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Fatty Acid Synthase Inhibition Triggers Apoptosis during S Phase in Human Cancer Cells[1]

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ABSTRACT

C75, an inhibitor of fatty acid synthase (FAS), induces apoptosis in cultured human cancer cells. Its proposed mechanism of action linked high levels of malonyl-CoA after FAS inhibition to potential downstream effects including inhibition of carnitine palmitoyltransferase-1 (CPT-1) with resultant inhibition of fatty acid oxidation. Recent data has shown that C75 directly stimulates CPT-1 increasing fatty acid oxidation in MCF-7 human breast cancer cells despite inhibitory concentrations of malonyl-CoA. In light of these findings, we have studied fatty acid metabolism in MCF7 human breast cancer cells to elucidate the mechanism of action of C75. We now report that: (*a*) in the setting of increased fatty acid oxidation, C75 inhibits fatty acid synthesis; (*b*) C273, a reduced form of C75, is unable to inhibit fatty acid synthesis and is nontoxic to MCF7 cells; (*c*) C75 and 5-(tetradecyloxy)-2-furoic acid (TOFA), an inhibitor of acetyl-CoA carboxylase, both cause a significant reduction of fatty acid incorporation into phosphatidylcholine, the major membrane phospholipid, within 2 h; (*d*) pulse chase studies with [^{14}C]acetate labeling of membrane lipids show that both C75 and TOFA accelerate the decay of ^{14}C-labeled lipid from membranes within 2 h; (*e*) C75 also promotes a 2–3-fold increase in oxidation of membrane lipids within 2 h; and (*f*) because interference with phospholipid synthesis during S phase is known to trigger apoptosis in cycling cells, we performed double-labeled terminal deoxynucleotidyltransferase-mediated nick end labeling and BrdUrd analysis with both TOFA and C75. C75 triggered apoptosis during S phase, whereas TOFA did not. Moreover, application of TOFA 2 h before C75 blocked the C75 induced apoptosis, whereas etomoxir did not. Taken together these data indicate that FAS inhibition and its downstream inhibition of phospholipid production is a necessary part of the mechanism of action of C75. CPT-1 stimulation does not likely play a role in the cytotoxic response. The continued ability of TOFA to rescue cancer cells from C75 cytotoxicity implies a proapoptotic role for malonyl-CoA independent of CPT-1 that selectively targets cancer cells as they progress into S phase.

INTRODUCTION

Altering fatty acid metabolism in cancer cells affords the possibility of cancer chemotherapy that does not substantially affect normal cycling cells. FAS,[3] the sole enzyme responsible for the *de novo* synthesis of fatty acids from carbohydrate, is highly expressed in common human tumors (1–20). Studies from our laboratory and others have shown high levels of FAS expression in most human cancers including breast, prostate, colon, lung, stomach, and skin without substantial elevation in normal tissues. C75, an inhibitor of FAS, induced apoptosis in cultured human breast cancer cells (21–24) and has shown significant antitumor activity against human breast (24), prostate (20), ovary,[4] and mesothelioma xenografts (25) in athymic mice. In addition to FAS inhibition, C75 has been shown recently to stimulate CPT-1 leading to increased fatty acid oxidation in cultured MCF-7 human breast cancer cells, nontransformed hepatocytes and adipocytes, and in diet-induced obese mice (26). The finding of CPT-1 stimulation by C75 in human breast cancer cells has led to a reassessment of the mechanism of action of C75 leading to apoptosis in human cancer cells.

FAS produces the 16-carbon fatty acid palmitate through successive NADPH-dependent condensations of acetyl-CoA with malonyl-CoA (27). C75, acting at the β-ketoacyl condensation step, blocks the covalent reaction of acetate with malonate in the active site (28). As a result of FAS blockade, high levels of malonyl-CoA continue to be generated by ACC, the rate-limiting enzyme of the fatty acid synthesis pathway (Fig. 1; Refs. 24, 29). Malonyl-CoA, in addition to its role as a substrate for FAS, is a competitive inhibitor of CPT-1 the rate limiting enzyme of fatty acid oxidation (30). CPT-1 esterifies long-chain acyl-CoAs to carnitine, thus allowing their entry into the mitochondria for oxidation (Fig. 1; Ref. 30). Thus, high levels of malonyl-CoA during lipogenesis inhibit CPT-1, preventing the oxidation of newly synthesized fatty acids.

We had proposed that the high levels of malonyl-CoA generated by FAS inhibition were proapoptotic based on a series of *in vitro* observations. TOFA, a competitive inhibitor of ACC, the enzyme responsible for the synthesis of malonyl-CoA, profoundly inhibits fatty acid synthesis but is essentially nontoxic to cultured human cancer cells (24). Moreover, treatment of human cancer cells with TOFA before C75 administration reproducibly rescued them from C75 cytotoxicity, and prevented the C75 induced increase in malonyl-CoA (24). Other studies found a physical association between CPT-1 and BCL-2 on the outer mitochondrial membrane providing an additional potential link between CPT-1 and the regulation of apoptosis (31). In light of the finding that C75 stimulates CPT-1 even in the setting of inhibitory concentrations of malonyl-CoA, it is unlikely that malonyl-CoA inhibition of CPT-1 is necessary for the cytotoxicity of C75 to human cancer cells. We have now focused on the consequences of C75 and TOFA on lipid metabolism and the cell cycle to generate a hypothesis that accommodates the divergent effects of these compounds on human breast cancer cells.

We now report that both C75 and TOFA inhibit phospholipid synthesis in MCF-7 cells. C75 also increases oxidation of membrane lipids in keeping with its CPT-1 stimulatory activity. Irreversible CPT-1 inhibition with etomoxir is nontoxic to cancer cells and also

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[3] The abbreviations used are: FAS, fatty acid synthase; CPT, carnitine palmitoyltransferase; ACC, acetyl-CoA carboxylase; TUNEL, terminal deoxynucleotidyltransferase (Tdt)-mediated nick end labeling; PEG, polyethyleneglycol; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt; TdT, terminal deoxynucleotidyl transferase; BrdUrd, bromodeoxyuridine.

[4] F. P. Kuhajda, J. N. Thupari, and M. L. Pinn, unpublished observations.

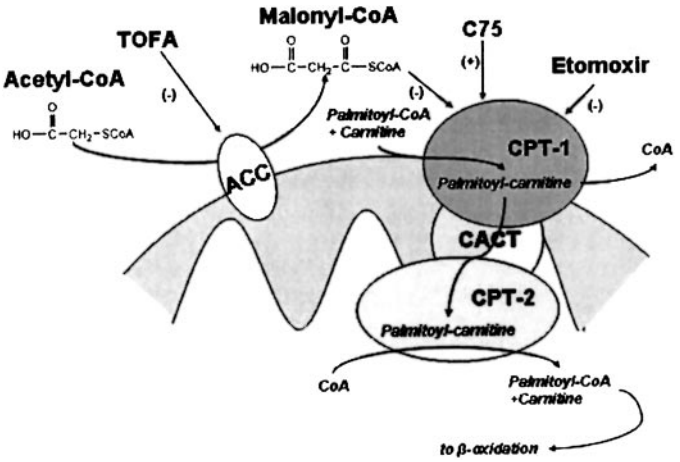


Fig. 1. Fatty acid synthesis and oxidation at the outer mitochondrial membrane. ACC, the rate-limiting enzyme of fatty acid synthesis, and CPT-1, the rate-limiting enzyme of fatty acid oxidation, both reside in the outer mitochondrial membrane. Oxidation of long chain fatty acids in the mitochondria is highly regulated by a transport system consisting of three proteins, CPT-1, CACT (carnitine acylcarnitine translocase), and CPT-2. CPT-1, regulated physiologically by competitive malonyl-CoA inhibition, transesterifies fatty acids from CoA to carnitine. The acylcarnitines are then transported to CPT-2 via CACT. CPT-2 reverses the transesterification of fatty acids, from acylcarnitines back to acyl-CoAs, for fatty acid oxidation. High levels of malonyl-CoA generated during fatty acid synthesis inhibit CPT-1 preventing oxidation of newly synthesized fatty acids. Etomoxir is an irreversible inhibitor of CPT-1 that binds at the same site on CPT-1 as malonyl-CoA. C75 stimulates CPT-1 activity even in the presence of inhibitory concentrations of malonyl-CoA.

fails to rescue cells from C75 cytotoxicity. C273, a chemically reduced form of C75 that is unable to inhibit purified human FAS or fatty acid synthesis, is nontoxic to MCF-7 cells. In the presence of C75, MCF-7 cells undergo apoptosis during S phase as demonstrated by BrdUrd and TUNEL labeling and TOFA preincubation blocks the C75-induced S phase apoptosis. Taken together these data indicate that: (*a*) inhibition of FAS is necessary for C75 induced cytotoxicity; (*b*) neither CPT-1 stimulation nor inhibition is proapoptotic in this system; and (*c*) C75 reduces BrdUrd incorporation and concomitantly induces apoptosis, which may be related to malonyl-CoA metabolism.

MATERIALS AND METHODS

Cell Culture, Chemicals, and Statistical Methods. MCF7 human breast cancer cells, obtained from the American Type Culture Collection (HTB-22), were cultured in DMEM with 10% FCS. MCF7 cells were used for all of the cellular assays. ZR-75–1 human breast cancer cells obtained from the American Type Culture Collection were cultured as above and used as a source for FAS purification. Cells were screened periodically for *Mycoplasma* contamination (Gen-probe). C75 was obtained from FASgen, Inc. Etomoxir was purchased from Horst P. O. Wolfe, Projeckt-Entwicklung, Konstanz, Germany. Etomoxir, as an irreversible inhibitor of CPT-1, blocks mitochondrial fatty acid oxidation but does not affect extramitochondrial α or ω fatty acid oxidation. TOFA, a reversible inhibitor of ACC, was synthesized by Craig A. Townsend at the Department of Chemistry at the Johns Hopkins University. C75 and TOFA, dissolved in DMSO, were added from 5 mg/ml stock solutions; the final DMSO concentration in cultures was $\leq$0.2%. Unless otherwise specified, data were analyzed using two-tailed, unpaired *t* tests using Graph Pad Prism software, version 3.0 for Windows (Graph Pad Software, San Diego, CA).

Clonogenic Assays. Four $\times$ 10^5 cells were plated in 25 cm^2 flasks. After overnight culture, cells were treated with C75 for 30 min or 6 h at concentrations listed. After drug treatment, cells were trypsinized, and equal numbers of treated and controls cells were plated in triplicate in 60-mm dishes at a density of 500 cells/dish in medium without C75. Clones were stained and counted after 7–10 days.

Synthesis of C273. C273 [4,5-*trans*-(±)-3-*cis*-Methyl-3-butyrolactone-5-octyl-4-carboxylic acid (*cis*- diastereomer = 3-methyl, 4-carboxylic acid are *cis*- to each other)] was produced by the reduction of C75 as follows. To a solution of C75 (100 mg, 0.39 mmol) in EtOAc (3.0 ml), Pd (30 mg, 10% on carbon), and H_2 (50 ψ) was added for 2 h. The mixture was filtered through Celite and evaporated to give a mixture of diastereomers (1.8:1, *trans:cis*-). Column chromatography (20%EtOAc/2%CH_3CO_2H/Hexanes) yielded the *trans*-diastereomer with an inseparable isomerized byproduct (3-methyl, 5-octyl,2-oxo,2,5-dihydro-furan,4-carboxylic acid; 3.8:1, 59.5 mg) and pure *cis*-isomer (32.7 mg; 92% overall yield).

Purification of Human FAS. Human FAS was purified from cultured ZR-75–1 cells. The procedure, adapted from Linn (32) and Kuhajda *et al.* (33), used hypotonic lysis, successive PEG precipitations, and anion exchange chromatography. Ten T150 flasks of confluent ZR-75–1 cells were lysed with 1.5 ml lysis buffer [20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, and 0.1% Igepal CA-630] and dounce homogenized on ice for 20 strokes. The lysate was centrifuged in a JA-20 rotor (Beckman) at 20,000 rpm for 30 min at 4°C and the supernatant brought to 42 ml with lysis buffer. A solution of 50% PEG 8000 in lysis buffer was added slowly to the supernatant to a final concentration of 7.5%. After rocking for 60 min at 4°C, the solution was centrifuged in a JA-20 rotor (Beckman) at 15,000 rpm for 30 min at 4°C. Solid PEG 8000 was then added to the supernatant to a final concentration of 15%. After the rocking and centrifugation was repeated as above, the pellet was resuspended overnight at 4°C in 10 ml of Buffer A [20 mM K_2HPO_4 (pH 7.4)]. After 0.45 μM filtration, the protein solution was applied to a Mono Q 5/5 anion exchange column (Pharmacia). The column was washed for 15 min with buffer A at 1 ml/min, and bound material was eluted with a linear 60-ml gradient over 60 min to 1 M KCl. FAS (M_r ~270,000) typically elutes at 0.25 M KCl. This procedure resulted in substantially pure preparations of FAS (>95%) as judged by Coomassie-stained PAGE.

Cytotoxicity Assay. To measure the cytotoxicity of specific compounds against cancer cells, 9 $\times$ 10^3 cells were plated per well in 96-well plates in DMEM medium with 10% fetal bovine serum, insulin, penicillin, and streptomycin. After overnight culture, the compounds, dissolved in DMSO, were added to the wells in 1 μl volume at the following concentrations: 80, 40, 20, 10, 5, 2.5, 1.25, and 0.625 μg/ml in triplicate. One μl of DMSO was added to triplicate wells as the vehicle control. C75 was run at 40, 20, 10, 15, 12.5, 10, and 5 μg/ml in triplicate as positive controls.

After 24 or 72 h, cells were incubated for 4 h with the XTT reagent as per manufacturer's instructions [Cell Proliferation kit II (XTT); Roche Diagnostics, Mannheim, Germany]. Plates were read at A^{490} and A^{650} on a Molecular Devices SpectraMax Plus Spectrophotometer. Three wells containing the XTT reagent without cells served as the plate blank. XTT data were reported as $A^{490} - A^{650}$. Averages and SE were computed using SOFTmax Pro software (Molecular Dynamics). The IC_{50} for the compounds was defined as the concentration of drug leading to a 50% reduction in $A^{490} - A^{650}$ compared with controls. IC_{50} was calculated by linear regression, plotting the FAS activity as percentage of control *versus* drug concentrations. Linear regression, best-fit line, r^2, and 95% confidence intervals were determined using Graph Pad Prism, version 3.0 (Graph Pad Software).

Cell Growth Assay. To asses cell growth during drug treatment, cells were plated in 24-well plates at 5 $\times$ 10^4/well and incubated overnight. After overnight culture, compounds added at concentrations indicated and vehicle controls were included for each plate. Each concentration was plated in quadruplicate. After 2, 4, and 24 h incubation with drug at 37°C, plates were stained with crystal violet (0.2% in 12% ethanol), solubilized, and absorbance was measured at 490 nm. Cell growth was expressed as percentage of control ± SE.

Metabolic Labeling. For measurement of fatty acid synthesis and pulse-chase studies, cells were cultured in 24-well plates at 5 $\times$ 10^4 per well and incubated overnight. For cells treated with C75, etomoxir, or TOFA alone, compounds were added for 2 h at concentrations listed before pulse labeling. When cells were treated with a combination of C75 and etomoxir, or C75 and TOFA, etomoxir or TOFA was added 1 h before the 2-h incubation with C75. Fatty acid synthesis was assayed by a 2 h pulse of [U^{14}C]acetate, 1 μCi/well, followed by Folch extraction and scintillation counting with the addition of drugs as described. Controls were treated with DMSO alone. For pulse-chase studies, cellular lipids were labeled for 24 h with [U^{14}C]acetate, 1 μCi/well. Labeled medium was then replaced with medium containing C75 at 10 μg/ml and 20 μg/ml for 2 h and 4 h, respectively; controls were treated with DMSO. Lipids from both cells and medium were Folch extracted and counted.

Lipid Characterization. Labeled lipids from cells and medium were characterized using single dimension TLC. Cells were cultured in 24-well plates at 1×10^5 per well, incubated overnight, and labeled with 1 μCi of [U^{14}C]acetate for 2 h, and then treated with C75 and/or TOFA for 4 h. After a saline wash, cells were harvested in 100 μl of ethanol. Culture medium was Folch extracted. After vortexing briefly, the samples were chromatographed on Silica Gel HL plates (Uniplate; Analtech) in chloroform:methanol:water (65:25:4). [^{14}C]phosphatidylcholine served as the control for phospholipids. Air-dried plates were scanned on a Molecular Dynamic PhosphorImager and quantitated with Image Quant software.

Fatty Acid Oxidation of Cellular Lipids. Cells were plated in T25 flasks fitted with plastic wells at 1×10^5 per flask in triplicate per time point. After overnight incubation at 37°C, the cellular lipids were labeled with 1 μCi of [U^{14}C]acetate for 24 h. Labeled medium was then replaced with medium containing C75 at 20 μg/ml, TOFA at 5 μg/ml, or DMSO alone, for 2 h and 4 h, respectively. After drug incubation, the reaction was stopped by adding 500 μl of 7% perchloric acid. Immediately, 400 μl of benzethonium hydrochloride was added to the center well to collect released [^{14}C]CO_2. After incubation at 37°C for 2 h, the benzethonium hydrochloride was removed and counted. For blanks, cells were killed immediately after rinsing with medium, and [^{14}C]CO_2 collection was performed.

BrdUrd Labeling. MCF-7 cells were plated on 4-chamber Permanox culture slides (Nalge Nunc International, Naperville, IL) at 1×10^5 per ml, 0.5 ml/chamber. After overnight incubation at 37°C, cells were labeled simultaneously with BrdUrd (1:1000; Roche Diagnostics, Indianapolis, IN) and treated with C75 and TOFA at concentrations indicated for various amounts of time (15′, 30′, 1 h, 2 h and 4 h). Cells were then fixed with Fixdenate (Roche Diagnostics) following the manufacturer's instructions. Cells were then washed in PBS and incubated in mouse α-BrdUrd (1:4000; Caltag Lab, Burlingame, CA) in 0.1% BSA/PBS for 30′ at 37°C. This was followed by washing in PBS and incubation with α-mouse immunoglobulin (Fab fragment) –AP (Roche Diagnostics) 30 min at 37°C. After washing, visualization was performed with nitroblue tetrazolium/X-phosphate in detection buffer (Boehringer Mannheim, Indianapolis, IN) for 20 min in the dark at room temperature. For total cell staining slides were counterstained using Nuclear Fast Red for 2–5 min (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instructions. For cell counts three rows of four ×40 fields spaced evenly over the area of the well were counted for each well. Values are reported as the mean ± SE number of BrdUrd-labeled nuclei/total nuclei per ×40 field. Cell counts were performed on 2-wells per treatment in duplicate experiments. Statistical significance was determined by 2-way ANOVA analysis of all of the treatments followed by Bonferroni post tests to compare individual treatments using Graph Pad Prism, version 4.0 (Graph Pad Software).

3′-TUNEL Analysis. MCF-7 cells were plated and treated with C75 as above. After fixation cells were washed thrice in water and once in 0.1 M Tris-HCl (pH 8.0). Positive control slides were then treated with RNase-free DNase (10 units/ml; Roche Diagnostics) in reaction buffer [30 mM Tris-HCl (pH 7.2), 140 mM Na Cacodylate, 4 mM $MgCl_2$, and 0.1 mM DTT] for 10 min at room temperature followed by washing in Tris-HCl (pH 8.0). All of the slides were then incubated in TdT buffer [30 mM Tris-HCl (pH 7.2), 140 mM Na Cacodylate, and 1 mM $CoCl_2$] for 15 min at room temperature. TdT enzyme was then added (200 units/ml; Sigma, St. Louis, MO) along with Biotin-16-dUTP (1:100; Roche Diagnostics), and slides were incubated for 1 h at 37°C. Negative control slides included all of the components except enzyme. After labeling, slides were washed for 15 min and 5 min in 2× SSC and 15′ in PBS. Staining was blocked with 3% normal horse serum (NHS)/PBS for 30′ at room temperature, and slides were incubated in Vectastain Elite avidin-biotin complex reagents (1:50; Vector Laboratories, Inc.) for 30 min at room temperature. Detection of signal was accomplished using development with 3,3′-diaminobenzidine/$NiCl_2$ reagent (Sigma). Slides were counterstained with Nuclear Fast Red as above. Cell counts and statistical analysis were performed as described above.

BrdUrd/TUNEL Double Labeling. Cells were plated and incubated for 24 h as described above. After incubation cells were treated with C75, TOFA, etomoxir, or combined TOFA for 2 h or etomoxir for 1 h followed by C75 for various time periods. BrdUrd was added to cultures 30 min before washing and fixation for each time point. Double labeling was performed as described above for each individual procedure except that TUNEL staining was amplified using a tyramide-biotin signal amplification kit (NEN Life Sciences, Boston, MA), and signal detection was developed with 3,3′-diaminobenzidine alone. BrdUrd labeling was performed one day before TUNEL analysis, because TUNEL analysis can destroy the antigenicity of some antigens. Cell counts and statistics were performed as described above except that results are reported as the mean ± SE of the percentage BrdUrd-positive cells that were also TUNEL positive.

RESULTS

C75 Inhibits Fatty Acid Synthesis in Human Cancer Cells. C75 inhibited purified human FAS (34) and has been reported to inhibit fatty acid synthesis *in vitro* by its inhibition of [U^{14}C]acetate incorporation into total lipids in human breast and colon cancer cells (23, 24). However, in light of the discovery that C75 stimulated CPT-1 and increased fatty acid oxidation, the interpretation of inhibition of [U^{14}C]acetate incorporation into total lipids became problematic because of the potential oxidation of newly synthesized labeled fatty acids. To determine whether C75 indeed inhibited endogenous fatty acid synthesis, we first blocked fatty acid oxidation in cancer cells with etomoxir, an irreversible inactivator of CPT-1 (35). By inhibiting CPT-1, etomoxir blocked mitochondrial β-oxidation of long-chain acyl-CoAs. Etomoxir does not affect mitochondrial short or medium chain fatty acid oxidation, or extramitochondrial α or ω fatty acid oxidation because they are not regulated by CTP-1. One h after applying etomoxir, we treated cells with C75 and measured [U^{14}C]acetate incorporation into total lipids. If C75 additionally reduced [U^{14}C]acetate incorporation into total lipids during fatty acid oxidation blockade, this would denote inhibition of fatty acid synthesis.

Fig. 2 compared [U^{14}C]acetate incorporation into total lipids in cells treated with C75 and etomoxir. C75 treatment at 10 μg/ml significantly inhibited [U^{14}C]acetate incorporation ($P = 0.012$). When C75 [5 μg] was added 1 h after etomoxir, there was an additional ~50% inhibition of fatty acid synthesis compared with etomoxir alone at any concentration level ($P = 0.003$, $P = 0.001$, and P = n.s., respectively) and ~75% additional inhibition at C75 (10 μg/ml; $P = 0.0008$, $P = 0.004$, and $P = 0.03$, respectively). Thus, in

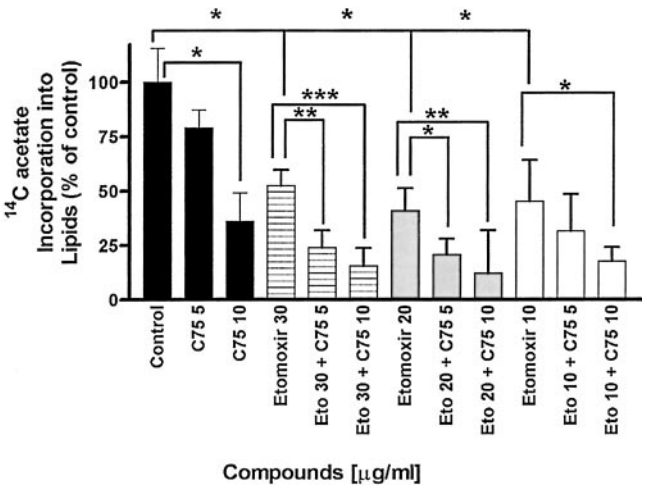


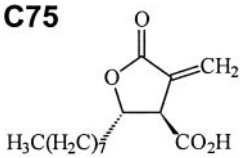
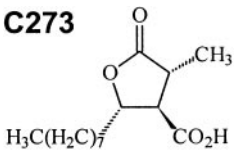
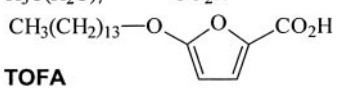
Fig. 2. C75 inhibited fatty acid synthesis during inhibition of fatty acid oxidation by etomoxir. C75 caused a significant, concentration-dependent inhibition of labeled acetate incorporation into lipids compared with controls, and also during irreversible inhibition of CPT-1 activity by etomoxir, demonstrating that C75 indeed inhibited fatty acid synthesis. Cells were treated with C75 or etomoxir alone for 2 h at concentrations indicated. In combination, etomoxir was added for 1 h before the 2-h incubation with C75. Compared with control, etomoxir alone also inhibited labeled acetate incorporation into total lipids in a nonconcentration-dependent fashion. *Error bars*, ±SE; all $n = 3$ (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

the presence of CPT-1 blockade, C75 inhibited [U^{14}C]acetate incorporation into total lipids demonstrating that C75 indeed inhibited fatty acid synthesis in human cancer cells in a concentration-dependent fashion. Etomoxir alone also inhibited fatty acid synthesis likely by increasing cellular acyl-CoA levels and reducing ACC activity (36).

C273 (Chemically Reduced C75) Did Not Inhibit Purified Human FAS or Fatty Acid Synthesis and Is Not Cytotoxic to MCF-7 Cells. C273 is structurally identical to C75 except for the single reduction of the exocyclic double bond group (Table 1). This site of unsaturation in C75 was critical for its inactivation of the β-ketoacyl synthase moiety of FAS. This change in oxidation state resulted in a compound that was unable to inhibit purified human FAS or fatty acid synthesis as measured by [^{14}C]acetate incorporation into total lipids. This compound was also nontoxic at concentrations up to and including 80 μg/ml that were negative in the XTT assay against MCF-7 cells. C273 confirmed the specificity of C75 for the FAS target and demonstrated that inhibition of FAS was required for the cytotoxicity of C75.

C75 and TOFA Inhibit Phospholipid Synthesis. Endogenous fatty acids produced in human cancer cells are incorporated predominantly into phospholipids, not into storage triglycerides (33). Cerulenin, a natural product inhibitor of FAS, inhibited phosphatidylcholine synthesis by reducing endogenous fatty acid synthesis, thereby limiting the diacylglycerol pool (37). We hypothesized that any inhibitor of endogenous fatty acid synthesis should reduce phosphatidylcholine levels by a similar mechanism. Using TLC to separate lipid classes, Fig. 3 demonstrated that C75 (10 and 15 μg/ml) inhibited [U^{14}C]acetate incorporation into phospholipids in a concentration-dependent fashion ($\sim$50% and 75%, $P < 0.0001$, respectively) after 2 h. TOFA, the ACC inhibitor, at [5 μg/ml], was more potent, reducing labeled acetate incorporation by 93% ($P < 0.0001$). Interestingly, TOFA pretreatment 1 h before the addition of C75 potentiated phospholipid synthesis inhibition compared with TOFA or C75 alone, causing 98% inhibition compared with C75 (10 μg/ml) alone ($P < 0.001$). Importantly, cell growth as measured by crystal violet staining showed no difference between C75 or TOFA treatment and controls at 2 h indicating that the reduction in phospholipid synthesis was not because of a reduction in cell number by C75 or TOFA (data not shown). Thus, inhibition of fatty acid synthesis with C75 or TOFA led to inhibition of phospholipid synthesis; combined inhibition of both FAS and ACC had an additive effect.

Table 1 *Structures of C75, C273, and TOFA*

Structure	Molecular weight	Fatty acid synthesis inhibition (IC_{50})	Cytotoxicity (LD_{50})
C75 	254	10.8 ± 2.7 μg/ml	10.7 ± 2.2 μg/ml
C273 	255	Negative	>80 μg/ml
TOFA 	324	4.0 ± 2.0	>20 μg/ml

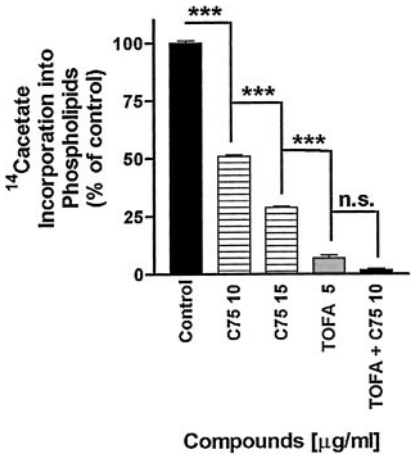


Fig. 3. C75 and TOFA significantly inhibit labeled acetate incorporation into phospholipids as determined by TLC. C75 and TOFA, a reversible inhibitor of ACC, both significantly inhibited [U^{14}C]acetate incorporation into phospholipids. TOFA and C75 together increased inhibition beyond TOFA or C75 treatment alone. Cells were treated with C75 or TOFA alone for 2 h. In combination, TOFA was added for 1 h before the 2-h incubation with C75. *Error bars*, ±SE; all $n = 3$ (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

C75 and TOFA Enhance the Loss of Endogenously Synthesized Membrane Lipids. Because both C75 and TOFA profoundly inhibit phospholipid production from endogenously synthesized fatty acids, this could affect the fate of *de novo* synthesized fatty acids in cellular membranes. After pulse labeling of endogenously synthesized membrane lipids with [^{14}C]acetate, we studied the decay of ^{14}C-labeled cellular lipids in the presence and absence of C75 and TOFA. Control cells lost 13.5% of labeled lipids within 2 h ($P = 0.012$ compared with time 0; Fig. 4*A*). Compared with control cells, C75 at 20 μg/ml caused a 32% loss of labeled lipids within 2 h, 18.5% more than control cells at 2 h ($P = 0.005$ compared with control at 2 h) and 16.5% more than control cells by 4 h ($P = 0.001$ compared with control at 4 h). TOFA treatment caused an even greater reduction of labeled lipids than C75; a 50% at 2 h, 34.5% greater loss compared with control cells at 2 h ($P < 0.0001$ compared with control at 2 h).

Because C75 is known to increase fatty acid oxidation of exogenously supplied palmitate (26), we hypothesized that increased fatty acid oxidation would be indiscriminate and, thus, also cause increased oxidation of membrane lipids. After pulse labeling of endogenously synthesized membrane lipids with [U^{14}C]acetate, C75 treatment at 20 μg/ml increased the oxidation of membrane lipids 2-fold over control levels after 2 h ($P = 0.005$; Fig. 4*B*). After 4 h, oxidation was still increased by $\sim$50% over control ($P = 0.012$). Changes in oxidation in control cells or those treated with TOFA over the course of the experiment were not statistically significant.

Etomoxir Does not Reverse C75 Reduction in Clonogenicity. To determine whether increased fatty acid oxidation of membrane lipids by C75 was responsible for its cytotoxicity, we blocked fatty acid oxidation with etomoxir, then treated cells with C75 and measured clonogenicity (Fig. 5). If fatty acid oxidation is a significant component of C75 cytotoxicity, prior treatment with etomoxir should rescue the cells. Compared with controls, C75 treatment for 6 h exhibited a concentration-dependent reduction in clonogenicity by 25% at 5 μg/ml to 55% at 10 μg/ml ($P = 0.007$ and $P < 0.0001$, respectively). Etomoxir alone at 5 and 10 μg/ml showed no significant cytotoxicity. Treatment of cells with etomoxir at either 5 or 10 μg/ml followed within 1 h by C75 at 5 or 10 μg/ml did not significantly alter clonogenicity compared with either concentration of C75 alone. Moreover, reversing the order of treatment or increasing the concentration of etomoxir to 100 μg/ml had no significant effect (data not

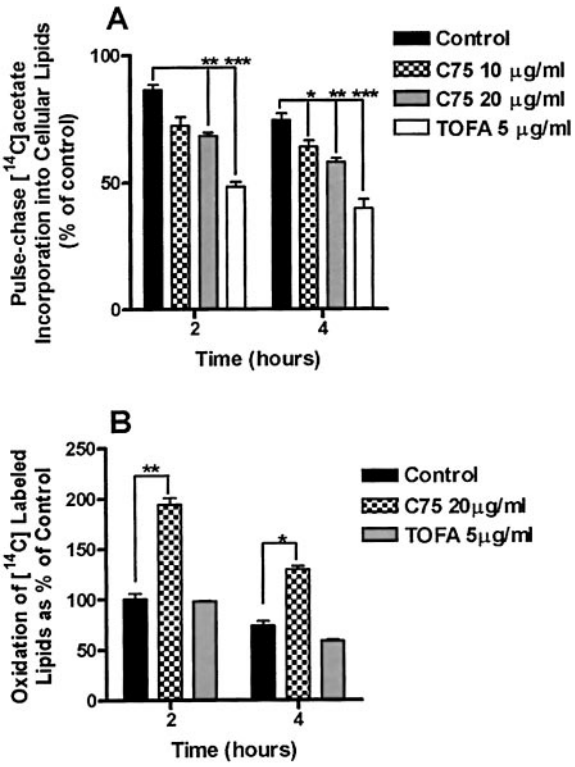


Fig. 4. C75 and TOFA both enhanced the loss of endogenously synthesized membrane lipids, whereas C75 increased membrane lipid oxidation. In *A*, control cells lost ~25.5% of endogenously synthesized membrane lipids over 4 h. C75 treatment at 10 μg/ml increased the loss of labeled membrane lipid compared with control value to 31.8% at 4 h ($P = 0.03$), whereas C75 at 20 μg/ml induced significant lipid loss compared with control values at all time points up to 42% at 4 h ($P = 0.0014$). TOFA treatment had a similar significant effect on loss of membrane lipid 2-h and 4-h time points. In *B*, C75 significantly increased the oxidation of membrane lipids as measured by released [$^{14}CO_2$] at both 2 h and 4 h ($P = 0.005$ and $P = 0.12$, respectively). TOFA did not increase fatty acid oxidation compared with control. *Error bars*, ±SE; all $n = 3$ (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

shown). In addition to concentration, the length of C75 exposure to the cells was significant. MCF7 cells exposed to C75 at 10 μg/ml for 30 min had only an 11% reduction in clonogenicity compared with 55% at 6 h. Whereas C75 stimulates fatty acid oxidation in cancer cells, increased fatty acid oxidation is not a significant component of its mechanism of cytotoxicity. Neither inhibition nor stimulation of fatty acid oxidation was toxic to MCF7 cells.

C75 Induces Apoptosis during S Phase. Because both C75 and TOFA: (*a*) inhibit *de novo* fatty acid synthesis; (*b*) inhibit phospholipid synthesis; and (*c*) accelerate the loss of labeled lipid from the membranes, this could lead to a net reduction in the availability of membrane lipid for cell division, which may not be reflected in the clonogenic assays. First, the effect of C75 on cumulative BrdUrd incorporation at various time points was examined over a 4-h time course (Fig. 6*A*). Within 1 h after treatment with C75, there was a statistically significant reduction in BrdUrd incorporation persisting over the remainder of the time course suggestive of an S phase arrest. We next examined the effect of C75 on apoptosis over the same time course via TUNEL labeling (Fig. 6*B*). After a 1-h exposure to C75, there was a slight increase in the number of apoptotic cells, which became statistically significant from control values within 2 h of exposure and remained so through the remainder of the time course (2 h, 13.2% increase over control values; 4 h, 9.6% increase over control values).

To directly investigate the relationship of apoptosis and S phase in C75-treated cells, we next performed BrdUrd/TUNEL double-labeling over the same 4-h time course. To reduce the number of background cells and limit our detection of BrdUrd to cells currently undergoing DNA synthesis, we labeled cells with BrdUrd for only 30 min before fixation. C75 caused a significant increase in the number of BrdUrd-labeled cells undergoing apoptosis at all of the time points, with the difference becoming greatest at the 2-h and 4-h time points with increases of 11% and 11.8% over control values, respectively (Fig. 6*C*). Both the timing and the magnitude of change suggest that increased numbers of cells undergoing apoptosis during S phase account for most of the increase in total numbers of apoptotic cells after exposure to C75. Application of TOFA caused no such increase in apoptosis during S phase, and application of TOFA 2 h before treatment with C75 was able to completely block the C75-induced increase in TUNEL and BrdUrd-labeled cells (Fig. 6*D*). Importantly, TOFA did not cause a significant change in BrdUrd or TUNEL labeling independent of C75 treatment (data not shown). These data support the notion that C75-treated cells undergo apoptosis as they move into S phas, and TOFA rescues the cells from this effect.

Etomoxir Does not Block C75 Induction of Apoptosis. Although etomoxir did not rescue cells from C75 in the clonogenic assay, we chose to determine whether etomoxir blocks early events of apoptosis in the BrdUrd/TUNEL double-labeling assay, which may not be detected by the clonogenic assay. In Fig. 7*A*, cells were treated with C75 and etomoxir alone. C75 treatment exhibited a concentration-dependent increase in apoptosis with a 35% increase in BrdUrd/TUNEL labeling with 20 μg/ml within 2 h ($P < 0.0001$). By 4 h, both concentrations of C75 caused a significant increase in apoptosis, whereas etomoxir alone at 10 and 20 μg/ml was similar to controls throughout the study. In Fig. 7*B*, cells were treated with both etomoxir and C75 before BrdUrd/TUNEL double-labeling to determine whether etomoxir would protect the cells from C75 induced apoptosis by blocking increased fatty acid oxidation. At all of the drug concentrations, etomoxir failed to rescue cells treated with C75; all of the drug combinations at 2 h and 4 h showed a significant increase in apoptosis over controls. Similar to the clonogenic assays, etomoxir did not rescue cells from C75, additionally supporting the hypothesis

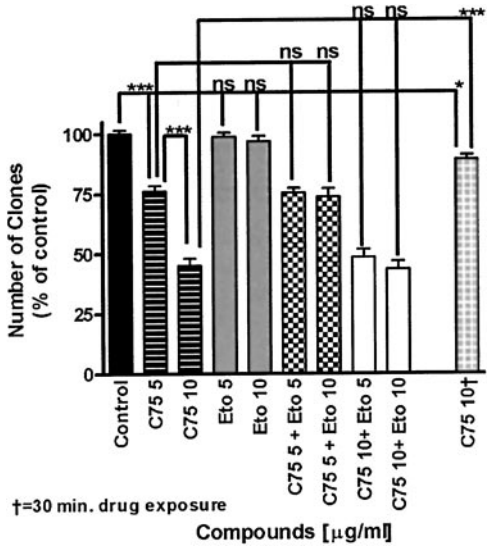


Fig. 5. Etomoxir did not rescue C75 cytotoxicity as measured by clonogenic assay. C75 caused a significant and concentration-dependent reduction in clonogenicity after 6 h and 30 min of treatment. Etomoxir, an irreversible inhibitor of CPT-1, alone did not reduce clonogenicity indicating that CPT-1 inhibition is not cytotoxic. Etomoxir in combination with C75 did not significantly reduce the clonogenicity of MCF7 cells compared with C75 treatment alone. These data indicate that CPT-1 stimulation was not responsible for C75 cytotoxicity. Cells were exposed to compounds for 6 h; † indicates 30-min drug exposure to cells. *Error bars*, ± SE; all $n = 3$ (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

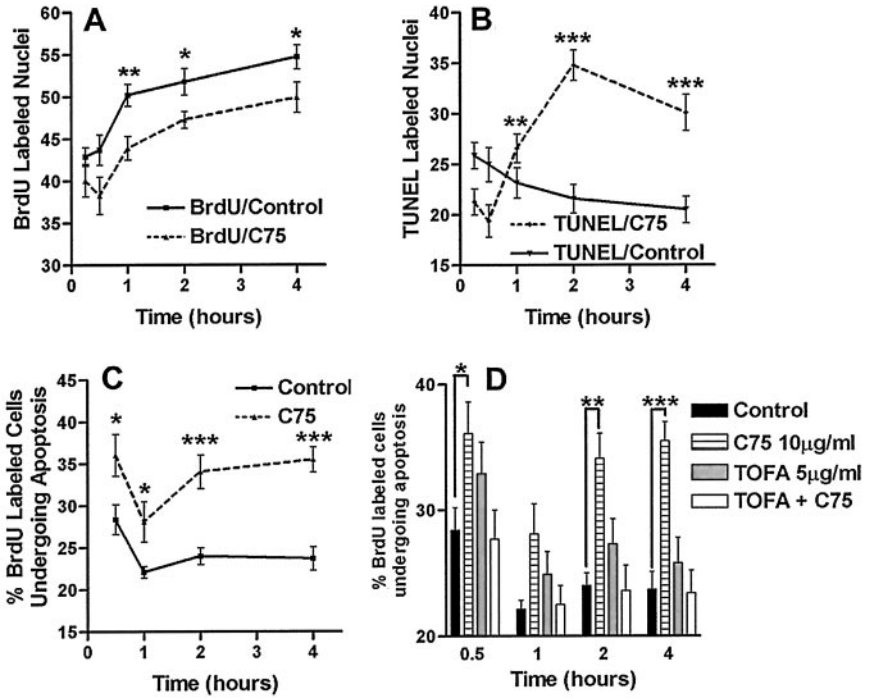


Fig. 6. C75 induced apoptosis during S phase that was rescued by TOFA treatment. In *A*, C75 (----) caused a reduction in BrdUrd-labeled nuclei within 30 min that reached statistical significance by 1 h compared with control (———) consistent with an S phase arrest. In *B*, apoptosis was measured over the same time course with TUNEL labeling. C75 (----) caused increased apoptosis within 1 h compared with control (———). In *C*, the relationship of apoptosis and S phase was examined with BrdUrd and TUNEL double-labeling. C75 (----) caused a significant increase in the number of BrdUrd-labeled cells undergoing apoptosis at all time points, with the difference becoming greatest at the 2- and 4-h time points over control values (———). In *D*, TOFA did not induce increased apoptosis during S phase, and application of TOFA before C75 prevented the occurrence of apoptosis during S phase. *Error bars*, ±SE; all $n = 3$ (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

that increased CPT-1 activity is not a significant mechanism of C75 cytotoxicity.

DISCUSSION

C75, the first synthetic inhibitor of purified human FAS, was developed to enable FAS inhibition to be tested for *in vivo* treatment of human cancer xenografts (34). Its mechanism of action was initially investigated in cultured human cancer cells where C75 was found to: (*a*) induce apoptosis (22, 23); (*b*) inhibit fatty acid synthesis as measured by [^{14}C]acetate incorporation into lipids (22); and (*c*) markedly elevate malonyl-CoA levels (24).

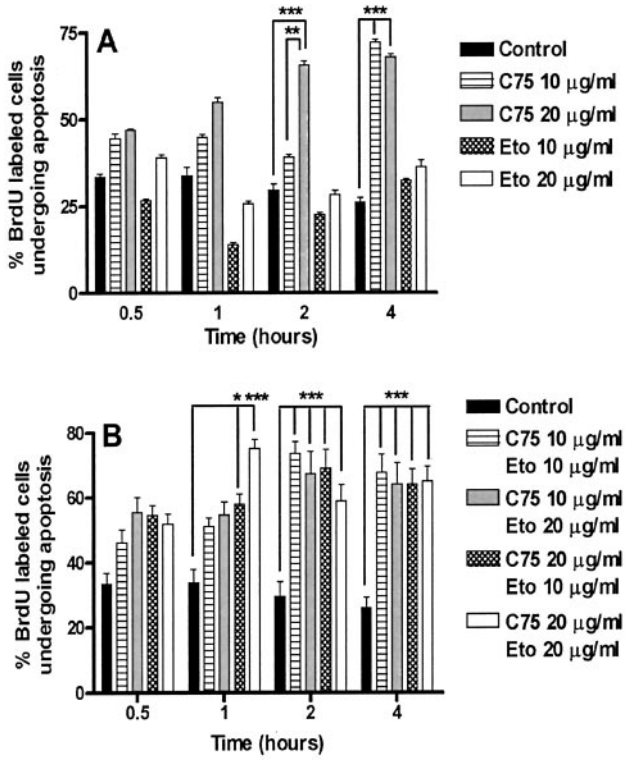


Fig. 7. Etomoxir failed to rescue cells from C75 induced apoptosis. In *A*, C75 treatment showed a concentration-dependent increase in TUNEL-positive cells in S phase, similar to Fig. 6. Etomoxir treatment alone showed no increase in apoptosis. In *B*, combinations of C75 and etomoxir at both concentrations showed no reduction in BrdUrd/TUNEL positivity. Etomoxir was unable to rescue cells from C75 induced apoptosis. *Error bars*, ±SE; all $n = 3$ (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Fatty acid synthesis inhibition can be achieved through impedance of a variety of enzymatic steps in the pathway including, for example, ACC, citrate lyase, FAS, and long-chain acyl-CoA synthase. We initially sought to determine whether C75 induced cytotoxicity was characteristic of fatty acid synthesis inhibition in general or specific to FAS inhibition. TOFA is a well-characterized competitive inhibitor of ACC, the rate-limiting enzyme of fatty acid synthesis. Because TOFA is a potent inhibitor of fatty acid synthesis in human cancer cells, we compared its activity with C75 in human cancer cells *in vitro*. Surprisingly, TOFA was noncytotoxic to cancer cells *in vitro*. Moreover, preincubation of cells with TOFA protected them against C75 cytotoxicity (24).

A key biochemical difference that distinguishes inhibition of fatty acid synthesis at the ACC step from the FAS step is changes in cellular malonyl-CoA levels. TOFA inhibits ACC, reducing malonyl-CoA, whereas C75 inhibits FAS, increasing malonyl-CoA levels (24). In addition to its role as a substrate for FAS, malonyl-CoA acts as a biochemical switch, regulating fuel consumption during energy excess and starvation. During lipogenesis, high levels of malonyl-CoA inhibit CPT-1, the rate-limiting enzyme of fatty acid oxidation, preventing the oxidation of newly synthesized fatty acids. On starvation, lipogenesis is rapidly down-regulated, malonyl-CoA levels fall, and CPT-1 inhibition is released, allowing the oxidation of storage fatty acids for fuel. Because C75 blocked FAS, increased malonyl-CoA levels, and triggered apoptosis, whereas TOFA blocked the C75 increase in malonyl-CoA protecting the cells from apoptosis, we hypothesized that high levels of malonyl-CoA appeared to be pro-apoptotic, most probably acting through CPT-1 inhibition (24). Contemporaneously, another group investigating CPT-1 and apoptosis found CPT-1 and BCL-2 to coimmunoprecipitate in cells overexpressing BCL-2, implying a physical association between CPT-1 and BCL-2, a modulator of apoptosis at the mitochondrial membrane (31).

This additionally supported a role for malonyl-CoA and CPT-1 in the generation of C75 induced apoptosis.

In vivo studies of C75 led to a second enzyme target of C75 action. Whereas C75 treatment caused significant antitumor activity against human xenografts (20, 24, 25), dosing was limited by dramatic, reversible weight loss (24). During pursuit of the mechanism responsible for the weight loss in mice, we discovered that C75 stimulated CPT-1 activity in a variety of cell lines including MCF-7 cells, hepatocytes, and skeletal muscle cells (26).[5] As a consequence of CPT-1 stimulation, fatty acid oxidation increased, along with cellular ATP levels, and fatty acid oxidation both *in vitro* and *in vivo* (26). Moreover, CPT-1 stimulation occurred even in the setting of inhibitory concentrations of malonyl-CoA (26). Thus, although C75 increased cellular malonyl-CoA levels, direct CPT-1 stimulation by C75 abrogated malonyl-CoA inhibition of CPT-1 as the primary mechanism of cytotoxicity.

C75 stimulation of CPT-1 required us to revisit C75 as a fatty acid synthesis inhibitor in the initial series of experiments. Inhibition of [^{14}C]acetate into lipids is a reliable measure of fatty acid synthesis inhibition provided the cells are not also concomitantly altering fatty acid oxidation. Increased fatty acid oxidation by C75 could remove labeled fatty acids from the cell as [$^{14}CO_2$], potentially mimicking fatty acid synthesis inhibition. To determine whether C75 truly inhibits fatty acid synthesis, we first specifically blocked mitochondrial long-chain fatty acid oxidation in cells with etomoxir, an irreversible CPT-1 inhibitor, then treated the cells with C75. Because C75 inhibited [^{14}C]acetate incorporation into lipids during CTP-1 blockade, it indeed inhibited fatty acid synthesis *in vitro*. Surprisingly, cells treated with etomoxir at concentrations known to substantially inhibit CPT-1 activity (30, 20, and 10 μg/ml) reduced [U^{14}C]acetate incorporation into total lipids by ~50% over controls ($P = 0.04$, 0.02, and 0.04, respectively). Etomoxir is not a direct inhibitor of enzymes involved in fatty acid synthesis, but it is known to increase cellular long-chain acyl-CoA levels by inhibition of fatty acid oxidation (36). Long-chain acyl-CoAs are potent inhibitors of ACC, the rate-limiting enzyme of fatty acid synthesis (38). Thus, etomoxir likely indirectly inhibits fatty acid synthesis through elevation of long-chain acyl-CoA levels and subsequent ACC inhibition. Because the concentrations of etomoxir we used substantially inhibited CPT-1, a nonconcentration-dependent inhibition of fatty acid synthesis was observed.

Because most endogenously synthesized fatty acids in cultured human cancer cells are incorporated into phospholipids, we turned our attention to phospholipid synthesis inhibition as a cause for C75 cytotoxicity. Inhibition of phospholipid synthesis by a variety of mechanisms has been shown to be cytotoxic to cycling cells (37). Blocking endogenous fatty acid synthesis with cerulenin, a FAS inhibitor, inhibited phospholipid synthesis indirectly by reducing diacylglycerides, the direct precursors for phospholipid synthesis (37). Through their inhibition of endogenous fatty acid synthesis, both C75 and TOFA also significantly inhibited phospholipid synthesis. Moreover, as a consequence of reduced phospholipid production, both C75 and TOFA accelerated the loss of labeled cellular lipids. These findings lead to the conclusion that fatty acid synthesis inhibition alone is not sufficient to account for the cytotoxicity of C75, because TOFA treatment led to nearly identical metabolic changes.

Stimulation of fatty acid oxidation occurs with C75 treatment but not with TOFA. C75 treatment caused a 2-fold increase in the oxidation of endogenously labeled cellular lipids. To determine whether increased fatty acid oxidation was responsible for cytotoxicity, we treated cells with C75 in the presence and absence of etomoxir to block CPT-1. Surprisingly, CPT-1 blockade did not rescue cells from C75 cytotoxicity, thus signifying that increased fatty acid oxidation alone is not a significant component of the mechanism of action of C75.

To additionally study the relationship of FAS inhibition to C75 cytotoxicity, we treated cells with C273, a reduced form of C75, which is unable to inhibit FAS or fatty acid synthesis. In concentrations up to 80 μg/ml, C273 was nontoxic to MCF-7 cells in contrast to C75 that has an IC_{50} of ~12 μg/ml. Because the reduction of the exocyclic double bond of C75 makes it unable to inactivate the β-ketoacyl moiety of FAS, C273 demonstrates that the cytotoxic effect of C75 requires inhibition of FAS. RNA interference-mediated down-regulation of FAS induced apoptosis recently in LNCaP human prostate cancer cells but not in nonmalignant skin fibroblasts (39). These studies together additionally confirm the pharmacological specificity of C75 for its FAS target and the requirement of FAS expression to maintain cancer cell growth.

Although inhibition of FAS with its resultant inhibition of phospholipid synthesis was required for C75 cytotoxicity, TOFA inhibition of ACC also inhibited fatty acid and phospholipid synthesis but was nontoxic in clonogenic assays (24). Phospholipid synthesis and its relationship to the cell cycle and apoptosis have been extensively studied. Most of the phospholipid accumulation in preparation for mitosis takes place during S phase (40). Thus, cells in S phase should be most sensitive to changes in phospholipid metabolism. In studies with HL60 human promyelocytic leukemia cells, C75 (10 μg/ml) inhibited fatty acid synthesis within 15–30 min followed by a reduction in [^{3}H]thymidine incorporation indicative of S phase arrest within 60–90 min (22). In these experiments, however, apoptosis as detected by flow cytometric detection of TUNEL labeling did not occur until 6 h after C75 treatment. In follow-up studies, flow cytometric studies of RKO human colon cancer cells detected an S phase block ~5 h after C75 treatment (23). Given the rapidity of fatty acid synthesis inhibition and subsequent reduction of thymidine incorporation observed in HL60 cells, we hypothesized that C75 or TOFA could cause apoptosis as cells traversed S phase within minutes of C75 treatment.

To study early time points and maximize sensitivity, we chose to double-label MCF-7 cells for BrdUrd incorporation and TUNEL labeling, and visually count cells. Similar to the studies reported previously, C75 inhibited BrdUrd incorporation within 30 min, reaching statistical significance at 60 min indicative of an S phase block. Within 30 min of C75 treatment (10 μg/ml), there was a statistically significant increase in cells showing both BrdUrd incorporation and TUNEL labeling indicative of apoptosis of cells in S phase. This remained throughout the 4-h duration of the study. All of the increase in apoptosis caused by C75 could be accounted for by these double-labeled cells indicating that C75 preferentially killed cells during S phase. Moreover, TOFA did not cause a decrease in BrdUrd labeling and did not cause an S phase block as detected by flow-cytometry (23). TOFA pretreatment of the cells prevented the decrease in BrdUrd labeling and rescued C75 induced cytotoxicity similar to the clonogenic assays reported previously. Inhibition of FAS by C75 caused a concomitant inhibition of phospholipid synthesis and a reduction in BrdUrd labeling or S phase block, which triggered apoptosis. Inhibition of ACC with TOFA also inhibited phospholipid synthesis, but failed to cause the S phase block and initiate apoptosis. Taken together, these studies again point to high levels of malonyl-CoA as proapoptotic.

We also treated cells with etomoxir and C75 alone and together during the rapid BrdUrd/TUNEL assay to determine whether blocking the C75 stimulation of fatty acid oxidation would rescue cells from apoptosis. Etomoxir treatment alone did not induce apoptosis nor did it rescue cells when combined with C75. These findings support the

[5] J. N. Thupari, unpublished observations.

clonogenic data indicating that neither inhibition nor stimulation of fatty acid oxidation were cytotoxic to MCF7 cells.

The mechanism of cytotoxicity of FAS inhibition in cancer cells involves an interplay of phospholipid synthesis inhibition and cell cycle events. C75 cytotoxicity is dependent both on the inhibition of FAS and a relative S phase block that is likely mediated by cyclin-dependent kinases (23). In RKO cells, C75 caused a marked reduction in cyclin A and B1 kinase activities within 4 h with subsequent suppression of DNA replication responsible for the S phase and G_2 block (41). Because the cyclin kinase activities were not assayed before 4 h, it is possible that these also could be activated within minutes of C75 treatment. How C75 leads to changes in cyclin kinases activities, however, is yet unknown. Because inhibition of ACC with TOFA rescued cells from C75, high levels of malonyl-CoA likely play a role in C75 cytotoxicity, but not mediated through CPT-1. These data provide a framework for the additional study of FAS as a pharmacological target and malonyl-CoA as its downstream effector for cancer treatment.

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