food intake in C75-treated mice (Fig. 4B), supporting the hypothesis that malonyl-CoA mediates feeding inhibition. In addition, the efficacy of centrally administered TOFA argues for a central nervous system (CNS) mechanism of action. ICV administration of C75 inhibited feeding by 82% (Fig. 4C), supporting the central target action of C75.

We have observed expression of both FAS and ACC in select neurons in the brain, most notably in the arcuate nucleus of the hypothalamus (15). Our studies with [5-³H]-C75 indicate that the drug enters the brain (Web fig. 5) (4). Thus, it is conceivable that these inhibitors act directly on the brain to control feeding, either in NPY-producing neurons or in neurons that act on them.

Fatty acid synthesis regulates fatty acid oxidation by a well-characterized regulatory mechanism (16). In this paradigm, malonyl-CoA levels rise during fatty acid synthesis and result in inhibition of carnitine palmitoyl transferase 1–mediated uptake of fatty acids into the mitochondrion. This results in elevation of cytoplasmic long-chain fatty acyl CoAs and diacylglycerol, molecules that may play a role in signaling, which leads to the proposal that malonyl-CoA levels act as a signal of the availability of physiological fuels (17). One such role proposed for malonyl-CoA is the mediation of nutrient-stimulated insulin secretion in the beta cell. Glucose-sensing neurons that regulate feeding in the hypothalamus share many features with the beta cell, including expression of glucokinase and the adenosine triphosphate–sensitive potassium channel (18). Our data support the prediction (17) that malonyl-CoA may signal fuel status in hypothalamic neurons.

Our studies provide evidence that FAS has a role in the control of feeding behavior and, thus, may represent a therapeutic target for the control of appetite and body weight.

Fig. 3. Leptin independent of action C75. (A). Male BALB/c mice ($n = 5$) were fasted (●) or received ip injections of RPMI (○) or of C75 (30 mg/kg) (■) and were free-fed for 24 hours, weighed, and then exsanguinated. Serum leptin was determined with a Quantikine murine leptin enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minnesota) and plotted against total body weight. (B) Male ob/ob (C57BL/6OlaHsd-Lep^ob) (Harlan, Oxon, England) mice ($n = 3$) were treated with ip injections of RPMI (○) or of C75 (22 mg/kg) (●) every third day (representative of two experiments). Change in body weight is displayed as mean ± SEM. (C) Representative vehicle- (left) and C75-treated (right) mice from (B) after 14 days of treatment. (D) Livers from vehicle- (control) and C75-treated mice were formalin fixed and paraffin embedded. Tissue sections (4 μm) were stained with hematoxylin and eosin. Bar, 50 μm.
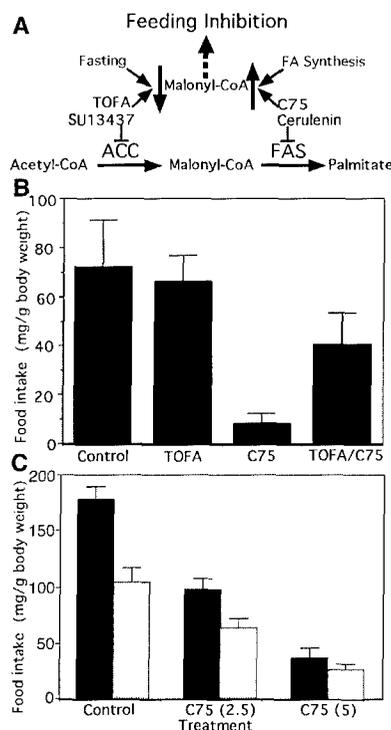

Fig. 4. Feeding regulation by malonyl-CoA. (A) Model of feeding regulation by inhibitors of FAS via malonyl-CoA. (B) BALB/c mice were anesthetized with metofane and received ICV injections of 2 μg of TOFA or dimethyl sulfoxide vehicle. After 2 hours recovery, mice received ip injections of C75 (15 mg/kg) or of RPMI vehicle and were monitored for total food intake over 2 hours. (C) Mice were anesthetized and received ICV injections of RPMI (2 μl) or of C75 (2.5 or 5 μg/μl), and food intake was monitored over 2 and 4 hours (gray and solid bars, respectively). (B) and (C) combine results from three experiments with $n = 3$ mice for each experiment (nine mice total).

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Increased Fatty Acid Synthase is a Therapeutic Target in Mesothelioma

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Running Title: FAS is a therapeutic target in mesothelioma.

Key words: mesothelioma, fatty acid synthase, immunohistochemistry

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Abstract

Many common human cancer tissues express high levels of fatty acid synthase, the primary enzyme for the synthesis of fatty acids, and the differential expression of FAS between normal and neoplastic tissues has led to the consideration of FAS as a target for anticancer therapy. To investigate the potential of targeting FAS for the treatment of pleural mesothelioma, we first determined whether FAS is overexpressed in human mesothelioma. By immunohistochemistry, we found 22 of 30 human mesothelioma tissue samples tested to express significantly increased levels of FAS compared to normal tissues, including mesothelium. To further explore FAS as a therapeutic target in mesothelioma, we established a nude-mouse xenograft model for human mesothelioma using the H-Meso cell line. Intraperitoneal xenografts of this cell line have high levels of FAS expression and fatty acid synthesis pathway activity, and grow along mesothelial surfaces in a manner similar to the growth pattern of human mesothelioma. Growth of these tumor xenografts was essentially abolished in mice treated with weekly intra-peritoneal injections of C75, a synthetic, small molecule inhibitor of FAS, at levels that resulted in no significant systemic toxicity except for reversible weight loss. These results suggest that FAS may be an effective target for pharmacological therapy in a high proportion of human mesotheliomas.

Introduction

Mesothelioma is an uncommon malignant neoplasm derived from mesothelial cells that is most often caused by exposure to asbestos. The incidence of mesothelioma in the United States is approximately 2,000-3,000 cases per year, and this incidence is steadily increasing (1). Mesothelioma, although slow growing, spreads diffusely along the mesothelial surfaces of the pleura, peritoneum, or pericardium and is thus difficult to treat by surgical resection. Furthermore, mesothelioma generally responds poorly to radiation therapy or conventional chemotherapy (2, 3) and the 3-year survival for this cancer is less than 5% (4). Thus, there is a need for novel therapeutic approaches to treat this cancer.

One therapeutic target that has not previously been considered for mesothelioma treatment is the pathway for the endogenous synthesis of fatty acid. Fatty acid synthase (FAS), the principal enzyme in this pathway, is highly expressed in many common human tumors (5). This is in contrast to normal human tissues where FAS is down-regulated due to our ingestion of high levels of dietary fatty acids. The preferential expression of FAS in cancer cells has recently been exploited as a target for anti-cancer chemotherapy. For example, significant anti-tumor activity against human breast (6) and prostate (7) xenografts that express high levels of FAS has been achieved using a novel pharmacological inhibitor of FAS, C75. The present study was undertaken to assess the feasibility of treating mesothelioma with pharmacological inhibitors of FAS. For this purpose, we first evaluated a series of mesothelioma tumors for FAS expression using immunohistochemistry. Encouraged by a finding that mesotheliomas frequently

overexpress this enzyme, we then developed a nude-mouse xenograft model of a FAS-overexpressing human mesothelioma cell line to test the hypothesis that FAS inhibition will exhibit anti-neoplastic activity.

Methods

Mesothelioma Tissues and Immunohistochemistry for FAS. Paraffin embedded samples from 30 cases of mesothelioma were obtained from surgical pathology, autopsy, and consultation files at the Johns Hopkins Bayview Medical Center. All patients had a previous history of exposure to asbestos. Immunohistochemistry for FAS was performed on tissue sections using a mouse monoclonal anti-human FAS antibody (8) at 1:2000 on the Dako Immunostainer using the LSAB2 detection kit. FAS expression was evaluated independently and semiquantitatively for both intensity and percent area of tumor stained by two pathologists (EG and FK). The fraction of positive cells was scored using a four-tiered scale (<10%=1, 11-50%=2, 51-80%=3, and >80%=4); and staining intensity was scored from 0-3+ as described (9). The overall FAS score was the product of both the intensity and fraction of positive cells score. Cases with an overall score ≤ 3 were considered negative. Adjacent lung and soft tissues and mesothelium were also stained for FAS and the same grading system was used for assessment.

Fatty acid synthesis inhibitor. C75 was synthesized in the laboratory of C.A. Townsend in the Department of Chemistry, at the Johns Hopkins University (10). C75 is an α-

methylene-γ-butyrolactone with an 8-hydrocarbon side chain. C75 is a slow-binding

inhibitor of mammalian FAS.

Cell lines, culture conditions, and metabolic labeling. The human mesothelioma cell

line, H-Meso (11) was maintained in DMEM with 10% fetal bovine serum. Cells were

screened periodically for *Mycoplasma* contamination (Gen-probe). All inhibitors were

diluted from stock 5 mg/ml solutions in DMSO. For fatty acid synthesis activity

determinations, 5×10^4 cells/well in 24 well plates were pulse labeled with [U-^{14}C]-acetate

after exposure to drug, or vehicle in triplicate for each concentration. Lipids were then

extracted and quantified as described previously (12). Pathway activity was determined

after 2 hours of inhibitor exposure.

Xenograft Studies. Intraperitoneal xenografts of the human mesothelioma cell line, H-

Meso in nu/nu female mice (Harlan) were used to study the anti-tumor effects of C75 *in*

vivo. All animal experiments complied with institutional animal care guidelines.

Approximately 10^7 (0.1 ml packed) H-Meso cells were xenografted from culture in

DMEM supplemented with 10% FBS.

To compare fatty acid synthesis activity in tumor to a normal tissue, tumor xenografts

and liver tissue from three mice were *ex vivo* labeled with [U-^{14}C], lipids were extracted

and counted as described (12). In a parallel experiment, to study FAS expression *in vivo*,

tumor and normal tissues from a xenograft were fixed in neutral-buffered formalin,

processed for routine histology, and immunohistochemistry for FAS was performed as

above. To test FAS inhibitory treatment on this mesothelioma xenograft model, we began intraperitoneal C75 treatment 2 weeks after tumor inoculation. Six mice were treated intraperitoneally with an initial dose of C75 at 40 mg/kg in 0.1 ml RPMI, then with weekly doses of C75 at 30 mg/kg in 0.1 ml RPMI. 5 mice were treated with vehicle control. Dosing was based on a single dose LD_{10} determination of 40 mg/kg in BALB/c mice; 30 mg/kg has been well tolerated in outbred nude mice . The experiment was terminated after one month as the control group underwent a 15% increase in weight due in part to a combination of tumor and malignant ascites.

Results and Discussion

By immunohistochemistry, 22 of the 30 (73%) mesothelioma cases were scored as FAS positive, while 8 (27%) were scored negative. Of the 22 FAS positive cases, 13 (43%) showed high levels of expression defined as an overall score ≥ 6, while 9 (30%) had moderate levels of FAS expression, with scores of 4-5 (Table 1). FAS expression is not limited to any histological subtype, as epithelial, mixed, and sarcomatous mesotheliomas in our series all displayed similar levels of FAS expression. Figure 1 illustrates immunohistochemical localization of FAS in clinical cases of malignant mesothelioma. Both epithelial (Figure 1 A & B) and sarcomatoid (Figure 1C) show intense cytoplasmic reactivity, while histologically benign mesothelial cells (Figure 1D) have undetectable levels of FAS. Variable FAS expression was noted in normal adipose tissue and in reactive Type II pneumocytes adjacent to the tumors in the lung (data not shown). In

6

comparison to carcinomas, high levels of FAS appear to be more common in mesothelioma than in breast cancer (8, 13, 14), but less frequent than in colon cancer (15), where high levels of expression are ubiquitous.

Based on the high levels of FAS expression in human mesothelioma, we chose the H-Meso human mesothelioma cell line as a model system both for its high level of endogenous fatty acid synthesis *in vitro* and *in vivo*, and for its ability to grow in athymic nude mice recapitulating human disease. One month post intraperitoneal inoculation of H-Meso cells, multiple tumors stud the internal surfaces of the abdominal peritoneum, bowel and mesentery, similar to the disease in humans (Figure 2A & C). In addition, the xenograft expresses high levels of FAS by immunohistochemistry similar to clinical tumor tissue (Figure 2C & D). These high levels of FAS expression by immunohistochemistry are reflective of high levels of endogenous fatty acid synthesis. The H-Meso xenograft has over a 25-fold increased fatty acid synthesis activity compared to liver as measured by *ex vivo* $[U^{14}C]$acetate incorporation into total lipids (Figure 3A). This level of fatty acid synthesis *in vitro* for mesothelioma is even higher than what has previously been observed in breast and prostate cancer cells (data not shown).

To establish our ability to pharmacologically inhibit FAS, we treated H-Meso cultures with cerulenin, a broad spectrum inhibitor of Type I and II FAS (16, 17), and C75, a novel, chemically stable, inhibitor of FAS (10). Both C75 and cerulenin inhibit fatty acid synthesis in H-Meso cells (by approximately 30% and 70% respectively) *in vitro* as

measured by [U^{14}C]acetate incorporation into lipids (Figure 3B). This level of fatty acid synthesis inhibition in other human cancer cells results in significant cytotoxicity *in vitro* (6). However, we were unable to demonstrate *in vitro* cytotoxicity of these agents on H-Meso cells using clonogenic assays due to the poor colony forming ability of the H-Meso cells. Hence, C75 was tested for anti-tumor activity directly in H-Meso xenografts.

To test the effect of C75 on H-Meso xenografts, tumor-bearing mice were treated with C75 beginning two weeks post-tumor inoculation. By one month, all control animals (Figure 1A) had widespread studding of the mesentery by tumor nodules, consisting of mesothelioma cell clusters ranging in size from <1 to 5 mm. One untreated animal also had 0.8 ml of malignant ascites. Among the treated animals, none had ascites, 2 had no evidence of tumor, and 3 had single tumor nodules ranging in mass from 0.1-1.1 g. None of the treated animals developed the multiple tumor seeding of the abdomen seen in control animals. A representative C75 treated animal with no gross or microscopic tumor is demonstrated in Figure 2.

Similar to the previous experience of treating breast and prostate xenografts (6, 7), transient reversible weight loss was noted. Histological analysis of normal host tissues failed to show evidence for significant acute or chronic toxicity other than a slight increase in fibrous adhesions in the abdominal cavity of treated mice. One animal in the C75 treatment group died within 24 h of the first dose; no deaths occurred with subsequent treatments. It could not be determined whether this death was due to the effect of the drug or other causes.

8

Our finding of FAS overexpression in mesothelioma tissues parallels observations of increased FAS expression in a variety of common human cancers including breast, prostate, colon, endometrium and ovary (5). While the biological advantage of endogenous fatty acid synthesis by human cancers has not yet been elucidated, inhibition of fatty acid synthesis at the FAS step ultimately leads to cancer cell death through inhibition of macromolecular synthesis and apoptosis (18, 19). The link between apoptosis and FAS inhibition is likely due to accumulation of the FAS substrate, malonyl-CoA rather than the reduction of fatty acid synthesis *per se* (6). Inhibition of FAS at doses used in this study leads to high levels of malonyl-CoA as measured in bacteria (20), cancer cell lines (6), and in mouse liver (21). The high levels of malonyl-CoA result from decreased utilization by FAS and continued synthesis by acetyl-CoA carboxylase driven in part by decreasing fatty acid synthesis.

While malonyl-CoA has only recently been implicated in apoptosis, this is not surprising given its key role as a regulator of intermediary metabolism. In addition to its role as a substrate for fatty acid synthesis, malonyl-CoA is a potent inhibitor of carnitine palmitoyltransferase-I (CPT-I), the rate limiting enzyme in fatty acid oxidation (22). CPT-I is located on the outer membrane of the mitochondria where it esterifies long-chain acyl-CoA's to carnitine allowing their entry into the mitochondria for oxidation. Physiologically, malonyl-CoA inhibits CPT-I during fatty acid synthesis to prevent the concomitant oxidation of newly synthesized fatty acid. Inhibition of CPT-I with etomoxir has produced apoptosis *in vitro* (23) and the recent association of CPT-1 with

9

Bcl-2 on the mitochondrial surface supports this observation (24). In our studies, inhibition of acetyl-CoA carboxylase with TOFA, prevented the rise in malonyl-CoA following FAS inhibition and significantly reduced the apoptotic response (6).

Most importantly, our studies suggest that C75 could have significant anti-cancer activity in a significant proportion of mesothelioma patients. The possibility of treating mesothelioma with pharmacological inhibitors of FAS has great significance because human mesothelioma remains a disease largely refractory to conventional treatment. Treatment of H-Meso xenograft mice with the FAS inhibitor C75 led to significant reductions in mesothelioma tumor burden in all treated animals and also altered the growth pattern of the tumor from diffuse abdominal involvement to more localized tumor masses. While direct intraperitoneal delivery of the C75 to the tumor xenografts may increase the efficacy of this agent, it is also notable that systemic C75 has been found to be effective in the treatment of subcutaneous human tumor xenografts (6, 7). Further experiments are need to determine whether local, systemic, or a combination of drug delivery modalities will be most effective for treatment of mesothelioma by inhibitors of FAS.

Acknowledgement

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Table 1: FAS Expression in Subtypes of Malignant Mesothelioma

	Epithelial	Mixed	Sarcomatous	Totals
High FAS	9 (30%)	2 (7%)	2 (7%)	13 (43%)
Low FAS	7 (23%)	1 (3%)	1 (3%)	9 (30%)
Negative FAS	5 (17%)	1 (3%)	2 (7%)	8 (27%)
Totals	21 (70%)	4 (13%)	5 (17%)	30 (100%)

Figure Legends

Figure 1: Immunohistochemical Expression of FAS in Human Mesothelioma

Panel A is a low magnification micrograph (40X) of an epithelial mesothelioma with

high FAS expression. Note the intense brown staining localizing FAS. Higher

magnification (400X)of the same tumor (Panel B), illustrates the cytoplasmic localization

of FAS in the tumor cells, which corresponds to the site of endogenous fatty acid

synthesis. Panel C depicts high levels of FAS expression in a sarcomatoid mesothelioma

(400X). Note the prominent spindle cell morphology with cytoplasmic localization of

FAS. In contrast, FAS is undetectable in histologically normal mesothelial cells (Panel

D, white arrow, 400X).

Figure 2: Mouse xenograft model of H-Meso cells treated with C75

Panel A shows a control animal with widespread studding of the mesentery by tumor

nodules consisting of clusters of mesothelioma cells (white arrows). Panel B shows a

C75 treated animal with no gross or microscopic tumor. High levels of FAS expression

by immunohistochemistry are present in the control tumor nodules (Panel C, white

arrows, 20X) in contrast to the surrounding mouse colon. Higher magnification of the

tumor nodules (Panel D, 400x) show intense FAS expression.

Figure 3. The H-Meso xenograft has high levels of fatty acid synthesis inhibited by C75 and cerulenin.

Panel A. Fatty acid synthesis is about 15-fold higher in the H-Meso xenograft compared to liver from the same animals as measured by *ex vivo* [U^{14}C]acetate incorporation into lipids (n=3, p<0.004, t-test). Within 2 h of drug administration, C75 at 10 μg/ml and cerulenin significantly inhibit fatty acid synthesis in H-Meso cells *in vitro* (Panel B). Student t-test comparing treated cells to control, in triplicate, are as follows: C75 [5 μg/ml], p=0.062; C75 [10 μg/ml], p=0.051; cerulenin [5 μg/ml], p=0.003; cerulenin [10 μg/ml], p=0.001 (GraphPad Prism Software). Error bars represent standard error of the mean.

Figure 1

Figure 2

Figure 3

A.

[¹⁴C] acetate
(cpm/mg wet tissue)

Liver   H-meso Xenograft

B.

[¹⁴C] acetate
Incorporation into
Lipids (% of control)

Control   C75 5µg/ml   C75 10µg/ml   Cerulenin 5 µg/ml   Cerulenin 10 µg/ml

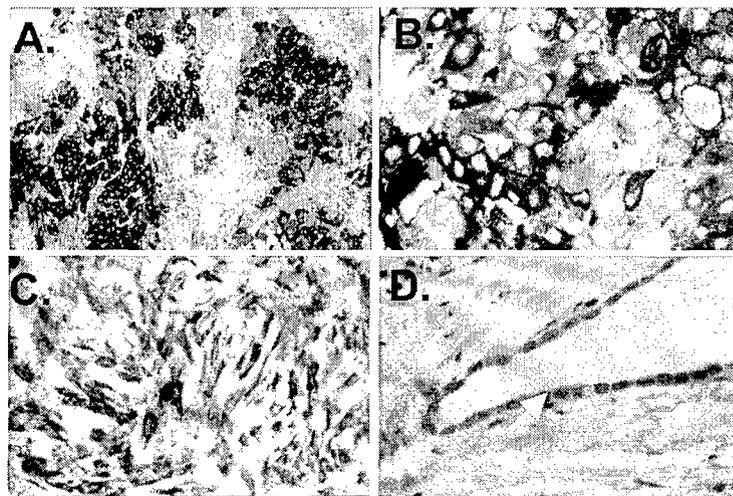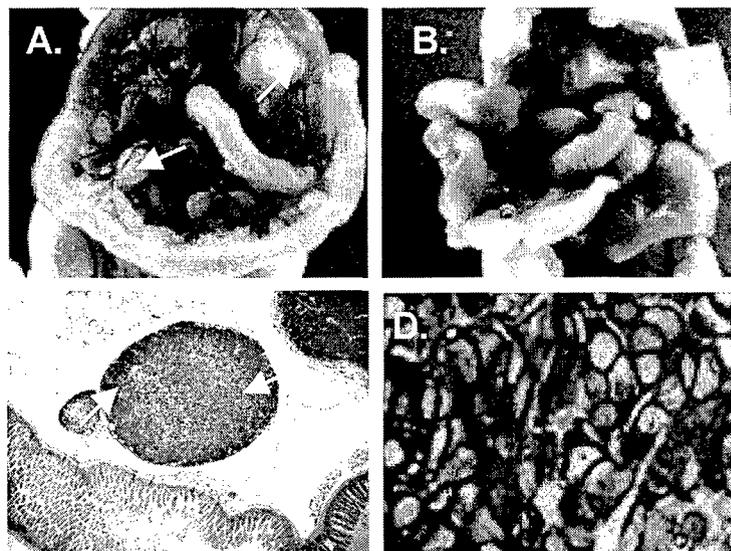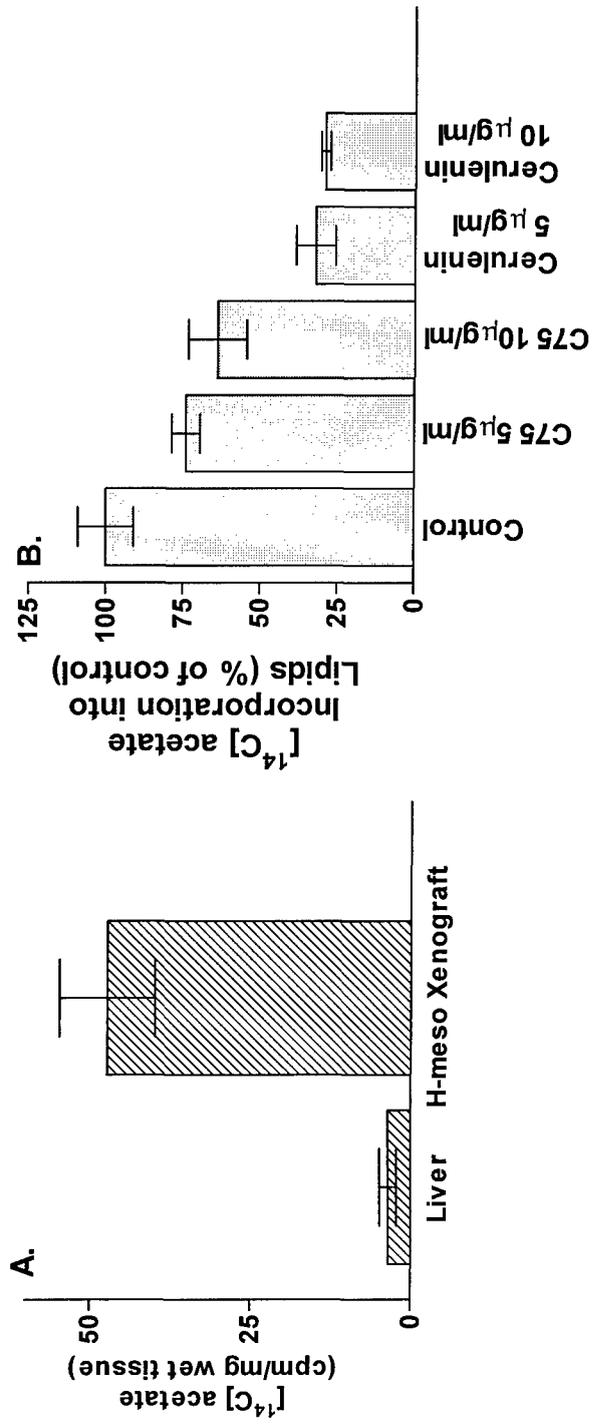**DEPARTMENT OF THE ARMY**
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