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Many common human cancers express high levels of fatty acid synthase (FAS), the primary enzyme responsible for the synthesis of fatty acids. Compared to cancer, fatty acid synthesis is highly down-regulated in normal tissues by dietary fat leading to a potential therapeutic index. Inhibition of FAS with cerulenin, a suicide inhibitor of FAS, induced apoptosis in cancer cells *in vitro,* demonstrating that cancer cells are dependent upon active fatty acid synthesis. To test the systemic effect of FAS inhibition on human cancer xenografts, synthesis of a chemically stable inhibitor was required. Based on the probable mechanism of action of cerulenin and the theoretical transition-state of the of the β-ketoacyl synthase reaction, we synthesized the first chemically stable inhibitor of FAS, C75. C75 is a slow-binding inhibitor of Type I mammalian FAS. In addition to its *in vitro* activity against cancer cells, C75 demonstrated significant anti-cancer activity in the MDA435/LCC6 and MCF-7 human breast cancer xenografts. C75 induced transient inanition which led to weight loss reversible with nutritional support. No other significant toxicity was identified. These data illustrates C75 anti-tumor activity, and demonstrate that the fatty acid synthesis pathway and FAS in particular are novel targets for breast cancer therapy.

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4. Table of Contents

5. Introduction:

Subject: Briefly. 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.

Purpose and Scope of the Research: 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 in this update during the last two 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:

[1] C75 is a slow-binding inhibitor of fatty acid synthase.
[2] C75 has activity against both estrogen receptor positive and negative human breast cancer xenografts.
[3] The only toxicity noted thus far is weight loss which is due to inanition and can be reversed by gavaged liquid diet.
[4] C75 and cerulenin appear to kill cancer cells by inhibition of fatty acid synthesis which leads to a ***secondary*** profound inhibition of DNA synthesis.

Our working hypothesis is that in cancer cells which are both cycling and synthesizing fatty acids inhibition of fatty acid synthesis is lethal. In normal cells which synthesize fat but do not readily enter the cell cycle such as liver, inhibition of fatty acid synthesis leads to decreased triglyceride synthesis, but no cytotoxicity. In normal cells that cycle but do not synthesize high levels of fatty acids such as normal colon crypt cells, there is also no cytotoxicity. This appears to be the basis of the therapeutic index of fatty acid synthesis inhibitors.

Most of the new data presented are also contained within the appendices. Appendix A is a paper in preparation for submission to Cancer Research that contains many of the figures and detailed experimental methods for this report. For example, the synthesis of C75 is exhaustively described in the Appendix along with references.

Appendix B contains important new work from one of our collaborators demonstrating that fatty acid synthesis leads to DNA synthesis which is the likely mechanism of cell death. That work is in press in Cancer Research.

6. BODY:

1. *C75 is a slow-binding inhibitor of fatty acid synthase (FAS).*

Rationale: At the time of the grant submission, C75 was known to inhibit fatty acid synthesis in tumor cells *in vitro*, however inhibition of purified mammalian FAS was not yet documented. Precise molecular determination of the target of C75 is crucial to understanding its mechanism of action and for structure-activity relationship data crucial to the future development of FAS inhibitors.

Experimental Methods: Contained in a paper submitted to *Nature Medicine,* we document that C75 is a slow-binding inhibitor of Type I mammalian fatty acid synthase. A preprint of the paper containing the methods and results are included in the Appendix. In brief, FAS was purified from rat liver without enzyme or protease inhibitors using stepwise ammonium sulfate precipitation, Sephacryl S-200 (Pharmacia) gel filtration, and Fast-Flow Q Sepharose (Pharmacia) anion exchange chromatography as described [21] FAS activity was measured spectrophotometrically monitoring oxidation of NADPH at 340 nm as described [22]. Briefly, 6.25 µg FAS, 50 µl of 1MK_2PO_4, pH 7.6 at 25°, 25 nanomoles of acetyl-CoA and 75 nanomoles of NADPH in 0.5 ml reaction volume were monitored at 340 nm in a heated chamber spectrophotometer at 37^0 for three minutes to measure background NADPH oxidation. Following the addition of 27 nanomoles of malonyl-CoA, the reaction was assayed for an additional 3 minutes to determine FAS dependent oxidation of NADPH. To detect the slow-binding inhibitor, FAS, C75, water and K_2PO_4 buffer were incubated at 37^0 for the specified time before commencing the standard FAS assay. Controls consisted of DMSO vehicle without enzyme.

Results and Discussion: 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. Figure 1 shows that increasing time of FAS and inhibitor contact by preincubation at 37° led to increasing enzyme inhibition up to 83% after 30 min. Slow-binding inhibition is a favorable pharmacological characteristic. In contrast to classical inhibitors, buildup of substrates is less likely to reverse inhibition due to isomerization of the enzyme-inhibitor complex.

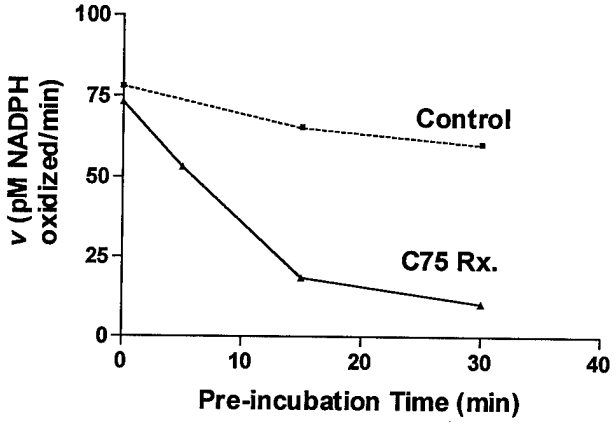


Figure 1. C75 is a slow-binding inhibitor of FAS.

Relationship of Studies to the Statement of Work: Task 1: Years 1-4. ***Synthesis of C-75 and tritiated C-75.***

- **a.** The slow-binding inhibitor studies while not outlined within Task 1, were deemed of sufficient importance to be accomplished.
- **b.** Synthesis C75 is ongoing and sufficient for all experiments.
- **c.** The synthesis of tritiated C75 is problematic. The level of radioactivity required is too great for the University laboratory and sufficient funds for commercial synthesis are not available. As a surrogate for labeled compound, we will use *in vivo* labeling with tritiated acetate to follow fatty acid synthesis during drug treatment. This will determine the half-life of the drug effect *in vivo* that is most useful for anti-cancer development.

2. C-75 is active against the MDA-435 xenograft model of human breast cancer.

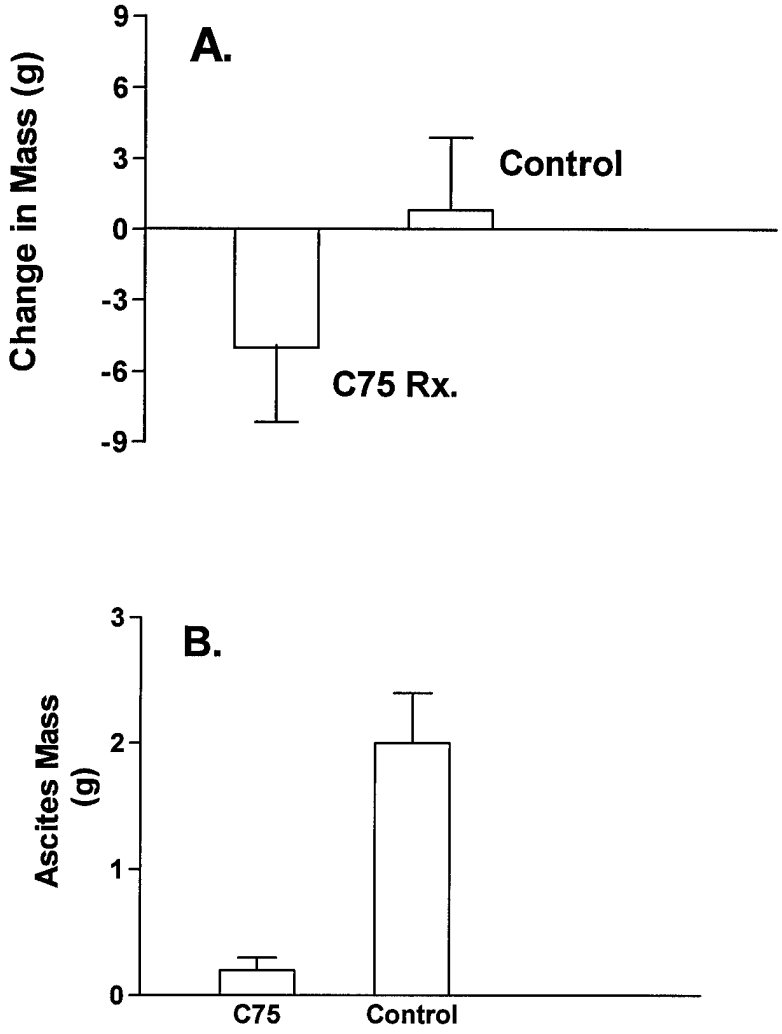
Rationale: The MDA-435/LCC6 breast cancer model has been chosen for reasons similar to the OVCAR-3 cells. The MDA-435/LCC6 cells are receptor negative cells, which have been adapted to grow as ascites or as solid tumor. These cells are sensitive to fatty acid synthesis inhibition *in vitro*, and undergo significant levels of fatty acid synthesis, similar to OVCAR-3 cells as demonstrated in Figure 3 of our grant proposal. While human breast cancer cells growing as ascites does not recapitulate clinical breast cancer progression, it will allow initial testing of fatty acid synthesis inhibitors to determine if breast cancer cells are susceptible to this therapy *in vivo.*

Experimental Methods: Six mice were inoculated with $2x10^6$ MDA435/LCC6 cells intraperitoneally. The xenograft was allowed to grow until the mice developed malignant ascites accounting for approximately 20% of body weight. Three mice were treated intraperitoneally with C75 at 100 mg/kg in RPMI and three received vehicle control. After 24h, mice were weighed, BrDU at 100 mg/kg was injected intraperitoneally and animals were sacrificed after 2h. Ascites was removed and measured, smears of ascites cells were fixed in 10% buffered formalin for immunohistochemistry, and aliquots of ascites cells were prepared for analysis of apoptosis. Apoptosis and DNA index were measured concurrently using 2 laser flow cytometry. Apoptosis was quantitated using merocyanine 540 staining[10μg/ml] added as a [1 mg/ml] stock in water directly to cells out of culture in medium (Sigma), which detects altered plasma membrane phospholipid packing that occurs early in apoptosis. The merocyanine 540 positive population was marked by an increase in red fluorescence, excited at 488 nm by an argon laser, and collected at 575 nm with a DF26 filter. DNA index was obtained using Hoechst 33342 staining excited at 350 to 366 nm by a crypton laser, and collected at 467 nm with a DF22 filter. Cytometry data were acquired on a Becton Dickinson FACStar^{Plus} with F12 optics, and analyzed with Cell Quest software The remainder of the animal was fixed in 10% buffered formalin for further studies.

Immunohistochemistry for BrDU, p53, and Ki-67 were performed on the tumor smears using Dako antibodies at 1:1000, 1:500, and 1:200 on the Dako Immunostainer using the LSAB2 detection kit.

Results and Discussion: C75 therapy significantly reduced malignant ascites volume in the MDA435/LCC6 xenograft. Figure 2A demonstrates that animals receiving one dose of C75 lost about 5 g of weight while control animals continued to gain weight due to accumulation of malignant ascites. Figure 2B shows that C75 treated mice had about 0.2 g of malignant ascites compared to 2 g. for controls.

Figure 2. C75 reduces malignant ascites in MDA435/LCC6 human breast cancer xenograft. One dose of C75 led to a 10-fold reduction in ascites.



Flow cytometry (Figure 3) performed by Dr.Pizer in the Department of Pathology at Johns Hopkins and Dr Chrest at the National Institute of Aging in Baltimore, confirmed C75 cytotoxicity against the xenograft. The figure depicts the ascites cells from representative

control and treated mice in 2 dimensional dot plots, with DNA content on the X-axis and merocyanine 540 (MC540) fluorescence on the Y-axis. Merocyanine 540 detects changes in plasma membrane phospholipid packing that occur early during entry into apoptosis (). The limited residual tumor present in the treated ascites demonstrated a reduction in the S-phase fraction in the MC540 negative (viable) population (region 1), an increase in the MC540 positive (early apoptotic) population (region 2), an influx of murine inflammatory cells (region 3) and an increase in cellular debris (sub G1 events). These features illustrate the manner of disappearance of the treated tumor. The early apoptotic fractions for the control mice are 3.42% +/- 1.28% versus 14.93% +/- 7.07% for C75 treated mice. In parallel with the reduced S-phase fraction, among the cancer cells remaining after therapy, immunohistochemistry demonstrated that surviving cells treated with C75 had a BrdU labeling index of about 2.5% compared to 20% for controls.

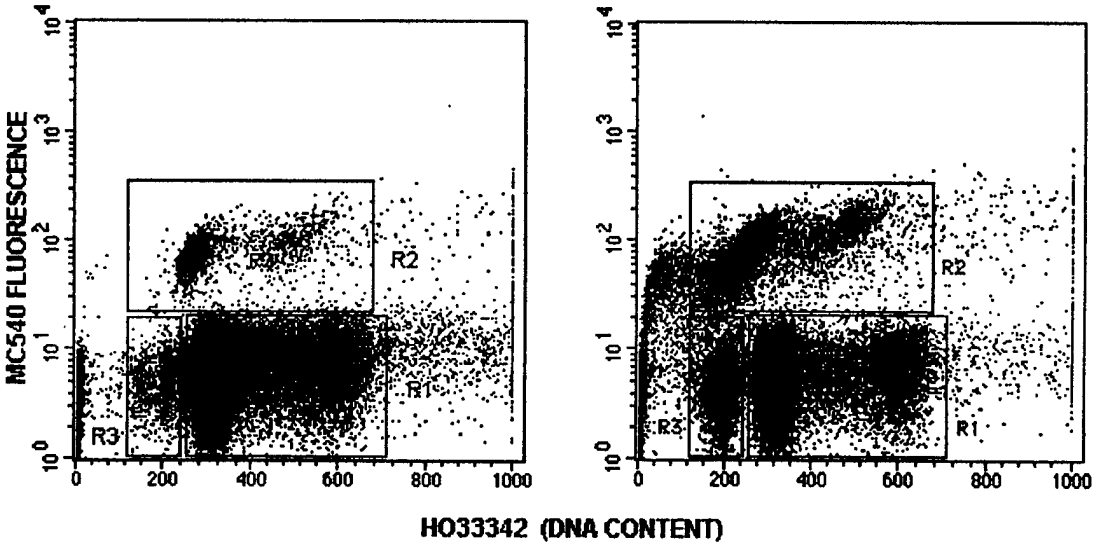


Figure 3. DNA Histogram of MDA435 Cells Treated with C75 *In Vivo*

These data suggest that C75 induces apoptosis and also inhibit DNA synthesis in tumor cells *in vivo*.

Relationship of Studies to the Statement of Work: Task 5: Months 9-18. *Treatment of the human breast cancer xenograft with C-75.*

- **a.** Given the lack of radiolabeled C75, we determined the maximum tolerated dose of C75 in outbred nude mice to be approximately 40 mg/kg. In the presence of significant ascites, up to 100 mg/kg was tolerated.
- **b.** The successful treatment of the MDA435/LCC6 cells in ascites demonstrates that C75 is cytotoxic to human breast cancer cells *in vivo* leading to apoptosis.

c. Flow cytometric and BrDU labeling studies suggest that C75 may act preferentially on dividing cells and C75 may actually inhibit DNA synthesis. ***In a later section, we will discuss the relationship of fatty acid synthesis inhibition and DNA synthesis inhibition which was discovered by my collaborator Dr. Ellen Pizer. These data impinge directly on the mechanism of action of C75 and other fatty acid synthesis inhibitors and as such will affect the future planned experiments.***

3. C-75 is active against the MCF-7 subcutaneous xenograft model.

Rationale: Since C75 was active against the ascites model, we wanted to move quickly to a more generally accepted and standard human breast cancer xenograft model. Thus, we chose the estrogen and progesterone receptor positive MCF-7 cell line injected subcutaneously into nude mice. This model would test C75 against a receptor positive cell line and also determine if C75 has truly systemic anti-tumor effects against a subcutaneous tumor. *In vitro* studies have determined that MCF-7 has high levels of fatty acid synthesis and is sensitive to C75 (data not shown).

Experimental Methods: Figure 4 is a preliminary experiment demonstrating activity of C75 against the MCF-7 xenograft. Five nude mice received 90 day slow release estrogen pellets subcutaneously. After five days, $2x10^6$ MCF-7 cells were injected subcutaneously in the left flank. Once tumors became measurable (approximately 3mm in diameter) C75 was administered at 30 mg/kg on the following dosing schedule to four mice. The dosing schedule was based on animal weight loss; animals were allowed to regain weight before a subsequent C75 dose was administered. (An experiment was attempted with the standard 40 mg/kg dose which led to death of the animals. Dose reduction to 30 mg/kg was well tolerated. One possible explanation is that estrogen administration led to increased drug sensitivity.) On the last day of the experiment, C75 was administered and animals were sacrificed 24 h later after a 2 h pulse with 100 mg/kg BrDU. All tissues were fixed in 10% buffered formalin for immunohistochemistry for FAS, estrogen receptor, and BrDU.

Results and Discussion: Figure 4 shows that the tumors were growing on the three days before C75 administration. Following C75 treatment on days 3 and 7, the tumors reduced in size and then grew at a slower rate compared to the control. This preliminary experiment demonstrates that C75 has activity against the slow growing MCF-7 xenograft. These data demonstrate that the C75 effect on the MDA435 cell line is not idiosyncratic and C75 has a systemic anti-tumor effect.

Immunohistochemistry for estrogen receptor was strongly positive in all tumor samples. Semi-quantitative analysis suggested that estrogen receptor positivity was reduced in the treated group compared to controls, but further studies will be required to confirm this observation. No difference was noted in FAS or BrDU reactivity between control or treated group. The lack of reduction in BrDU incorporation into DNA in the treated tumors only represents a single time point. We must repeat BrDU labeling at different times following C75 administration in this model to determine if BrDU incorporation into DNA is altered.

Relationship of Studies to the Statement of Work: This work accomplishes more of Task 5 moving to a subcutaneous xenograft model. We are in the process of testing intravenous and oral administration of C75, but since the intraperitoneal approach has a systemic effect, we will

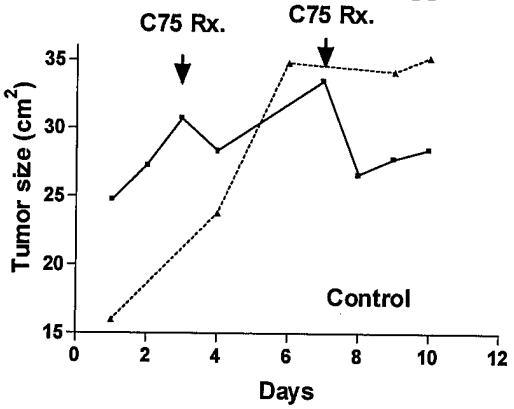


Figure 4. C75 has an anti-tumor effect on the MCF-7 human breast cancer xenograft.

continue to use i.p. injections to finish the initial xenograft studies with MDA435 and MCF-7. Concerning tasks 6-8 on pathway status in treated animals, we are changing the experimental approach to reflect the new data concerning cell cycle arrest and DNA synthesis inhibition by C-75. These plans will be addressed in the final section.

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

Rationale: In all of our *in vivo* studies, C75 induces weight loss in the animals and this appears to be the limiting toxicity. The weight loss is reversible and no tissue damage has been identified in the gastrointestinal tract or in other sites. Using metabolic cages monitoring input and output of mice on C75 therapy, a marked reduction in food consumption was noted. We also compared the rate of weight loss in animals given only water with C75 treatment to determine if the drug induced weight loss is consistent with inanition or other additional mechanisms such as increased basal metabolic rate may be involved. In addition, we began to study gavaging liquid diet to mice during therapy to reduce weight loss. Originally, we proposed to study C75 effects of animals placed on high and low fat diets. Since animals do not eat during therapy, these data have changed our approach to dietary manipulation as will be outlined below.

Experimental Methods: Three Balb-C mice were placed in each of two metabolic cages. Once group received C75 at 40 mg/kg i.p. in RPMI while the controls received vehicle only. Food and water consumption and urine and feces production was monitored along with animal weight. In parallel experiments, three mice were given water for three days and weight was monitored daily. We have also obtained a high calorie (3 kCal/ml) liquid diet from Dyets and gavaged three mice following C75 treatment three times a day with about 1 ml per meal in an attempt to blunt the weight loss.

Results and Discussion: Figure 5A demonstrates that mouse food consumption as measured by fecal production is markedly reduced within 24 h of C75. By day 3, fecal production increases and control levels are reached by day 4. Water consumption (Figure 5B) is also reduced and parallels fecal production. C75 thus induces inanition in the animals which persists for about 48

h. Importantly, the animals remain active during the period of inanition. The experiment in Figure 5C seeks to determine if the weight loss observed with C75 therapy was due to the inanition or other factors. The rate and magnitude of weight loss in C75 treated mice was similar

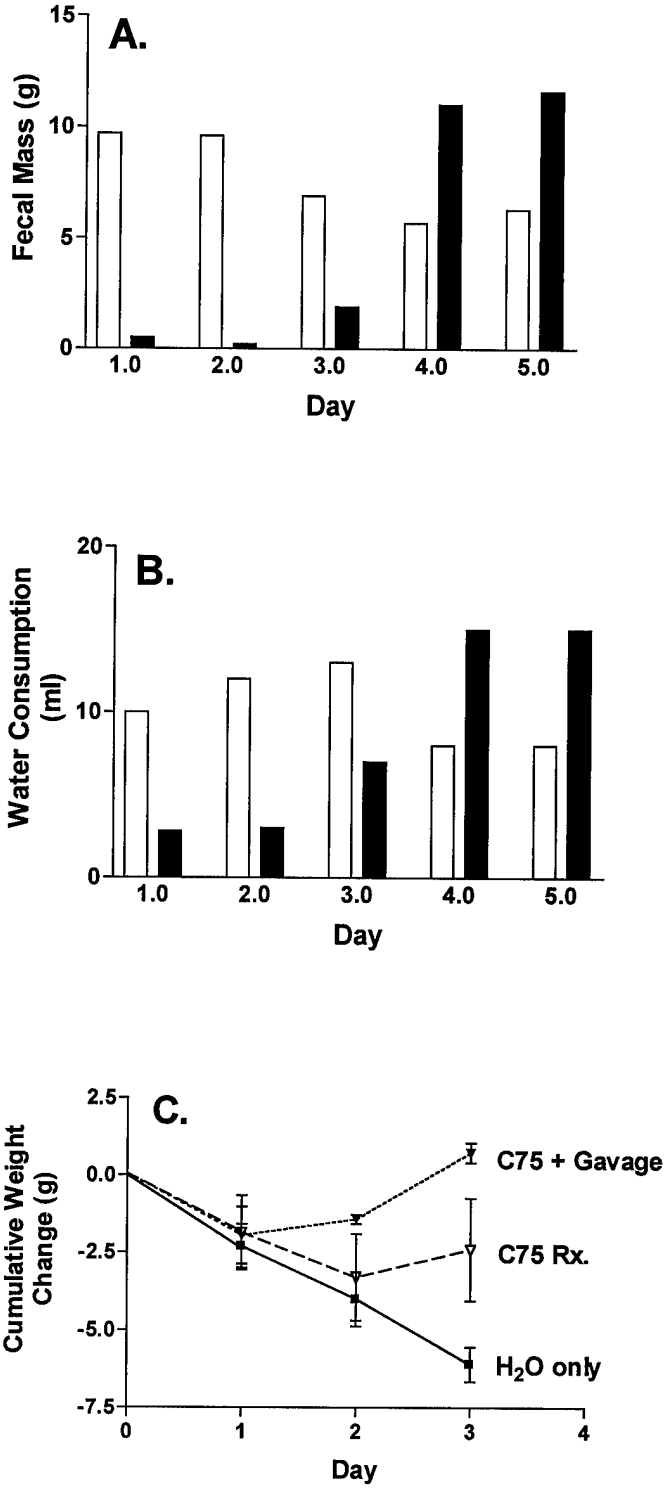


Figure 5. C75 weight loss is caused by inanition. Gavage of liquid diet reduces the weight loss.

to mice given water only over the first 48 h. This suggests that C75 induced weight loss is due primarily to inanition rather than other metabolic consequences of fatty acid synthesis inhibition. By day 3, animal weight increased in the C75 treated group with onset of feeding. Gavaging the mice with 3 kcal/day of liquid diet blunted the C75 induced weight loss and demonstrates that gastrointestinal function remain intact. We plan to combine gavage with anti-tumor treatment to increase dosing of C75 to maximize the anti-tumor effect.

In parallel studies not funded from this grant, we have found FAS immunostaining in neurons of the adult mouse in olfactory nuclei. Our working hypothesis is that FAS inhibition leads to decreased appetite and subsequent weight loss in the mouse. If fatty acid synthesis is active in these nuclei, this pathway could be used to monitor energy status centrally and influence appetite or olfactory perception of food in the rodent. We intend to pursue these studies in collaboration with the Departments of Neuroscience (Drs. Ronnett and Williams) and Department of Anatomy (Dr. Molliver). If our hypothesis is correct, the weight loss associated with this therapy is less significant to cancer patients since they could simply eat or be given hyperalimentation to easily prevent the weight loss.

Relationship of Studies to the Statement of Work: Task 9: Months 36-48. ***Examine the effects of high and low fat diets on the anti-tumor effect of C-75 and its effect on pathway status in the tumor and normal tissues of xenograft model as a means to potentiate the anti-tumor effect of C-75.*** Initially, we will use our liquid diet to increase the anti-tumor effect of C75 through gavage. If this is successful, we can compare high and low fat liquid diets to optimize treatment.

5. Pharmacological Inhibition of Mammalian Fatty Acid Synthase Activity Also Leads to Inhibition of DNA Replication.

Rationale: As part of her work under a clinician scientist award, Dr. Ellen Pizer from the Department of Pathology at Johns Hopkins, has been studying the mechanism of action of cancer cell death by fatty acid synthesis inhibition *in vitro*. Over the last 18 months, she has noted that inhibition of fatty acid synthesis by either cerulenin or C75 leads to profound inhibition of DNA synthesis. Furthermore, the inhibition does not appear to be a direct effect of the drugs on DNA synthetic machinery but rather is a consequence of fatty acid synthesis inhibition. This suggests links between the cell-cycle, DNA synthesis and fatty acid synthesis in tumor cells.

Experimental Design: The experiments and related figures are contained in the enclosed paper in the Appendix which is in press at *Cancer Research.*

Results and Discussion: The paper in the appendix details the results and discussion. In summary, both cerulenin and C75 produce profound inhibition of DNA replication and S-phase progression in human cancer cells. The dose responses for fatty acid synthesis inhibition and DNA synthesis inhibition are similar. The kinetics of both effects are rapid, with fatty acid synthesis inhibition occurring within 30 minutes and DNA synthesis inhibition occurring within 90 minutes of drug exposure. Apoptotic changes are not detected until approximately 6 hours.

Fatty acid synthetic pathway activity and the magnitude of DNA synthesis inhibition by FAS inhibitors are increased in parallel by withdrawal of lipid-containing serum from the cultures. The mechanism of DNA synthesis inhibition by cerulenin is indirect, because expression of viral oncogenes rescues DNA synthesis/S-phase progression in cerulenin exposed cells. The data suggest a direct linkage at a regulatory level, between fatty acid synthesis and DNA synthesis in proliferating tumor cells.

In the above (part 3) *in vivo* experiment with MDA435 cells, decrease in S-phase fraction was demonstrated by flow cytometry and BrDU labeling was significantly decreased. Both of these tests demonstrate that tumor DNA synthesis is inhibited by C75 *in vivo*. In another study, we sought to determine if DNA synthesis would be inhibited by C75 in normal cells. Twelve Balb-C mice were treated with C75 at 40 mg/kg i.p. followed by BrDU labeling at 1,2,4,8, and 24 h. No differences in labeling of the small intestinal crypts (proliferation compartment) with BrDU was identified. Thus, in normal tissues with high levels of proliferation without high levels of fatty acid synthesis, C75 does not inhibit DNA synthesis.

Our working hypothesis is that fatty acid synthase inhibitors such as C75 are targeting cells that are simultaneously proliferating and synthesizing fatty acids such as tumor cells. In normal proliferating cells, DNA synthesis is not significantly affected because they are not synthesizing high levels of fatty acids.

Relationship of Studies to the Statement of Work: Task 6: Months 9-11. ***Determination of pathway status in normal tissues of mice without tumor.*** **Task 7: Months 11-24.** ***Determination of pathway status in normal tissues and tumor cells in the presence of increasing mass of tumor.*** **Task 8: Months 24-36.** ***Compare the effect of C-75 on pathway status in normal tissues from mice without tumor, versus normal tissues and tumor cells from mice with increasing mass of tumor.*** Given these compelling *in vitro* and *in vivo* evidence of a link between fatty acid synthesis and DNA synthesis, we will continue to study the effects of C75 administration on fatty acid synthesis rate, FAS expression, DNA synthesis rate, and apoptosis in the breast cancer xenografts. We will also feed animals liquid diet during C75 therapy to reduce weight loss, increase C75 dose schedule, and increase efficacy of therapy.

7. Conclusions:

1. C75 is a slow inhibitor of fatty acid synthase. We have demonstrated that C75 inhibits fatty acid synthesis by inhibition of fatty acid synthase. In addition, C75 has features of a slow binding inhibitor which is a favorable pharmacological characteristic.

2. C75 is active against the MDA435/LCC6 human breast cancer xenograft. One dose of C75 led to a 10-fold reduction in malignant ascites in this model. Flow studies demonstrated that C75 induces apoptosis *in vivo*. In addition, it appears that tumor cell *but not normal cell* DNA synthesis, was also inhibited by C75. This is in keeping with the recent *in vitro* data from our collaborators that demonstrate that inhibition of fatty acid synthesis leads to DNA synthesis inhibition which is the likely mechanism of cell death.

3. C75 is active against the MCF7 human breast cancer xenograft. Preliminary studies indicate that C75 was active against subcutaneous MCF-7 xenografts. This indicates that:

[a] C75 is active against more than one breast cancer xenograft.

[b] C75 is active against an estrogen receptor positive slow-growing xenograft.

[c] C75 has systemic anti-tumor activity.

4. C75 induced weight loss occurs through inanition. One dose of C75 causes animals to cease eating solid chow for about 48 h. It appears that weight loss is due to inanition rather than an intrinsic metabolic abnormality secondary to fatty acid synthesis inhibition such as alteration in basal metabolic rate, uncoupling oxidative phosphorylation, or other catabolic process. Gavaging the animals with liquid diet reverses the weight loss indicating that the gastrointestinal tract is functioning normally. Since fatty acid synthase expression has been identified by our group in the CNS, it is possible that C75 is altering the appetite in the animals. This has implication for treating patients in the future since an adequate diet will need to be maintained during treatment but the therapy should not worsen the catabolic state in cachectic patients.

5. Pharmacological Inhibition of Mammalian Fatty Acid Synthase Activity Also Leads to Inhibition of DNA Replication. ***Our working hypothesis is that fatty acid synthase inhibitors such as C75 are targeting cells that are simultaneously proliferating and synthesizing fatty acids such as tumor cells.*** In normal proliferating cells, DNA synthesis is not significantly affected because they are not synthesizing high levels of fatty acids.

APPENDIX A.

Synthesis and Anti-tumor Activity of a Novel Inhibitor of Fatty Acid Synthase

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Abstract

Many common human cancers including carcinoma of the colon [1], prostate [2,3], ovary [4], breast [5, 6, 7] and endometrium [8], express high levels of fatty acid synthase (FAS, E.C. 2.3.1.85), the primary enzyme responsible for the synthesis of fatty acids [9]. Compared to cancer, fatty acid synthesis is highly down-regulated in normal tissues by dietary fat [10] leading to a potential therapeutic index. Inhibition of FAS with cerulenin, a suicide inhibitor of the β-ketoacyl synthase reaction on FAS [11], induced apoptosis in cancer cells *in vitro* [12, 13] demonstrating that cancer cells are dependent upon active fatty acid synthesis for survival. To test the systemic effect of FAS inhibition on human cancer xenografts, synthesis of a chemically stable inhibitor was required. Based on the probable mechanism of action of cerulenin [11] and the theoretical transition-state of the of the β-ketoacyl synthase reaction, we synthesized the first chemically stable inhibitor of FAS, C75. C75 is a slow-binding inhibitor of Type I mammalian FAS. In addition to its *in vitro* activity against cancer cells, C75 demonstrated significant anti-cancer activity in the NIH:OVCAR3 human ovarian cancer and MDA435/LCC6 human breast cancer xenografts. C75 induced transient inanition which led to weight loss reversible with nutritional support. No other significant toxicity was identified. This study describes the synthesis of C75, illustrates its anti-tumor activity, and demonstrates that the fatty acid synthesis pathway and FAS in particular are novel targets for anti-cancer therapy development.

Introduction

Fatty acid synthesis is common to most organisms. In well-nourished humans, however, the fatty acid synthetic pathway is down-regulated due to high levels of dietary fat. While 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, E.C. 2.3.1.85) in a wide variety of human malignancies and their precursor lesions including carcinoma of the colon[1], prostate[2,3], ovary[4], and breast[5-7]. FAS is the principal synthetic enzyme of fatty acid synthesis which catalyzes the NADPH dependent condensation of malonyl-CoA and acetyl-CoA to produce the 16-carbon saturated free fatty acid, palmitate[9-14]. *Ex vivo* measurements in tumor tissue have shown that high levels of FAS connote high levels of fatty acid synthesis indicating that the entire genetic program is functional consisting of some 25 enzymes from hexokinase to FAS.

The widespread expression of FAS in human cancer and its association with aggressive disease in breast[5-7], prostate[2-3], and ovarian cancer[4], 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 suicide inhibitor of the β-ketoacyl synthase reaction on FAS, led to cell death via apoptosis demonstrating that cancer cells with highly active fatty acid synthesis require a functional pathway[22]. Recent *in vivo* data from our laboratory has shown that cerulenin treatment of the NIH:OVCAR-3 human ovarian cancer xenograft led to a reduction in malignant ascites development and a modest increase in xenograft survival[18].

While cerulenin achieved some local control of tumor, it is chemically unstable *in vivo* likely as a result of its reactive epoxide moiety. To study the systemic effects of fatty acid synthesis inhibition, a more stable inhibitor of fatty acid synthesis was required. Because of the metabolic specificity of the β-ketoacyl synthase reaction in FAS, we chose to target this enzymatic activity for inhibition. Based on the probable mechanism of action of cerulenin[11] and the theoretical transition-state of the of the β-ketoacyl synthase reaction, we synthesized an inhibitor of FAS with systemic anti-cancer activity. This study describes the first *de novo* synthesis and biological activity of a chemically stable Type I FAS inhibitor and its *in vitro* and *in vivo* effects on human cancer.

Materials and Methods

Synthesis of C-75. The design of inhibitors of FAS was predicated upon both an understanding of the mechanism of fatty acid biosynthesis, in particular, that of the critical ketosynthase or "condensing enzyme" domain of this polyfunctional enzyme[9,11] and, the well-studied inactivation of FAS by the natural product cerulenin. We have conducted simple molecular modeling exercises and structure-based searches based on the mechanism outlined in (Figure 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 (Figure 1, Panel 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 [15]. The mechanisms of action for

either of these activities are unknown. Nonetheless, the latter observation led us to prepare several compounds (Figure 1, Panels 3 and 4), having various side chain lengths to test their anti-tumor and FAS inhibitory behavior.

While 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 [16] achieved this goal and is set out in Figure 1. Deprotonation of *p*-methoxybenzyl itaconate (Figure 1, Panel 1) at low temperature gave the dianion (Figure 1, Panel 2), which underwent aldol reaction with a series of aldehydes, RCHO, to give on strongly acidic workup a mixture of γ-lactones (Figure1, Panel 3 and 4). Separation on silica gel and crystallization afforded the pure diastereomers.

Two equivalents of lithiumhexamethyldisilyl amide (LiHMDS) were added to a solution of *p*-methoxybenzyl itaconate[16] dissolved in dry tetrahydrofuran (THF) at -78 °C. After 1 h, an equivalent of 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 (Figure 1, Panels 3 and 4) 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. The following analytical data were collected for C75 (Figure 1, Panel 3, R = n-C_8H_{17}) and are representative: *trans*-isomer (Figure 1, Panel 3, R = n-C_8H_{17}): mp 76-77 °C; IR (film) 3000-3400, 2924, 2852, 1743, 1717, 1660, 1621, 1460 cm^{-1}; ^1H NMR ($CDCl_3$) 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, 1H, J = 6, 12.8 Hz), 6.0 (d, 1H, J = 2.8 Hz), 6.4 (d, 1H, J = 3.2 Hz); ^{13}C NMR ($CDCl_3$) δ 14.0, 22.6, 24.7, 29.1, 29.14, 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.

Growth Inhibition of α-methylene-γ-butyrolactones and Cerulenin in SKBR3 Cells and Normal Human Fibroblasts. Both SKBR3 cells and fibroblasts were plated in 96 well plates at a density of 5,000 cells per well in RPMI with 10% fetal bovine serum. After overnight growth at 37°C with 5% CO_2, compounds solubilized in DMSO were added at concentrations ranging from 0.08-40 μg/ml in volume of 100 μl. Each concentration was plated in quadruplicate. After 72 h incubation with drug at 37°C, plates were stained with crystal violet (0.2% in 2% ethanol) and read at 490 nm. ID_{50} was computed using linear regression. 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 using stepwise ammonium sulfate precipitation, gel filtration, and anion exchange chromatography as described[21]. FAS was >95% pure as

estimated from SDS-PAGE with Coomasie blue staining. FAS activity was measured spectrophotometrically monitoring oxidation of NADPH at 340 nm as described (ref). Briefly, 6.25 μg FAS, 50 μl of 1MK_2PO_4, pH 7.6 at 25°, 25 nanomoles of acetyl-CoA and 75 nanomoles of NADPH in 0.5 ml reaction volume were monitored at 340 nm in a heated chamber spectrophotometer at 37^0 for three minutes to measure background NADPH oxidation. Following the addition of 27 nanomoles of malonyl-CoA, the reaction was assayed for an additional 3 minutes to determine FAS dependent oxidation of NADPH. One O.D. unit is equivalent to 80.5 nanomoles of NADPH oxidized. To detect the slow-binding inhibitor, FAS, water and K_2PO_4 buffer were incubated at 37^0 for the specified time before commencing the standard FAS assay. Controls consisted of DMSO vehicle without drug.

Measurement of Fatty Acid Synthesis in HL60 Cells. HL60 human promyelocytic leukemia cells were grown in serum and fatty acid free medium as described (ref). C75 or vehicle alone (10 μg/ml) was added to cultures of $5x10^5$ cells. After 2 h, cells 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 described (ref). Labeled lipids are subjected to thin layer chromatography (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 controls were run for each of the lipid classes. Following chromatography, lipid classes were identified on a Molecular Dynamics phosphorimager and quantitated with ImageQuant™ software.

Measurement of Fatty Acid Synthesis in Liver. Three Balb-C mice maintained on Purina Mouse Chow *ad libitum* were treated intraperitoneally with C75 40 mg/kg in RPMI and three received vehicle alone. After 4 h, mice were labeled intraperitoneally with 0.5 mCi [U-^{3}H]-acetate for 2 h. Mice were sacrificed and total lipids were extracted from liver as described (ref). Labeled lipids from 100 mg of liver per mouse were subjected to thin layer chromatography as above. Following chromatography, lipid classes were identified using tritium screens on a Molecular Dynamics phosphorimager and quantitated with ImageQuant™ software.

Metabolic Studies: To determine the effect of C75 on food consumption and weight loss, six Balb-C mice were maintained in metabolic cages where water and food consumption and urine and feces production were measured daily. Three mice received a single intraperitoneal dose of C75 40 mg/kg in RPMI and were fed Purina mouse chow *ad libitum*; three mice received vehicle alone with a similar diet. To compare the rate and magnitude of C75 induced weight loss to abstinence from food and to liquid diet supplementation, three Balb-C mice received a single intraperitoneal dose of C75 40 mg/kg in RPMI with Purina mouse chow available, three mice received the same dose of C75with a liquid mouse meal of 1 ml (3 kcal) gavaged thrice daily, and three mice received water only. Mice were weighed daily and the experiment was terminated after three days.

Xenograft Studies. The NIH:OVCAR-3 human ovarian cancer cell line was maintained by serial passage in outbred nude mice (Harlan). For disease progression studies, 0.1 ml packed NIH:OVCAR-3 cells passed in outbred nude mice (Harlan), were inoculated

intraperitoneally on day 0. On days 1,3,and 5 post-inoculation, 10 animals were treated with 40mg/kg C75 and 10 received vehicle alone. Mouse weight was monitored and mice were sacrificed when achieving 30% weight increase due to malignant ascites. For treatment of established disease, OVCAR-3 was inoculated as above into 6 mice and allowed to progress until approximately 15% increase in average weight was achieved due to malignant ascites. Three mice were treated intraperitonally with 100 mg/kg C75 and three with vehicle control. Mice were subsequently weighed for three days after which the experiment was terminated.

The human breast cancer xenograft, MDA435/LCC6 was utilized to explore the mechanism of action of C75 *in vivo*. Six mice were inoculated with $2x10^6$ MDA435/LCC6 cells intraperitoneally. The xenograft was allowed to grow until the mice developed malignant ascites accounting for approximately 20% of body weight. Three mice were treated intraperitoneally with C75 at 100 mg/kg in RPMI and three received vehicle control. After 24h, mice were weighed, BrdU at 100 mg/kg was injected intraperitoneally and animals were sacrificed after 2h. Ascites was removed and measured, smears of ascites cells were fixed in 10% buffered formalin for immunohistochemistry, and aliquots of ascites cells were prepared for flow cytometric analysis of apoptosis.

Flow-cytometric and Immunohistochemical Studies. Immunohistochemistry for BrdU, and Ki-67 were performed on the tumor smears using Dako antibodies at 1:1000, and 1:200 on the Dako Immunostainer using the LSAB2 detection kit. Apoptosis and DNA index were measured concurrently using 2 laser flow cytometry. Apoptosis was

quantitated using merocyanine 540 staining[10μg/ml] added as a [1 mg/ml] stock in water directly to cells out of culture in medium (Sigma), which detects altered plasma membrane phospholipid packing that occurs early in apoptosis. The merocyanine 540 positive population was marked by an increase in red fluorescence, excited at 488 nm by an argon laser, and collected at 575 nm with a DF26 filter. DNA index was obtained using Hoechst 33342 staining excited at 350 to 366 nm by a crypton laser, and collected at 467 nm with a DF22 filter. Cytometry data were acquired on a Becton Dickinson FACStar^{Plus} with F12 optics, and analyzed with Cell Quest software (Becton Dickinson).

Results

C75 Selectively Growth Inhibits Human Cancer Cells In Vitro. Table I summarizes the structure and growth inhibition obtained for a representative selection of the α-methylene-γ-butyrolactones. Based on: **[1]** selective *in vitro* growth inhibition between SKBR3 cells, a human breast cancer cell line with high levels of fatty acid synthesis (ref) (ID_{50}= 5.0 +/- 0.1 μg/ml), and normal fibroblasts (ID_{50}= 21.6 +/- 1.4 μg/ml); **[2]** aqueous solubility; and **[3]** similar alkyl side chain length to cerulenin; C75, (Figure 1, Panel 3, where R = *n*-C_8H_{17}) was chosen for further study. Interestingly, adding the double bonds to C75 in the same location of the alkyl side chain as cerulenin (Table 1, C271) or altering the α-methlyene lactone to a four-carbon ring (Table 1, C43) abolished growth inhibitory activity.

C75 is a Slow Binding Inhibitor of FAS. Figure 2A demonstrates that C75 exhibits characteristics of a slow binding inhibitor of purified mammalian 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[17]. In the published assay, where FAS, inhibitor, and substrates are in contact for approximately 6 min at 37°, C75 is inactive whereas cerulenin is active. Increasing time of FAS and inhibitor contact by preincubation at 37° led to increasing enzyme inhibition up to 83% after 30 min. Slow-binding inhibition is a favorable pharmacological characteristic. In contrast to classical inhibitors, buildup of substrates is less likely to reverse inhibition due to isomerization of the enzyme-inhibitor complex [17].

C75 Inhibits Fatty Acid Synthesis in both Cancer Cells and Normal Liver. HL60 human promyelocytic leukemia cells proliferate readily in serum and fatty acid-free culture media and as such provide a convenient *in vitro* model for fatty acid synthesis [18]. In this environment, the cells are dependent upon endogenous fatty acid synthesis for the production of storage and structural lipids. As shown in Figure 2B, after 4h exposure to drug at 5 μg/ml, [U-^{14}C] acetate incorporation into phospholipids and triglycerides was inhibited by 87% and 89% respectively. Label into the free fatty acid pool was also reduced by 44% Presence of serum derived fatty acids in the culture medium did not prevent C75 inhibition of fatty acid synthesis. In studies with MCF7 and SKBR3 human breast carcinoma cells grown with 10% fetal bovine serum (~0.1 mM fatty acid), incorporation of [U-^{14}C] acetate into phospholipids was also inhibited by C75 (data not shown). Recent studies demonstrate that C75 inhibition of fatty acid synthesis *in vitro* is superior to comparable doses of cerulenin[23]. Both Tunnel labeling (ref) and pulse-field gel electrophoresis (data not shown) demonstrated that C75 induces apoptosis in cancer cells. Clonogenic assays with C75 confirmed its cytotoxic effect *in vitro* (data not

shown). To test if C75 inhibited fatty acid synthesis *in vivo*, Balb-C mice were treated intraperitoneally with 40 mg/kg C75 followed by metabolic labeling. Figure 2C shows that C75 inhibited uptake of [U-^{3}H] acetate into hepatic triglyceride by nearly 30%.

C75 Induces Weight Loss by Inanition which is Reversible with Supplemental Feeding: Figure 3A demonstrates that mouse food consumption as measured by fecal production is markedly reduced within 24 h of C75. By day 3, fecal production increases and control levels are reached by day 4. Water consumption (Figure 3B) is also reduced and parallels fecal production. C75 thus induces inanition in the animals which persists for about 48 h. Importantly, the animals remain active during the period of inanition. The experiment in Figure 3C seeks to determine if the weight loss observed with C75 therapy was due to the inanition or other factors. The rate and magnitude of weight loss in C75 treated mice was similar to mice given water only over the first 48 h. This suggests that C75 induced weight loss is due primarily to inanition rather than other metabolic consequences of fatty acid synthesis inhibition. By day 3, animal weight increased in the C75 treated group with onset of feeding. Gavaging the mice with 3 kcal/day of liquid diet blunted the C75 induced weight loss and demonstrates that gastrointestinal function remain intact.

C75 Increased Survival of the NIH:OVCAR-3 Human Ovarian Cancer Xenograft. C75 treatment of the NIH:OVCAR3 human ovarian cancer xenograft model demonstrated selective cytotoxicity against cancer cells. The NIH:OVCAR-3 xenograft is an excellent model of human ovarian cancer. Intraperitoneal inoculation of tumor cells leads to development of malignant ascites with late metastases similar to human disease

[19]. NIH:OVCAR3 is multiply drug resistant to cytoxan, adriamycin, and cis-platin [20] and undergoes high levels of endogenous fatty acid synthesis both *in vitro* and *in vivo* [18]. Figure 4A demonstrates that C75 treatment increased xenograft survival by 50%. The median survival of treated mice was 61.5 days compared to 30 days for control ($p=0.0008$ log rank statistic, hazard ratio 3.68). Moreover, 1 animal exhibited long-term survival and appeared cured of disease. Cytotoxicity was dose dependent as treatment reduction from 3 to 2 doses reduced survival below statistical significance without long-term survival (data not shown). Dose-dependent weight loss was the only toxicity yet identified with C75 treatment. Figure 4B demonstrates that C75 reduced malignant ascites in xenografts with established disease. The significant weight loss following one dose of C75 was reflected in marked reduction of ascites. Bloody ascites precluded meaningful quantitation three days after treatment. Histological examination of organs from treated mice showed reduction of abdominal and cutaneous adipose tissue without evidence of tissue damage.

C75 Induced Apoptosis in the MDA435/LCC6 Human Breast Cancer Xenograft. The MDA-435/LCC6 breast cancer model is better suited to study the effects of C75 on established disease. The MDA-435/LCC6 cells are estrogen and progesterone receptor negative cells that have been adapted to grow as ascites. These cells are sensitive to fatty acid synthesis inhibition *in vitro*, undergo significant levels of fatty acid synthesis, similar to OVCAR-3 cells, and grow as single cells which are particularly suitable for flow-cytometric analysis. C75 therapy significantly reduced malignant ascites volume in the MDA435/LCC6 xenograft (Figure 5A). Mean ascites volume in treated animals was 0.2

ml compared to 2 ml in controls. Treated animals also lost an average of 5 g while controls continued to gain weight over 24 h. Flow cytometry (Figure 6) confirmed C75 cytotoxicity against the xenograft. The figure depicts the ascites cells from representative control and treated mice in 2 dimensional dot plots, with DNA content on the X-axis and merocyanine 540 (MC540) fluorescence on the Y-axis. Merocyanine 540 detects changes in plasma membrane phospholipid packing that occur early during entry into apoptosis (). The limited residual tumor present in the treated ascites demonstrated a reduction in the S-phase fraction in the MC540 negative (viable) population (region 1), an increase in the MC540 positive (early apoptotic) population (region 2), an influx of murine inflammatory cells (region 3) and an increase in cellular debris (sub G1 events). These features illustrate the manner of disappearance of the treated tumor. The early apoptotic fractions for the control mice are 3.42% +/- 1.28% versus 14.93% +/- 7.07% for C75 treated mice. In parallel with the reduced S-phase fraction, among the cancer cells remaining after therapy, immunohistochemistry demonstrated that surviving cells treated with C75 had a BrdU labeling index of about 2.5% compared to 20% for controls.

Discussion

The synthetic strategy utilized to for FAS inhibitor design led to C75, an α methylene γ butyrolactone with a number of advantages over cerulenin. C75 synthesis was accomplished in two steps resulting in high yields of a pure crystalline stereoisomer with aqueous solubility. While synthesis of *r*-cerulenin, the active isomer, has been performed, the method is impractical for large scale production and solubility remains problematic requiring organic solvents (ref). C75 also lacks the highly reactive epoxide

present on cerulenin enhancing chemical stability and specificity. The slow binding inhibitor kinetics of C75 is another favorable pharmacological characteristic. In contrast to classical inhibitors, buildup of substrates is less likely to reverse inhibition due to isomerization of the enzyme-inhibitor complex [17].

C75 is an effective inhibitor of FAS *in vitro* and *in vivo*. C75 significantly inhibited radiolabeled acetate incorporation into phospholipids and triglycerides in cultured cancer cells and inhibition occurred in culture conditions with or without exogenous lipid. Importantly, since these biochemical measurements took place within 4 h of drug exposure, they represented pathway inhibition in viable cells before the onset of early apoptotic events as measured by flow cytometry[23]. C75 also inhibited hepatic fatty acid synthesis. In contrast to dividing cancer cells that synthesize both triglycerides and phospholipids, the liver incorporated label predominantly into triglycerides reflecting hepatic synthesis of lipoproteins. Moreover, there was no evidence of hepatocellular injury or inflammation following C75 administration. Although toxicological studies are not yet complete, the only toxicity yet observed was weight loss with concomitant reduction of adipose tissue.

While we have not found histologic evidence of toxicity of C75, the animals undergo profound weight loss following C75 administration. Metabolic studies have demonstrated that the weight loss is due to inanition, which persists for about 48 h following a single dose. The weight loss was partially reversible with gavaged liquid diet indicating that gastrointestinal function was not impaired. The mechanism of weight loss

is important with a view toward treating cancer patients. If weight loss occurred despite adequate nutrition, it would suggest another metabolic abnormality inherent to fatty acid synthesis inhibition, which would likely enhance cachexia. While yet preliminary, these data indicate that nutritional support may eliminate the loss of body mass due to C75 administration.

C75 demonstrated significant activity against human cancer xenografts. In the multiply drug resistant NIH:OVCAR-3 xenograft, cerulenin affected local tumor growth by reduction in ascites development[18]. C75 treatment led to long-term survival of the xenograft suggesting a systemic anti-tumor effect. Transient weight loss has been the only discernable toxicity in the xenograft. Treatment of established malignant ascites in the MDA435/LCC6 human breast cancer xenograft led to a rapid loss of malignant ascites with induction of apoptosis as measured by flow cytometric analysis of plasma membrane conformational changes. BrdU labeling was also markedly reduced following C75therapy. These *in vivo* data support the recent *in vitro* observation that inhibition of fatty acid synthesis leads to DNA synthesis inhibition in cancer cells[23] providing further evidence of a possible biochemical link between fatty acid synthesis and DNA synthesis in proliferating cancer cells.

C75 is the first synthetic, chemically stable inhibitor of Type I FAS. As a single agent, it exhibited profound cytotoxic effect on both human breast and ovarian cancer xenografts without significant toxicity. As cytotoxicity of FAS inhibition parallels pathway activity in many types of cancer cell lines[12,18,22], the anti-tumor activity will likely generalize to

include any malignancies that undergo high levels of fatty acid synthesis regardless of the site of origin. Furthermore, since this is a novel target pathway, combination therapy with C75 and standard agents are likely to improve their cytotoxicity against many types of cancers.

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Figure Legends

Figure 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 acyl carrier protein (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.

[The iterative two-carbon chain extension characteristic of fatty acid biosynthesis takes place at the β-ketoacyl synthase (KAS) domain [9,14]. 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 (Figure 1, A.). Decarboxylation of the malonyl-ACP takes place to give a reactive enolate anion (Figure 1, B.), which attacks the acyl-ACP in a thioester Claisen condensation. The transiently generated tetrahedral oxyanion (Figure 1, C.) rapidly decomposes to generate the resting state of KAS and the homologated β-ketoacyl intermediate bound to ACP (Figure 1, D.).]

Figure 2. ***C75 inhibits FAS and fatty acid synthesis.*** **A.** C75 is 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 to control (dotted line). This is characteristic of a slow-binding inhibitor. **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 free fatty acids is reduced by about 50%. **C.** C75 inhibits hepatic fatty acid synthesis in mice. In contrast to cancer cells, C75 reduced [U ^{3}H] acetate incorporation predominantly into triglycerides while phospholipids were unaffected. All error bars represent standard error of the mean.

Figure 3. ***C75 Induces Weight Loss by Inanition which is Reversible with Supplemental Feeding.*** **A.** Balb-C mice treated intraperitoneally with 40 mg/kg C75 (black bars) show marked reduction in average fecal mass excretion compared to vehicle controls (white bars) for 48 h. Fecal mass measurement parallels food ingestion and is more accurately measured. **B.** Average water consumption in C75 treated mice (black bars) is reduced compared to controls (white bars) and parallels fecal excretion. **C.** Weight loss in C75 treated mice (large dashed line) is similar to mice received water alone (solid line) over the first 48 h. During the subsequent 24 h, C75 treated mice being eating and weight loss is reversed. C75 treated mice receiving supplemental liquid meals 1 ml (3 kcal/ml) gavaged thrice daily show reduction in weight loss. All error bars represent standard error of the mean.

Figure 4. ***C75 prolongs survival of the NIH:OVCAR-3 xenograft and reduces malignant ascites.*** **A.** Kaplan-Meier life table analysis demonstrates that C75 significantly increases the survival of the NIH:OVCAR-3 xenograft (solid line) compared

to vehicle controls (dashed-line). One animal achieved long term survival consistent with cure. **B.** C75 treatment of mice with established disease demonstrate a rapid reduction of malignant ascites as reflected in loss of body mass (solid-line) compared to vehicle controls (dotted-line). Error bars represent standard error of the mean.

Figure 5. ***C75 Reduced Malignant Ascites in the MDA435/LCC6 Human Breast Cancer Xenograft.*** **A.** C75 treated xenografts with established disease lose five grams within 24 h compared to vehicle controls which gained from 1-3.5 g. **B.** Ascites mass was reduced tenfold in the C75 treated animals compared to controls. Error bars represent standard error of the mean.

Figure 6. ***C75 Induced Apoptosis In Vivo.*** Merocyanine 540 staining on the Y-axis and DNA staining on the X-axis demonstrates both a reduction in the S-phase fraction of in viable cells (merocyanine negative) in region 1 and an increase in merocyanine positive cells in region 2 following C75 treatment of the MDA435/LCC6 xenograft. These features demonstrate significant apoptosis with a reduction in S-phase of the remaining viable cells.

Table I: *In vitro* Growth Inhibition of Compounds Against SKBR3 Human Breast Cancer Cells

Compound	Akyl 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
C 77	$-C_9H_{19}$	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.A.*
C43*	$-C_8H_{17}$	57.1 +/- 10.1	N.A.
DMSO control		N.A.	N.A.

N.A.: No toxicity identified.
*4-carbon ring.

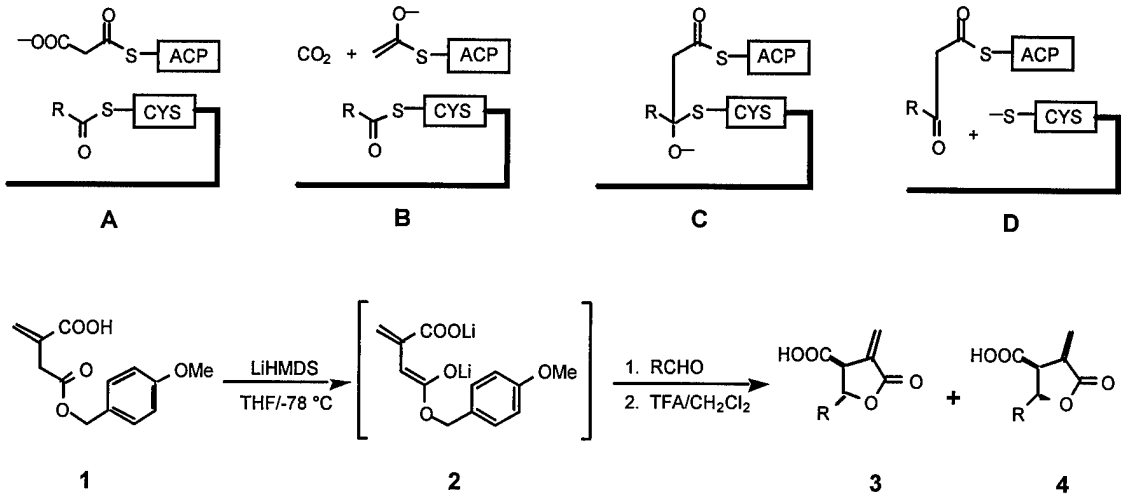

ACP
CYS
A
B
C
D
COOH
OMe
LiHMDS
THF/-78 °C
COOLi
OLi
1. RCHO
2. TFA/CH2Cl2
HOOC
1
2
3
4

Figure 1.

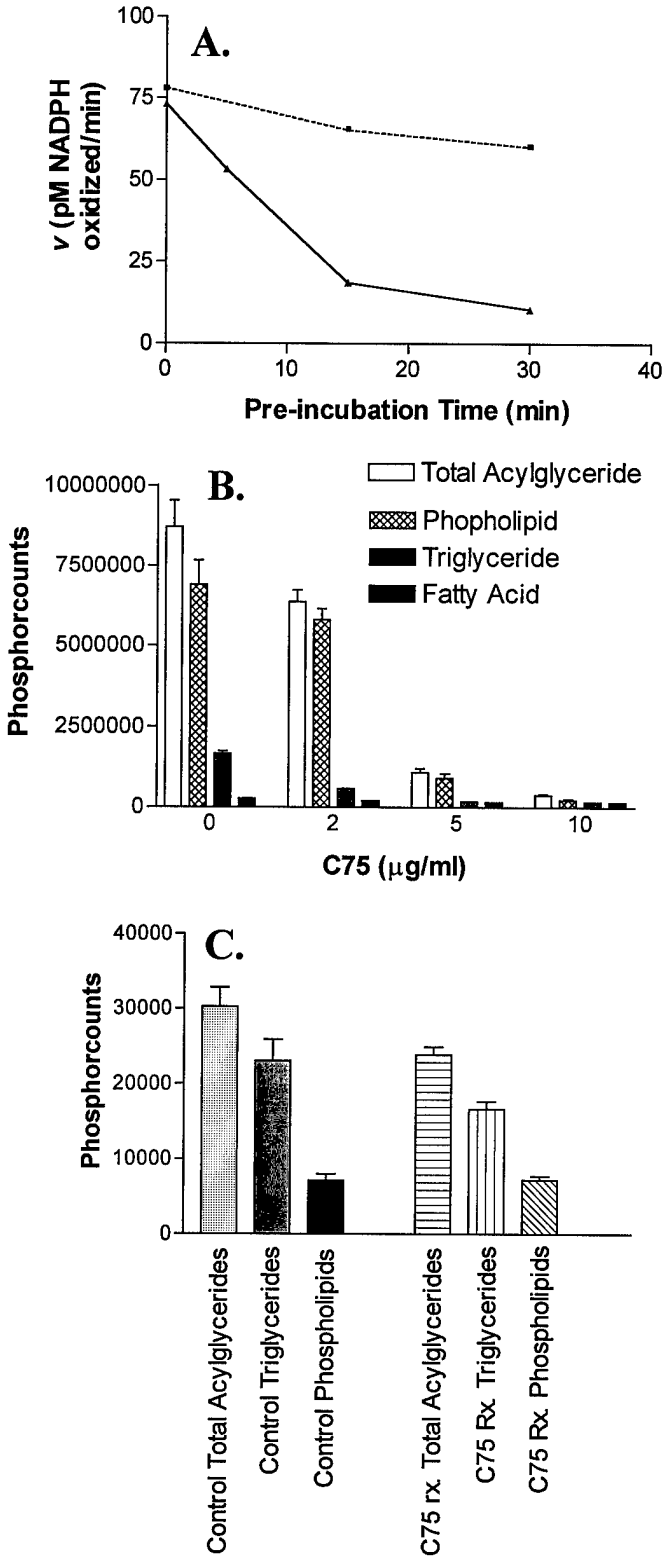
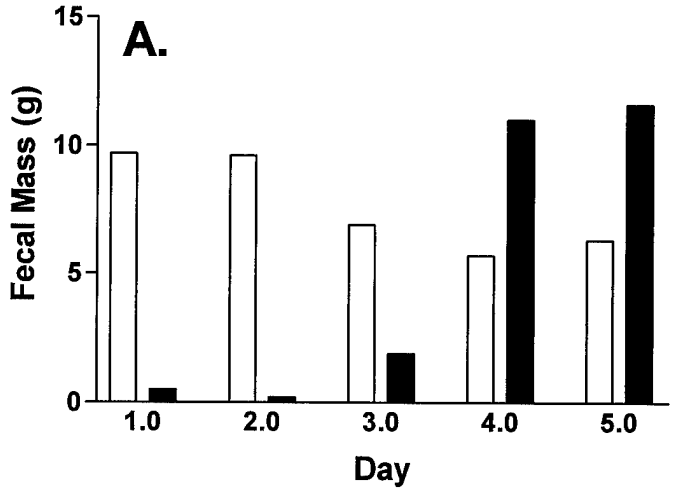
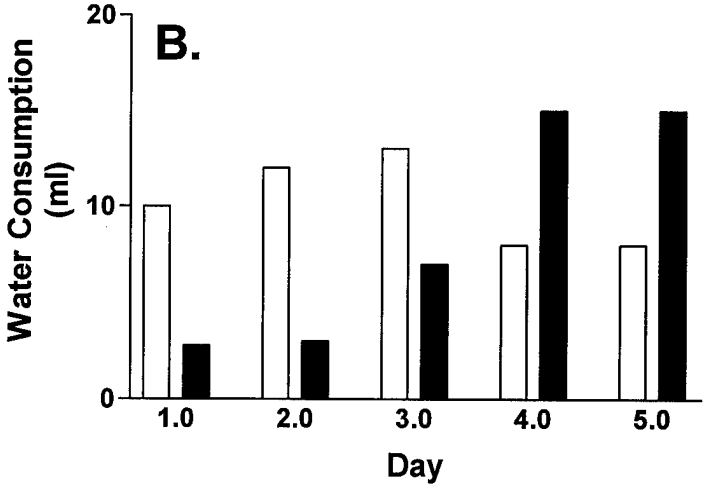

A.
v (pM NADPH oxidized/min)
100
75
50
25
0
0
10
20
30
40
Pre-incubation Time (min)
B.
Total Acylglyceride
Phopholipid
Triglyceride
Fatty Acid
Phosphorcounts
10000000
7500000
5000000
2500000
0
0
2
5
10
C75 (µg/ml)
C.
Phosphorcounts
40000
30000
20000
10000
0
Control Total Acylglycerides
Control Triglycerides
Control Phospholipids
C75 rx. Total Acylglycerides
C75 Rx Triglycerides
C75 Rx Phospholipids

Figure 2


A.
Fecal Mass (g)
15
10
5
0
1.0
2.0
3.0
4.0
5.0
Day


B.
Water Consumption (ml)
20
10
0
1.0
2.0
3.0
4.0
5.0
Day

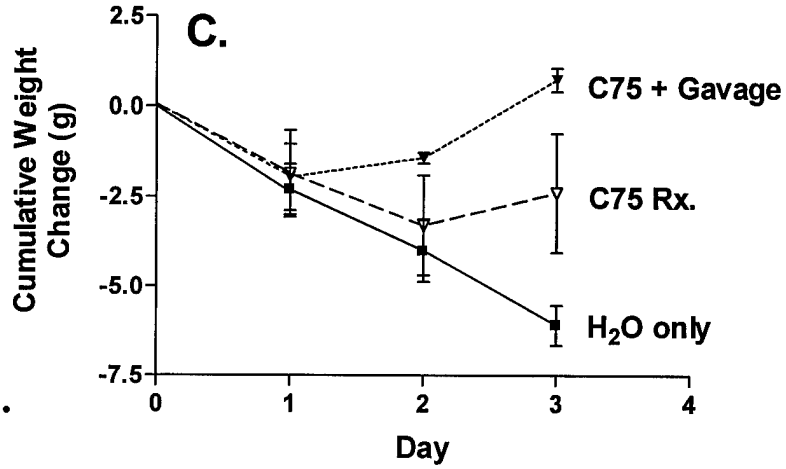
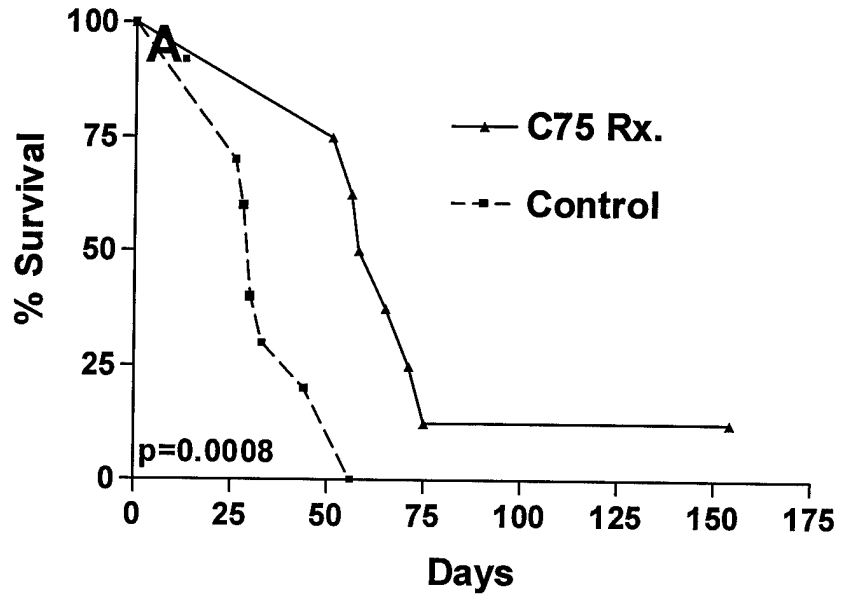

C.
Cumulative Weight Change (g)
2.5
0.0
-2.5
-5.0
-7.5
0
1
2
3
4
Day
C75 + Gavage
C75 Rx.
H2O only

Figure 3.


A.
100
75
50
25
0
% Survival
C75 Rx.
Control
p=0.0008
0
25
50
75
100
125
150
175
Days

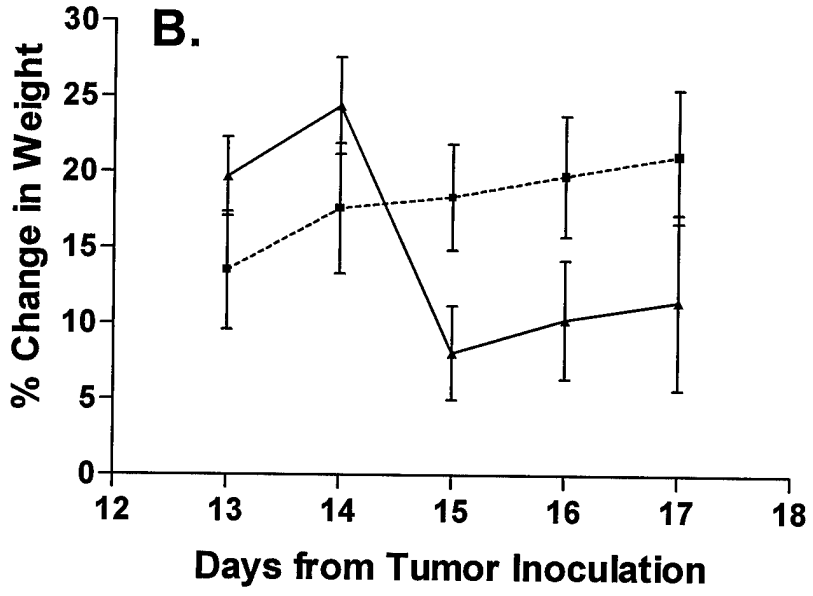

B.
30
25
20
15
10
5
0
% Change in Weight
12
13
14
15
16
17
18
Days from Tumor Inoculation

Figure 4.

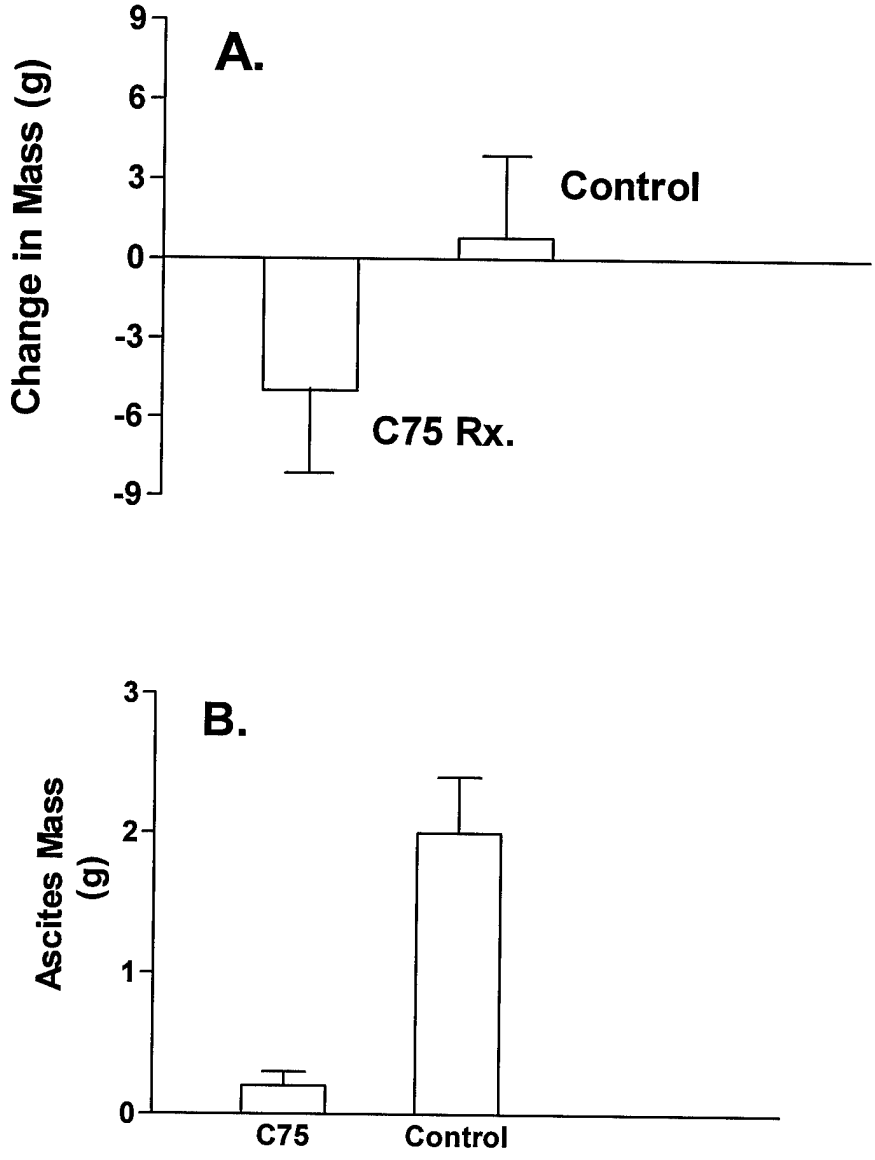

A.
Change in Mass (g)
9
6
3
0
-3
-6
-9
Control
C75 Rx.
B.
Ascites Mass (g)
3
2
1
0
C75
Control

Figure 5.

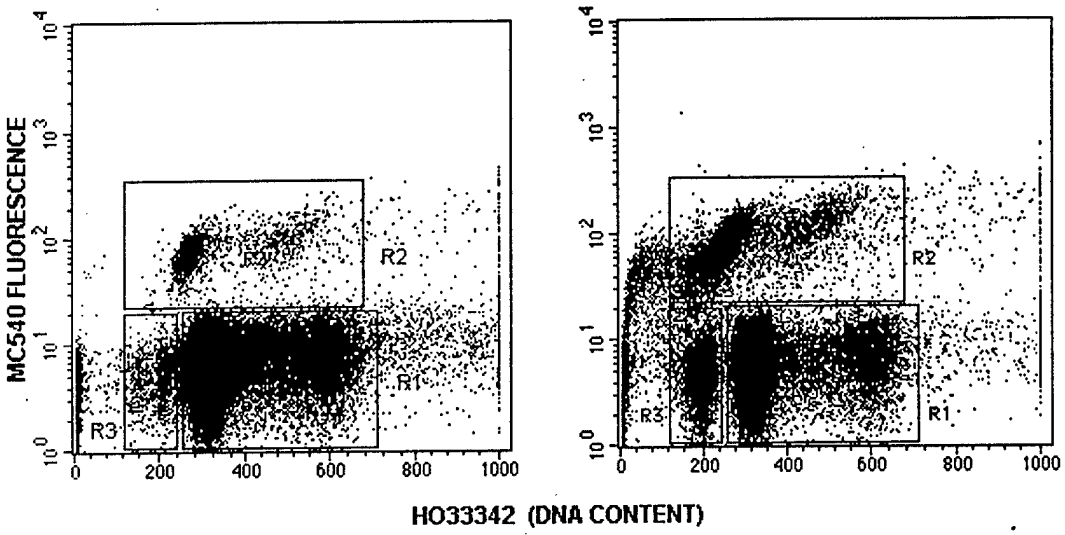


Figure 6.

APPENDIX B.

Pharmacological Inhibition of Mammalian Fatty Acid Synthase Activity Also Leads to Inhibition of DNA Replication.

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Running Title: Inhibition of DNA Replication by Fatty Acid Synthase Inhibitors

Key Words: Fatty Acid Synthase, cerulenin, DNA replication, metabolism, chemotherapy

Table of Contents

Abstract

Pharmacological inhibitors of the anabolic enzyme, fatty acid synthase (FAS), including the natural product cerulenin and the novel compound c75, are selectively cytotoxic to cancer cells via induction of apoptosis, apparently related to the tumor cell phenotype of abnormally elevated fatty acid synthetic metabolism. As part of a larger effort to understand the immediate downstream effect of FAS inhibition that leads to apoptosis, the effects of these inhibitors on cell cycle progression were examined. Both FAS inhibitors produce rapid, profound inhibition of DNA replication and S phase progression in human cancer cells. The dose responses for fatty acid synthesis inhibition and DNA synthesis inhibition are similar. The kinetics of both effects are rapid, with fatty acid synthesis inhibition occurring within 30 minutes and DNA synthesis inhibition occurring within 90 minutes of drug exposure. Meanwhile, apoptotic changes are not detected until 6 hours or later after inhibitor exposure. Fatty acid synthetic pathway activity, and the magnitude of DNA synthesis inhibition by FAS inhibitors are increased in parallel by withdrawal of lipid-containing serum from the cultures. The mechanism of DNA synthesis inhibition by cerulenin is indirect, because expression of certain viral oncogenes rescues DNA synthesis/S phase progression in cerulenin exposed cells. The data suggest a direct linkage at a regulatory level, between fatty acid synthesis and DNA synthesis in proliferating tumor cells.

Introduction.

The anti-metabolite, cerulenin, is a natural product of cephalosporium caerulens. Cerulenin, (2R, 3S)-2,3-epoxy-4-oxo-7,10-trans,trans- dodecadienamide, has been known since the 1960s as a specific inhibitor of fatty acid synthesis across a broad phylogenetic spectrum (1, 2). Cerulenin irreversibly inhibits the enzyme, fatty acid synthase (FAS), by binding covalently to the active site cysteine of the beta keto acyl synthase moiety, which performs the condensation reaction between the elongating fatty acid chain and each successive acetyl or malonyl residue. We have recently developed a novel small molecule inhibitor of FAS, called c75, with comparable inhibitory effects on fatty acid synthesis[1].

Cerulenin and c75 are selectively cytotoxic to human cancer cells, apparently related to the tumor cell phenotype of abnormally elevated fatty acid synthetic metabolism (3, 4). Exposure of human cancer cells to cerulenin or c75 triggers apoptosis (5). However, little is known of the immediate downstream effects of FAS inhibition that precede activation of the cell death program in tumor cells. Cerulenin has been previously reported to produce inhibition of DNA synthesis in bacteria, in addition to its well characterized effects on fatty acid synthesis, but the reported work did not distinguish a specific inhibitory effect on DNA synthesis from a more general cytotoxic or cytostatic effect (6). We report here that two chemically distinct FAS inhibitors produce rapid, profound inhibition of DNA replication and S phase progression in human cancer cells that precedes their cytotoxic effect. This result is consistent with an indirect mechanism secondary to fatty acid synthesis inhibition.

[1] Synthesis and Anti-tumor Activity of a Novel Inhibitor of Fatty Acid Synthase. Kuhajda, F.P., Pizer, E.S., Mani, N.S., and Townsend, C.A. Submitted 6/98.

Materials and Methods:

Cell lines and culture conditions: With the following exceptions, cell lines were obtained from the American Type Culture Collection. RKO colon carcinoma cells and stably transfected RKO clones were provided by Dr. M. Kastan (7). The stably transfected MCF7 clone was provided by Dr A. Fornace (8). Cells were cultured in DMEM with 10% fetal bovine serum (Hyclone). HL60 cells were cultured in RPMI with 10% fetal bovine serum or in serum-free medium as previously described (9). Cells were screened periodically for mycoplasma contamination (Gen-probe). Cerulenin (Sigma) and c75 were added as stock 5mg/ml solutions in DMSO. The final concentration of DMSO in cultures was at or below 0.2%.

Metabolic labeling: For pulse labeling experiments, cells were plated at $5x10^4$/well in 1 ml in 24 well plates and incubated overnight. DNA synthesis was assayed with a 2 hour pulse of ^{3}H-thymidine, 1μCi/ well, followed by precipitation in 10% trichloroacetic acid, 2 washes in 95% ethanol, solublization in 0.1 N ammonium acetate, and scintillation counting. Fatty acid synthesis was assayed with a 2 hour pulse of [U-^{14}C]-acetic acid, 1μCi/ well, followed by Folch extraction and scintillation counting (10). All determinations were in triplicate. Kinetic labeling studies of fatty acid synthesis in HL60 cells were performed by addition of 5μCi/ well of [U-^{14}C]-acetic acid to 1.8 ml suspensions of HL60 cells at 3x 10^6/ml. Aliquots of 0.1 ml were removed in duplicate at stated time points and quantitated as above. Kinetic labeling studies of DNA synthesis in HL60 cells were performed by addition of 5μCi/ well of ^{3}H-thymidine to 2.5ml suspensions of HL60 cells at 3x 10^6/ml. Aliquots of 0.1 ml were removed in triplicate at

stated time points and quantitated as above. Data are presented as mean values with bars showing the standard error. Calculations and graphing were performed in Prism 2.0 (GraphPad).

Detection of apoptosis: HL60 cells were exposed to cerulenin [10μg/ml] or c75 [10μg/ml] for the indicated intervals, then analyzed by TUNNEL labeling of DNA as previously described (11). Detection of exposed phosphatidyl serine on the outer surface of the plasma membrane was by decoration with merocyanine 540 according to the manufacturer's instructions (Sigma). Cytometry data were acquired on a Becton Dickinson FACSscan and analysed with LYSYS11, version 1.1.

Results and Discussion:

Several lines of evidence suggest linkage between tumor cell proliferation and elevated fatty acid synthesis (9, 12). In order to determine whether cell cycle progression is altered after inhibition of fatty acid synthesis, we measured incorporation of thymidine into DNA after exposure of tumor cells to cerulenin or c75 (Figure 1A and B). These experiments demonstrated that fatty acid synthesis inhibitors produce a profound inhibition of DNA replication within 2 hours. Since S phase typically requires at least 6 hours, this effect is consistent with inhibition of S phase progression rather than inhibition of the G1/S transition. Figure 1A and B demonstrate that the dose responses of fatty acid synthesis and DNA synthesis to cerulenin and c75, 2 hours after drug exposure, are similar for HCT116 colon carcinoma cells. Cerulenin [10μg/ml] and c75 [10μg/ml] both produce inhibition of DNA synthesis to less than 10% of control levels. Pulse

labeling at later time points confirmed that DNA synthesis remained inhibited to less than 1% of control levels at 8 and 18 hours after drug exposure (Figure 1C).

In order to determine how rapidly fatty acid synthesis and DNA synthesis are inhibited by these drugs, suspension cultures of HL60 promyelocytic leukemia cells adapted to serum-free medium were metabolically labeled with [U-^{14}C]-acetic acid or ^{3}H-thymidine after exposure to drug and serially sampled to determine labeling rates. Inhibition of fatty acid synthesis was very rapid, with divergence of labeling between control and drug treated cells occurring between 15 and 30 minutes (Figure 2a). Thymidine labeling demonstrated partial inhibition of DNA synthesis during the first 60 to 90 minutes after drug exposure, with maximal inhibition occurring after 60 minutes of exposure to c75 or after 90 minutes of exposure to cerulenin (Figure 2b). The fatty acid synthesis and DNA synthesis rates during each time interval after addition of drug, determined as a percentage of control, are shown below the graphs. Meanwhile, fragmentation of chromosomal DNA, a definitive sign of apoptosis, was not detectable until at least 6 hours after drug exposure (Figure 2c, 11). Similar kinetics of apoptosis induction were observed in HL60 cells using exposure of phosphatidyl serine on the outer surface of the plasma membrane as the indicator of early entry into apoptosis (data not shown). The rate at which apoptosis occurs is variable among cell lines, with most carcinoma lines that we have studied requiring more than 6 hours after drug exposure before phosphatidyl serine is exposed on the outer surface of the plasma membrane (data not shown).

We have previously observed that adaptation of HL60 cells from medium containing 10% fetal bovine serum to serum-free, fatty acid-free medium produces

elevation of FAS enzyme expression (9). Metabolic labeling of endogenously synthesized lipids performed in medium with 10% fetal bovine serum or in serum-free, fatty acid-free medium reveals comparable modulation of fatty acid synthesis pathway activity, probably because cells deprived of the exogenous lipid available in serum increase endogenous fatty acid synthesis to produce membrane lipids required for growth (Table 1). Despite an approximately 20-fold difference in overall pathway activity in the two media, both cerulenin and c75 inhibit fatty acid synthesis to approximately half of control levels in both media. However, the fractional inhibition of DNA synthesis by cerulenin or c75 is much greater in serum-free, fatty acid-free medium than in medium with 10% fetal bovine serum (approximately 20% of control levels versus approximately 80% of control). When the same experiment was performed in 5% serum, an intermediate level of DNA synthesis inhibition occurred (data not shown). The parallel modulation of fatty acid synthesis pathway activity and sensitivity to DNA synthesis inhibition suggests a functional linkage between inhibition of fatty acid synthesis and S phase arrest. Of note, carcinoma cells do not demonstrate comparable plasticity in their response to serum and exogenous lipid concentrations.

Replication of DNA in mammalian cells is a highly complex process that requires coordinate activity of numerous metabolic pathways for nucleotide synthesis, active basal replication machinery and S phase cyclins and cyclin dependent kinases (13). The early genes of certain DNA tumor viruses are able to abrogate higher order S phase regulation, and facilitate passage of infected cells through S phase in order to accomplish viral replication (14). In order to broadly distinguish between cellular targets that are directly

required for DNA replication and those that indirectly affect replication, we compared the effect of FAS inhibitors on cells before and after introduction of three viral oncogenes.

Expression of viral oncogenes from SV40 or Human Papilloma Virus relieved the inhibitory effect of cerulenin on DNA replication, though fatty acid synthesis inhibition was similar. CV-1, a monkey kidney cell line, served as the parent of COS-1, a stably transfected, transformed line that expresses SV40 T antigen (15). The dose response of fatty acid synthesis to cerulenin is similar for CV-1 cells and COS-1 cells, indicating that the drug is able to enter the cells and inactivate FAS comparably (figure 3a). The dose response of DNA synthesis to cerulenin for CV-1 cells was similar to that of HCT116 colon carcinoma cells. However, the inhibitory effect of cerulenin on DNA synthesis in COS-1 cells was markedly reduced, with thymidine incorporation remaining at 66% of control levels after exposure to cerulenin [10μg/ml] (figure 3b). Similar results were obtained when RKO colon carcinoma cells were compared to stably transfected RKO clones that expressed Human Papilloma Virus (HPV)16 E6 or E7 genes (figure 3c and d), and when MCF7 breast carcinoma cells were compared to a stably transfected MCF7 clone that expressed the HPV 16 E6 gene (figure 3e and f). The RKO-HPV16 E6 clone shown in figure 3 is representative of 3 clones tested. An RKO clone transfected only with neomycin phosphotransferase behaved similarly to the parent cell line, but with slightly delayed drug response kinetics (not shown). The differential effects of c75 on DNA replication in this panel of cell lines was variable (not shown).

The mechanism(s) by which cerulenin and c75 inhibit DNA replication remain to be elucidated. However, the above data support the model that DNA replication is inhibited as a consequence of fatty acid synthesis inhibition. Two chemically distinct

FAS inhibitors both inhibit DNA replication with dose responses similar to their effects on FAS. The inhibition of DNA replication follows rapidly after fatty acid synthesis inhibition, and co-modulates with fatty acid synthesis pathway activity in response to serum. The mechanism likely does not involve a direct effect of drug on the DNA molecule, or on the basal replication machinery. Rather, inhibition probably occurs at the level of higher order cell cycle regulation, and may involve p53 function, since expression of SV40 T antigen, HPV 16 E6 or HPV16 E7 abrogated the inhibition of DNA replication by cerulenin. This differential effect on DNA synthesis occurred despite similar fatty acid synthesis inhibition in parent and viral oncogene expressing cells. Our model postulates a direct mechanistic linkage between fatty acid synthesis and DNA synthesis. This model proposes a novel regulatory pathway in proliferating mammalian cells, that could have a major role in maintaining normal cellular growth. An analogous growth arrest response in bacteria, the stringent response, coordinately inhibits macromolecular synthesis after a variety of nutrient deprivation stresses, including cerulenin exposure (16), and similar physiological effects occurred after restriction of phospholipid synthesis (17). The importance of this effect in the ultimate cytotoxic outcome of fatty acid synthesis inhibition in human cancer remains to be determined.

Acknowledgements: We thank F. Kuhajda, A. Owens and L. Pizer for helpful discussions of this work and for review of the manuscript. We thank M. Kastan for the RKO cell lines, and for advice on experimental design. The MCF7 E6 cell line was supplied by A. Fornace. Compound 75, (c75), was supplied by C. Townsend.

Figure 1. **Inhibition of fatty acid synthesis and of DNA replication by FAS inhibitors have similar dose responses.** (A and B) Triplicate cultures of HCT116 colon carcinoma cells were exposed to cerulenin or c75 for 4 hours. During the last 2 hours, cells were pulse labeled with either ^{3}H-thymidine or [U-^{14}C]-acetic acid as described in materials and methods. Label incorporation is shown as percent of control. (C) Similar pulse labeling with ^{3}H-thymidine was performed from 6 to 8 or from 16 to 18 hours after drug exposure. Label incorporation is shown as DPM per $5x10^{4}$ cells. Bars = Standard Error of the Mean.

Figure 2. **Inhibition of DNA replication occurs rapidly after inhibition of fatty acid synthesis and in the early interval before cell death.** (A and B) Suspension cultures of HL60 promyelocytic leukemia cells in serum free medium were sampled at intervals during drug treatment and metabolic labeling, as described in materials and methods. Bars = SEM. The fatty acid synthesis and DNA synthesis rates during each time interval after addition of drug, are expressed as a percentage of control, and are shown below the graphs. (C) A time course for induction of apoptotic changes is displayed.

Figure 3. **Expression of certain viral oncogenes abrogates the inhibitory effect of cerulenin on DNA replication.** Triplicate cultures of the indicated cells were exposed to cerulenin for 4 hours. During the last 2 hours, cells were pulse labeled with either [U-^{14}C]-acetic acid (A, C and E) or ^{3}H-thymidine (B, D, and F) as described in materials and methods. Label incorporation is shown as percent of control. Bars = Standard Error of the Mean.

Table 1

Modulation of Fatty Acid Synthesis and DNA Replication Inhibition by Serum Concentration

Serum	10% FBS[a]		None[a]	
FA synthesis inhibitor	Cerulenin	c75	Cerulenin	c75
Fractional inhibition of fatty acid synthesis[b]	45%	65%	31%	58%
Fractional inhibition of DNA synthesis[b]	76%	87%	21%	21%

[a] Fatty acid synthesis pathway activity (^{14}C-acetate incorporation into extractable lipid/cell/hour) is 20 fold higher in medium without serum than in 10% FBS.

[b] mean percent of control after maximal inhibition.

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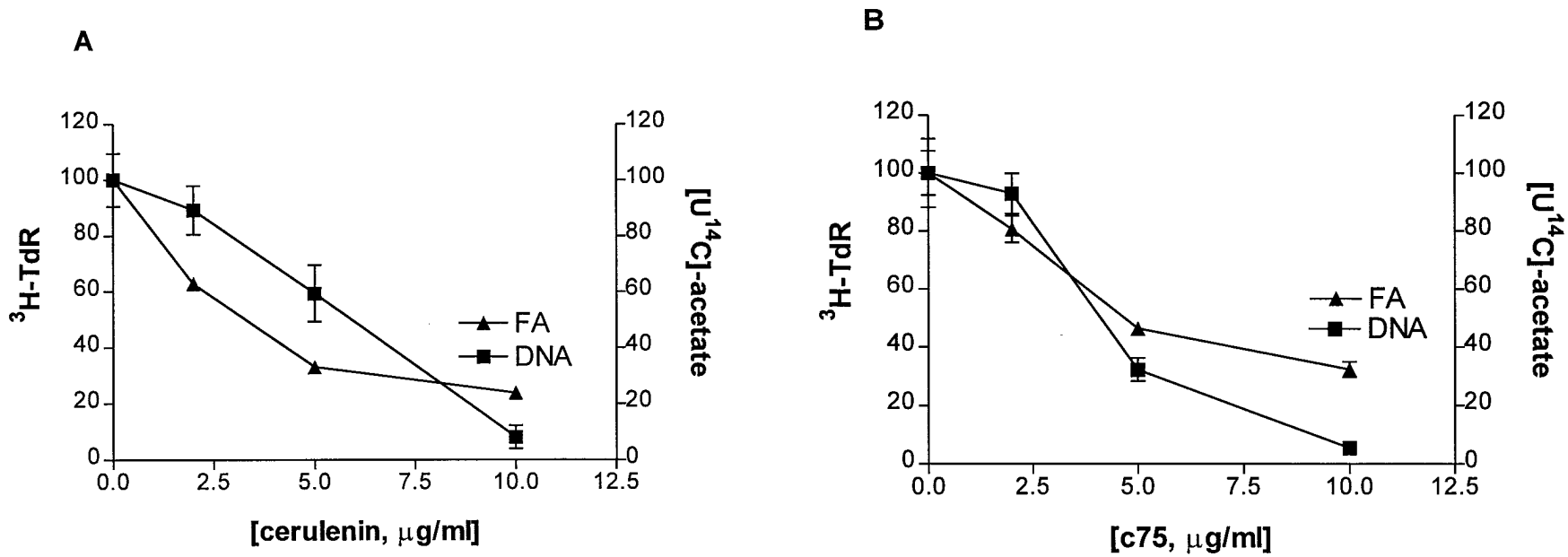
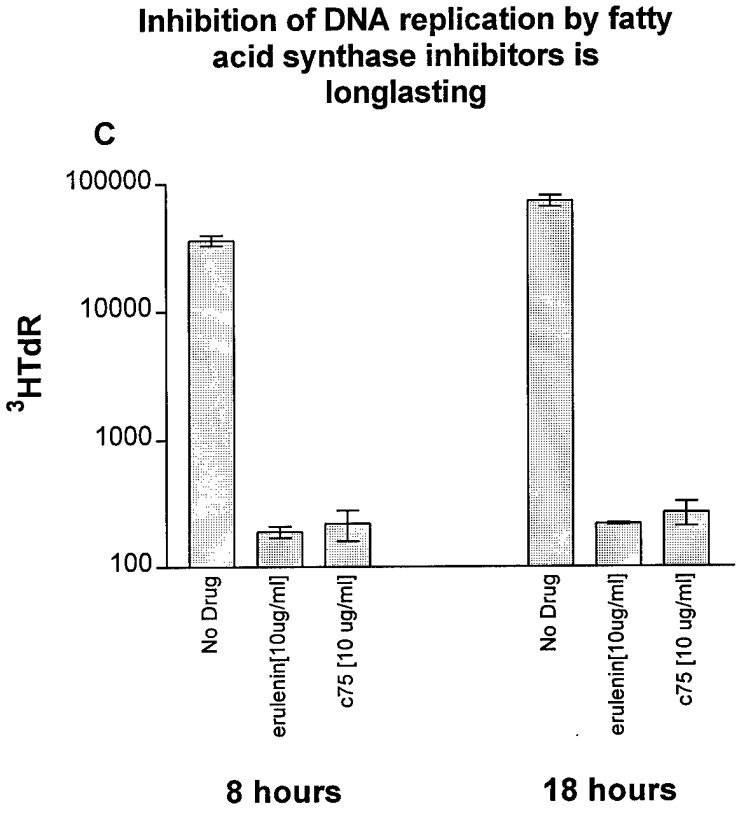
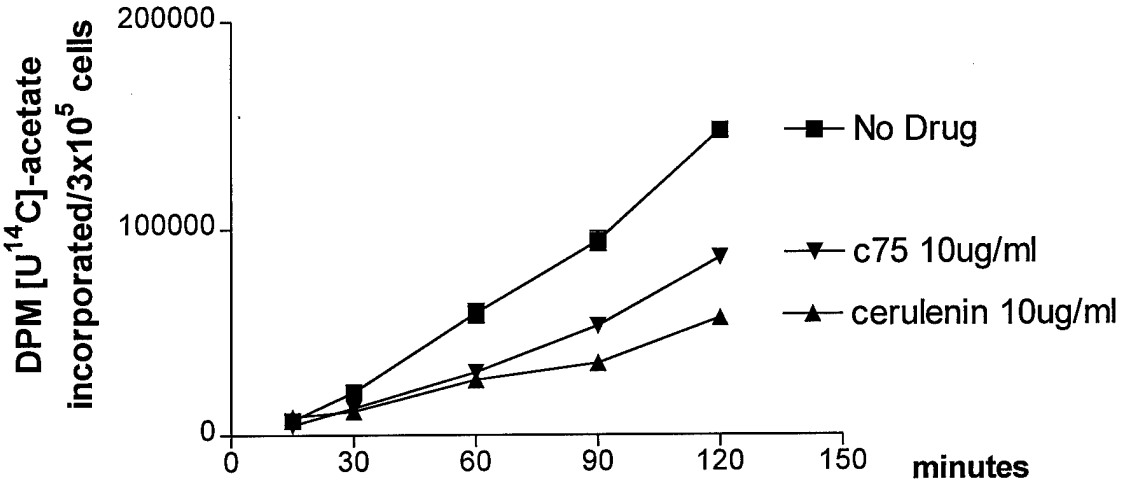
Inhibition of fatty acid synthesis and DNA replication by Fatty Acid Synthase inhibitors have similar dose responses



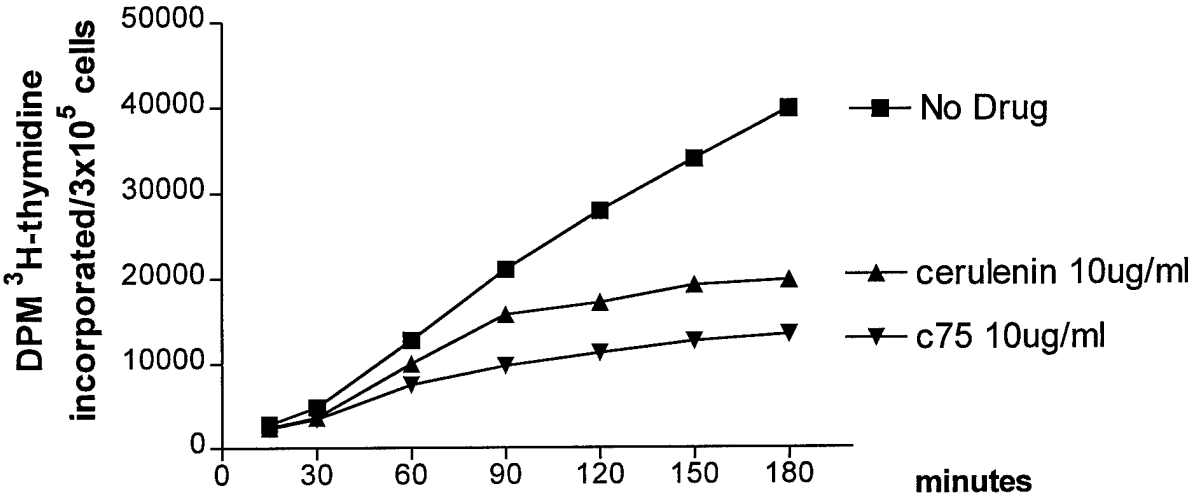

Inhibition of DNA replication by fatty acid synthase inhibitors is longlasting
C
100000
10000
1000
100
^{3}HTdR
No Drug
erulenin[10ug/ml]
c75 [10 ug/ml]
No Drug
erulenin[10ug/ml]
c75 [10 ug/ml]
8 hours
18 hours

A



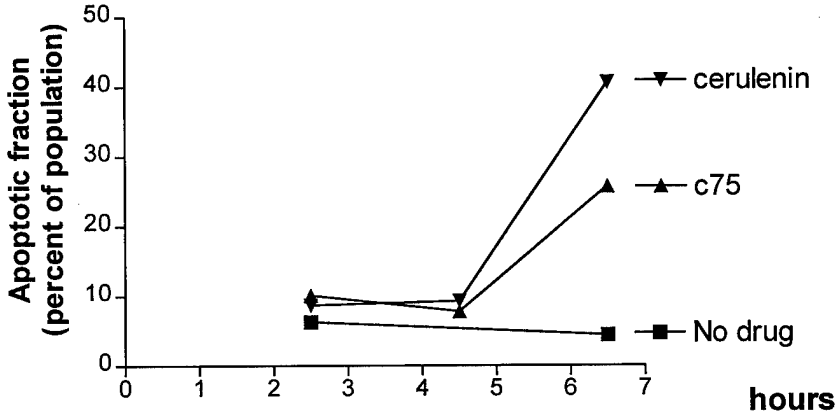
	minutes	15	30	60	90	120
FA synthesis rate	c75	69	59	46	65	62
(percent of control)	cerulenin	120	20	40	23	41

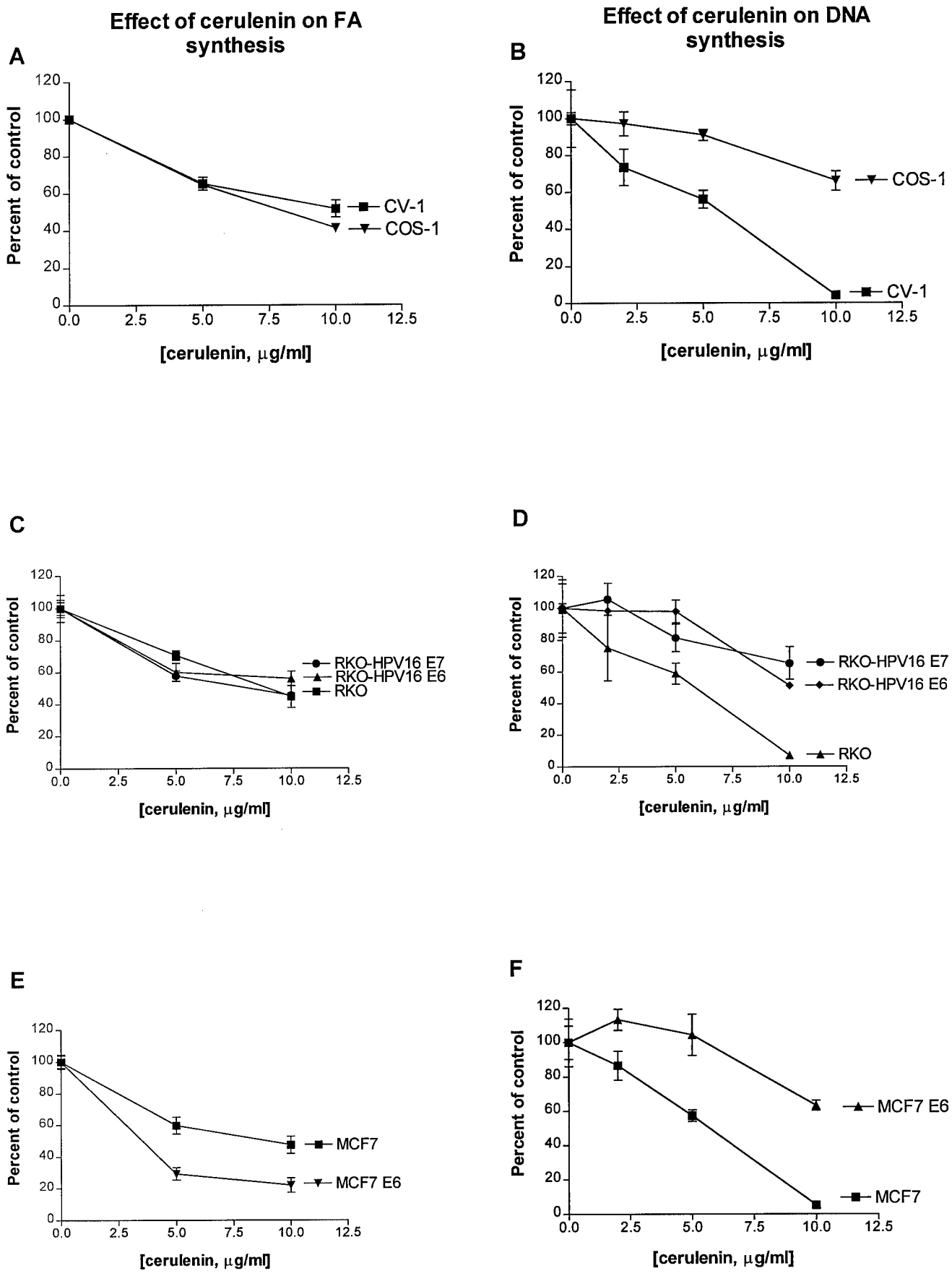
B



	minutes	15	30	60	90	120	150	180
DNA synthesis rate	c75	82	56	52	27	22	23	13
(percent of control)	cerulenin	86	61	80	70	20	33	10

C




A
Effect of cerulenin on FA synthesis
Percent of control
120
100
80
60
40
20
0
0.0
2.5
5.0
7.5
10.0
12.5
[cerulenin, μg/ml]
CV-1
COS-1
B
Effect of cerulenin on DNA synthesis
Percent of control
[cerulenin, μg/ml]
COS-1
CV-1
C
Percent of control
[cerulenin, μg/ml]
RKO-HPV16 E7
RKO-HPV16 E6
RKO
D
Percent of control
[cerulenin, μg/ml]
RKO-HPV16 E7
RKO-HPV16 E6
RKO
E
Percent of control
[cerulenin, μg/ml]
MCF7
MCF7 E6
F
Percent of control
[cerulenin, μg/ml]
MCF7 E6
MCF7