

# Pharmacological Inhibition of Fatty Acid Synthase Activity Produces Both Cytostatic and Cytotoxic Effects Modulated by p53<sup>1</sup>

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## ABSTRACT

Fatty acid synthetic metabolism is abnormally elevated in tumor cells, and pharmacological inhibitors of the anabolic enzyme fatty acid synthase (FAS), including the natural product cerulenin and the novel synthetic compound c75, are selective inhibitors of tumor cell growth. We have recently reported that these two FAS inhibitors both produce rapid, potent inhibition of DNA replication and S-phase progression in human cancer cells, as well as apoptotic death. Here we report an additional characterization of the cellular response to FAS inhibition. RKO colon carcinoma cells were selected for study because they undergo little apoptosis within the first 24 h after FAS inhibition. Instead, RKO cells exhibited a biphasic stress response with a transient accumulation in S and G<sub>2</sub> at 4 and 8 h that corresponds to a marked reduction in cyclin A- and B1-associated kinase activities, and then by accumulation of p53 and p21 proteins at 16 and 24 h and growth arrest in G<sub>1</sub> and G<sub>2</sub>. The response of RKO cells to FAS inhibition resembled a genotoxic stress response, but DNA damage did not appear to be an important downstream effect of FAS inhibition, because none was detected using the single cell gel electrophoresis assay (comet assay) to assess DNA damage. p53 function is probably important in protecting RKO cells from FAS inhibition because, similar to many other tumor lines, RKO cells expressing a dominant negative mutant p53 gene underwent extensive apoptosis within 24 h after FAS inhibition. Sensitization of cells to FAS inhibitors by the loss of p53 raises the possibility that these agents may be clinically useful against malignancies carrying p53 mutations. Whereas induction of apoptosis appeared related to accumulation of the substrate, malonyl-CoA, after FAS inhibition, the cytostatic effects were independent of malonyl-CoA accumulation and may have resulted from product depletion.

## INTRODUCTION

FAS,<sup>3</sup> (E.C. 2.3.1.85) is the major biosynthetic enzyme for the synthesis of fatty acids from small carbon substrates. FAS is a multifunctional enzyme that performs seven sequential reactions to convert acetyl-CoA and malonyl-CoA to palmitate. Elevated expression of FAS and abnormally active endogenous fatty acid synthetic metabolism are frequent phenotypic alterations in many human cancers, including carcinomas of breast, prostate, endometrium, and colon (1–5). The function(s) that active fatty acid synthesis provides for tumor cells appears linked to proliferation, and the bulk of endogenously synthesized fatty acids are incorporated into membrane lipids by proliferating tumor cells (4, 6, 7). Endogenous fatty acid synthetic activity occurs in tumors despite available dietary fatty acid, which down-regulates the pathway in most normal tissues (8–10).

The biological basis for this phenotypic alteration is not clear. However, altered fatty acid metabolism represents a novel target for anti-metabolite therapy because pharmacological inhibition of FAS is

selectively cytotoxic for tumor cells, triggering their programmed cell death (11, 12). The cytotoxic mechanism of FAS inhibition appears to result from accumulation of the committed substrate, malonyl-CoA, or from related biochemical consequences of the inhibition of an active metabolic pathway, inasmuch as pathway down-regulation before FAS inhibition rescues tumor cell survival (13). To gain additional insight into the biological role of the fatty acid synthetic pathway for tumor cells and the nature of the growth inhibition resulting from the inhibition of FAS, we have examined the cellular events that follow inhibition of FAS and precede cell death.

Two chemically distinct inhibitors of FAS were studied in parallel to provide a generic picture of the consequences of the loss of FAS function. Cerulenin, (2R, 3S)-2,3-epoxy-4-oxo-7,10-trans, trans-dodecadienamide, a natural product of *Cephalosporium caerulens*, is a specific inhibitor of FAS enzymes across a broad phylogenetic spectrum (14–16). Cerulenin irreversibly inhibits 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. In *Saccharomyces cerevisiae*, a point mutation in FAS that confers a 30-fold reduction in affinity of the enzyme for cerulenin also abolishes the growth inhibitory effects of the drug accordingly, demonstrating that FAS is a critical target for cytotoxic effects of the drug (17). Recently we have synthesized a novel small-molecule inhibitor of FAS, an  $\alpha$ -methylene- $\gamma$ -butyrolactone with a C7 hydrocarbon side chain, called c75, with comparable inhibitory effects on fatty acid synthesis (18).

We have recently reported that FAS inhibitors produce rapid, profound blocks of DNA replication and S-phase progression in human cancer cells (19). Fatty acid synthesis inhibition occurred within 30 min and DNA synthesis inhibition occurred within 90 min of drug exposure, and induction of apoptosis followed several hours later. The suppressive effect of fatty acid synthesis inhibition on DNA replication was indirect, because expression of certain viral oncogenes alleviated it. Here, we report an additional characterization of the cellular response to FAS inhibition. RKO colon carcinoma cells were selected for study because they undergo little apoptosis within the first 24 h after FAS inhibition. Instead, RKO cells exhibit a biphasic stress response marked by early loss of S phase and G<sub>2</sub> cyclin-dependent kinase activity and the subsequent accumulation of p53 and p21 proteins, which may protect RKO cells from the cytotoxic effects of FAS inhibition.

## MATERIALS AND METHODS

**Cell Lines and Culture Conditions.** RKO colon carcinoma cells and RKO clones stably transfected with a dominant negative mutant p53 gene were provided by Dr. M. Kastan (St. Jude Children's Research Hospital, Memphis, TN) (20). The MCF7 clone stably transfected with the *human papillomavirus 16 E6* gene was provided by Dr. A. Fornace (National Cancer Institute, NIH, Bethesda, MD) (21). Other cells were obtained from the American Type Culture Collection. Most cells were cultured in DMEM with 10% fetal bovine serum (Hyclone). Cells were screened periodically for *Mycoplasma* contamination (Gen-probe). Cerulenin (Sigma), c75, and TOFA, dissolved in DMSO,

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<sup>3</sup> The abbreviations used are: FAS, fatty acid synthase; TOFA, 5-(tetradecyloxy)-2-furoic acid; BrdUrd, bromodeoxyuridine; cdk, cyclin-dependent kinase.

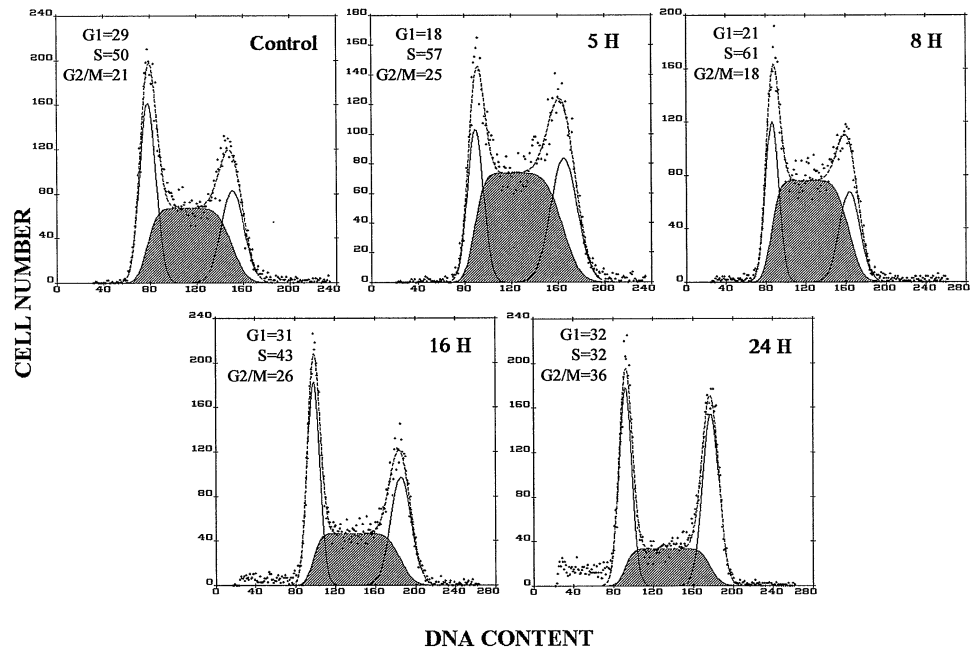


Fig. 1. Cell cycle progression after FAS inhibition is transiently blocked in S and G<sub>2</sub>-M and then in G<sub>1</sub> and G<sub>2</sub>-M. DNA content of RKO cells was analyzed by flow cytometry after the indicated time periods of exposure to cerulenin (10  $\mu$ g/ml).

were added from 5 mg/ml stock solutions; the final concentration of DMSO in cultures was  $\leq 0.2\%$ .

**Flow Cytometry and Detection of Apoptosis.** Cells were exposed to cerulenin or *c75* for the indicated doses and time intervals and then detached from plastic with trypsin for flow cytometry analysis. Apoptosis and DNA content were measured concurrently by multiparameter flow cytometry using a FACStar<sup>Plus</sup> flow cytometer equipped with argon and krypton lasers (Becton Dickinson). Apoptosis was quantified using 10  $\mu$ g/ml merocyanine 540 (Sigma), which detects altered plasma membrane phospholipid packing that occurs early in apoptosis (19, 22–24). Merocyanine 540-positive cells were identified using 488-nm excitation from an argon laser and a 575-nm DF26 bandpass filter for collection of events with increased red fluorescence. DNA content was simultaneously measured from cells stained with 10  $\mu$ M Hoechst 33342 (HO33342; Molecular Probes). Cells treated with HO33342 were excited with 350–356-nm light from a krypton laser, and emitted light was collected using a 467-nm DF22 bandpass filter. Data were collected and analyzed using CellQuest software (Becton Dickinson). Determination of the percentages of cells in G<sub>1</sub>, S and G<sub>2</sub>-M was done with Multicycle software (Phoenix Flow Systems). Figures show representative results of at least two independently performed experiments.

**BrdUrd Detection by Laser Scanning Cytometry.** Dual-parameter detection of BrdUrd labeling and DNA content was performed using a laser scanning cytometer (Compucyte Corp.). Cell cultures were pulse-labeled for 20 min with 10  $\mu$ M BrdUrd and chased for the indicated times in the absence or presence of drug, then detached from plastic with trypsin, ethanol-fixed, and applied to glass slides. Cells were subjected to standard heat-induced epitope retrieval (DAKO) before staining with anti-BrdUrd antibody (DAKO) and FITC-conjugated goat antimouse antibody (CALTAG, DAKO Autostainer). DNA content was assessed after staining with 0.5% propidium iodide. Data were collected and analyzed using WinCyte software (Compucyte Corp.).

**Immunoprecipitation and Immunocomplex-Kinase Assay.** RKO cells ( $5 \times 10^6/100$ -mm plate) were treated with 10  $\mu$ g/ml cerulenin or *c75* for the indicated time intervals. The control cells received equivalent amounts of DMSO. After drug treatment, the plates were washed once and lysed with immunoprecipitation buffer [150 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% NP40, 1 mM ethyleneglyco-bis-tetraacetic acid, 0.2 mM sodium vanadate, and 0.2 mM phenylmethylsulfonyl fluoride]. Protein concentration was measured using the BCA Protein Assay Kit (Pierce). One hundred  $\mu$ g of protein from each sample were incubated at 4°C for 1 h with 1  $\mu$ g of primary antibody (antihuman cyclin A rabbit polyclonal antibody or antihuman cyclin B1 monoclonal antibody; Santa Cruz) and then overnight after the addition of Protein A or protein G-Sepharose (Santa Cruz). The immunoprecipitates were washed twice with immunoprecipitation buffer and once with kinase buffer [10

mM Tris (pH 7.4), 150 mM NaCl, 10 mM MgCl<sub>2</sub>, and 0.5 mM DTT] and resuspended in 40  $\mu$ l of kinase buffer containing 1  $\mu$ g of histone H1, 25  $\mu$ M of ATP, and 2.5  $\mu$ Ci of  $\gamma$ -<sup>32</sup>P-ATP. After a 30-min incubation at 30°C, the reaction was terminated by adding 40  $\mu$ l of 2 $\times$  Laemmli sample buffer. Samples were resolved by electrophoresis through 12% SDS-polyacrylamide gels and quantitated on a Storm 820 system (Molecular Dynamics). All samples were run in duplicate, and each experiment was performed at least twice. Means and standard errors of one representative experiment are shown in Fig. 3B.

**Immunoblot Analysis.** One million cells/60-mm plate were treated with 10  $\mu$ g/ml cerulenin or *c75* in duplicate for the indicated time intervals; control cells received equivalent amounts of DMSO. After drug treatment, cells were lysed with 200  $\mu$ l Laemmli sample buffer and boiled. Ten  $\mu$ l of each lysate per lane was separated by SDS-PAGE, transferred to nitrocellulose, and exposed to antibodies against p53 (Pab1801; Oncogene Research Products), p21 (6B6; PharMingen), or actin (I-19; Santa Cruz), and then horseradish peroxidase-conjugated goat antimouse or rabbit antigoat antibody (Pierce), enhanced chemiluminescence (Amersham), and autoradiography.

**Determination of Fatty Acid Synthesis Pathway Activity.** Cells were plated at  $5 \times 10^4$ /well in 1 ml in 24-well plates and incubated overnight. Fatty acid synthesis was assayed with a 2-h pulse of [U-<sup>14</sup>C]acetic acid, 1  $\mu$  Ci/well, before Folch extraction and scintillation counting (6). For determination of residual pathway activity after FAS inhibitor exposure (Fig. 7B), a 3-h pulse of U-[<sup>14</sup>C]acetic acid, 1  $\mu$  Ci/well, was performed after 2 h of drug exposure. All determinations were in triplicate. Data are presented as mean values, with bars showing the SE. Calculations and graphing were performed in Prism 2.0 (GraphPad).

**Clonogenic Assays.** Subconfluent cells were exposed to the indicated drug concentrations for 6 h, then detached from plastic with trypsin, counted, and replated for colony formation. Clones were fixed, stained with crystal violet (0.1%; Sigma) and counted 1 week later. Data are presented as mean values, with bars showing the SE. Calculations and graphing were performed in Prism 2.0 (GraphPad).

**Single Cell Gel Electrophoresis (Comet) Assay.** MCF7 breast cancer cells were treated with cerulenin or *c75* for 3 h at doses bracketing 75% survival at 24 h. All experiments were repeated three times, and duplicate slides from each experiment were prepared and scored.

The comet assay was performed under alkaline conditions, essentially as described (25), with some modifications. In brief, cells were suspended in 0.5% low-melting point agarose (LMA; Trevigen) and spread on glass microscope slides precoated with 1% normal agarose. After immersion in lysis solution (Trevigen) at 4°C for a minimum period of 1 h to remove cellular proteins, the slides were immersed in electrophoresis buffer [300 mM NaOH,

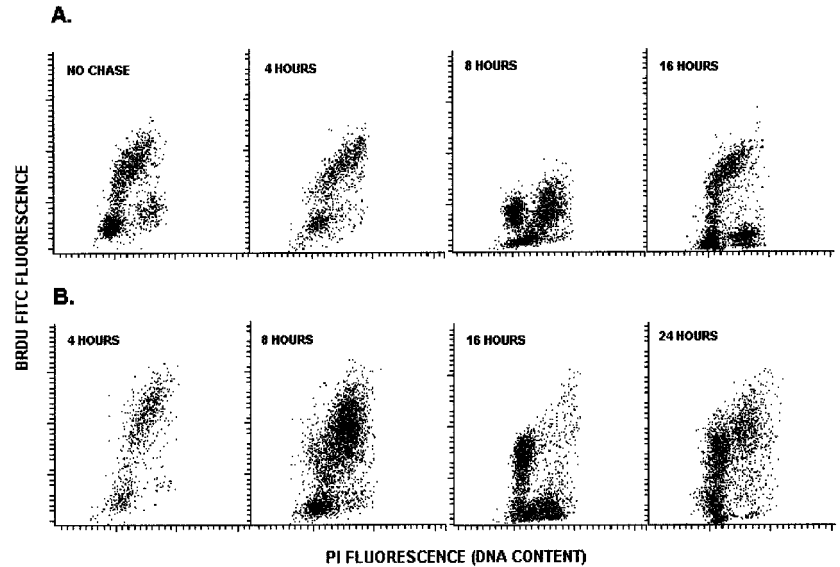


Fig. 2. BrdUrd pulse/chase analysis of cell cycle progression after FAS inhibition. *A*, two-dimensional analysis of DNA content versus BrdUrd content in pulse-labeled RKO cells chased for the indicated time periods in the absence of FAS inhibitors. The BrdUrd-positive S-phase fraction at time 0 progresses through the cell cycle at later time points. *B*, similar cell preparations were chased in the presence of cerulenin (10  $\mu\text{g/ml}$ ).

1 mM EDTA (pH > 13)] for unwinding DNA, and subjected to electrophoresis (25 V; 300 mA) for 20 min. Neutralized, dehydrated slides were stained with ethidium bromide (2 ng/ml) and comets scored under a Nikon fluorescence microscope (with TRITC filters) coupled to KOMET 4.0 software (Kinetic Imaging, Ltd). The comet parameters, "Olive Tail Moment," "Tail Length" (DNA migration), and "percentage DNA in the tail," were used as indicators of DNA damage. One hundred consecutive cells were scored from the middle of each slide, and the means calculated. The final results were expressed as the (mean of the individual means)  $\pm$  (SD of the means).

## RESULTS

**FAS Inhibitors Induce Delays in Cell Cycle Progression.** Recent studies of the role of endogenous fatty acid synthesis in tumor cell biology suggested linkage of fatty acid synthesis to proliferation (6, 7, 26), and the fatty acid synthase inhibitors cerulenin and *c75* to suppressed DNA synthesis and inhibited S phase progression (19). Additional investigation by flow cytometric analysis of serial samples taken after FAS inhibition demonstrated a biphasic effect on the cell cycle progression of RKO colon carcinoma cells (Fig. 1). When proliferating cells were exposed to 10  $\mu\text{g/ml}$  cerulenin, there was a redistribution of cells into S phase and G<sub>2</sub>-M during the early time points, at 5 and 8 h, compatible with inhibited progression through these cell cycle phases. Later, at 16 and 24 h, the S-phase fraction decreased substantially, with a redistribution of cells into G<sub>1</sub> and G<sub>2</sub>-M. This effect was characteristic of both cerulenin and *c75* treatment on RKO cells, as well as on other cell lines that had limited apoptotic responses to FAS inhibitors (not shown).

A similar experiment measured cell cycle progression by pulse/chase labeling with bromodeoxyuridine (BrdUrd, Fig. 2). The progress of BrdUrd pulse-labeled RKO cells through the cell cycle was monitored over 24 h without inhibition of FAS, or during exposure to cerulenin (10  $\mu\text{g/ml}$ ) or *c75* (10  $\mu\text{g/ml}$ ). A 20-min exposure of proliferating cells to BrdUrd labeled the S-phase population at time 0. Chase samples were collected at 4, 8, 16, and 24 h. In control cultures (Fig. 2A), the BrdUrd-labeled population progressed through G<sub>2</sub>-M, first reappearing in the G<sub>1</sub> population in the 8-h chase sample. By 16 h, the BrdUrd-labeled population was in G<sub>1</sub> and S phase again, indicating a complete cell cycle traverse time of approximately 16 h for RKO cells. By 24 h, labeled and unlabeled populations were distributed throughout the cell cycle, indicating continued progression and loss of synchronization (not shown). RKO cells treated with FAS

inhibitors demonstrated substantial delays in cell cycle progression that corresponded with the flow cytometry single-parameter cell cycle results (Fig. 1). The cerulenin-treated samples are shown in Fig. 2B; *c75*-treated populations exhibited a similar response (not shown). The treated 8-h chase sample showed no BrdUrd-labeled cells yet reappearing in G<sub>1</sub>, which was in agreement with our observation, seen in Fig. 1, that cells redistribute into the S and G<sub>2</sub>-M phases. By 16 h, most of the BrdUrd-labeled cells had reentered G<sub>1</sub>, but very few had entered S phase, and by 24 h most cells, labeled and unlabeled, were in G<sub>1</sub> or G<sub>2</sub>-M and were still synchronized, indicating that cell cycle progression had slowed down substantially.

**FAS Inhibitors Induce Inhibition of S Phase and G<sub>2</sub> cdk Activity during the Early Period of Exposure.** Cell cycle progression is regulated through the sequential activation and inactivation of

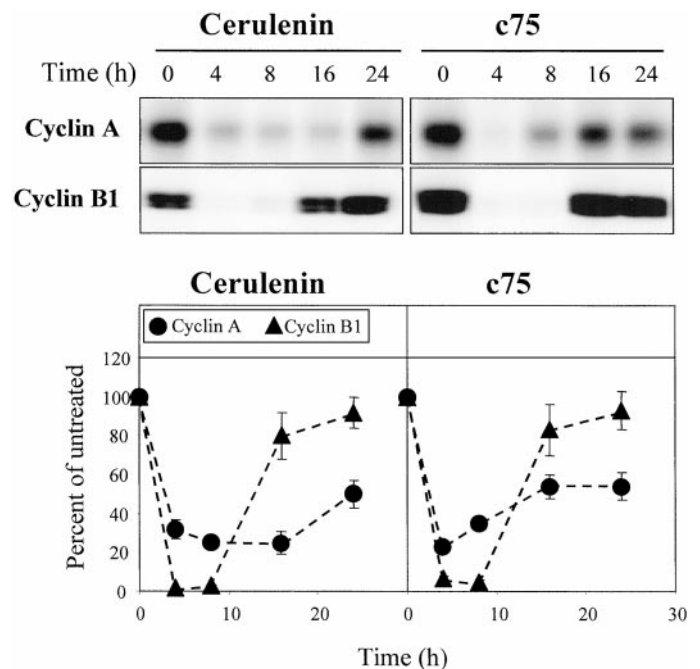


Fig. 3. FAS inhibition induces a marked reduction of S- and G<sub>2</sub>-M-associated cdk activity during the early period of exposure. After RKO cells were exposed to FAS inhibitors for the indicated time periods, cyclin A- and cyclin B1-associated kinase activities were determined by an immunocomplex-kinase assay.



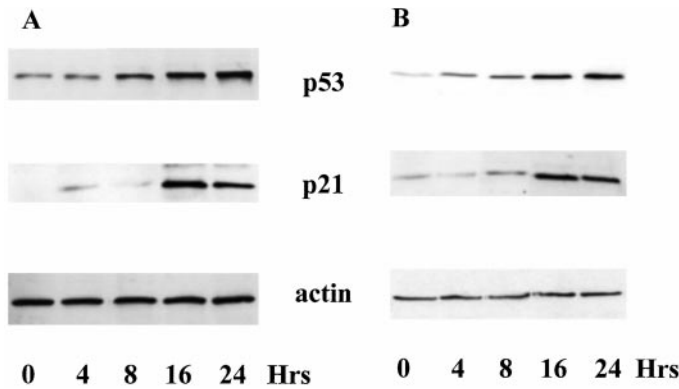


Fig. 4. Accumulation of p53 and p21 is induced in RKO colon carcinoma cells by pharmacological inhibitors of FAS. Cells were treated with cerulenin (10  $\mu\text{g/ml}$ ; A) or c75 (10  $\mu\text{g/ml}$ ; B) for the stated exposure times and analyzed by immunoblotting for p53 and p21 protein content using actin as an internal control.

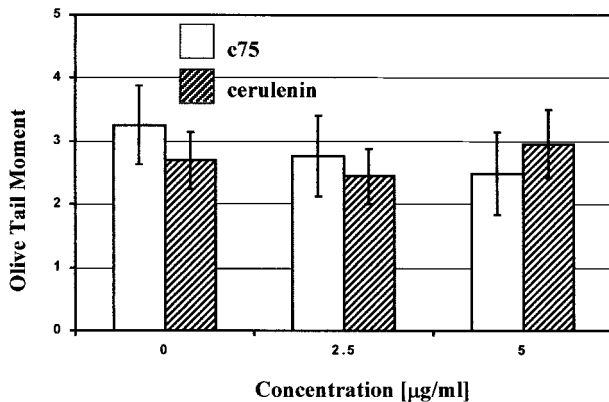


Fig. 5. FAS inhibition does not produce significant DNA damage. Cerulenin- or c75-treated MCF7 breast carcinoma cells were subjected to alkaline single cell gel electrophoresis (comet assay). Olive tail moment indicates electrophoretic mobility of DNA induced by DNA damage. Lymphoblasts exposed to 0 or 1 Gy  $\gamma$  irradiation had olive tail moments of  $0.9 \pm 0.3$  and  $7.1 \pm 0.8$  in this experiment. Exposure to 5 cGy  $\gamma$  irradiation typically produces an olive-tail moment of twice the control.

cdks that, in turn, phosphorylate key regulatory proteins (27). Cyclin A/cdk2 complex activity is required for efficient DNA replication, and the activity of complexes containing cdc2 and cyclins A and B is required for passage through  $G_2$  and mitosis. The effect of FAS inhibitors on the activity of cyclin/cdk complexes in RKO cells was determined in a time-course analysis. As shown (Fig. 3), the kinase activity associated with immunoprecipitated complexes containing cyclin A decreased to  $<40\%$  of control levels at 4 and 8 h after exposure to either cerulenin or c75, then increased moderately at later time points. The kinase activity associated with immunoprecipitated cyclin B decreased to  $<5\%$  of control levels by 4 and 8 h after exposure to either cerulenin or c75, then increased to  $>80\%$  of control levels at 16 and 24 h. These changes in S and  $G_2$  cdk activity correlated well with the biphasic pattern of cell cycle distribution demonstrated in Fig. 1 and 2. Immunoblots of cyclin A and B levels performed in parallel with the experiment in Fig. 3 demonstrate that, unlike the associated kinase activities, the cyclin levels do not decrease until 24 h (not shown).

**FAS Inhibitors Induce p53 and p21 Accumulation, but Not DNA Damage.** The delays in cell cycle progression, with redistribution of cells into  $G_1$  and  $G_2$  after FAS inhibition, were suggestive of cell cycle checkpoint activation by the tumor suppressor p53, which occurs after genotoxic or other cellular stresses (28). Accumulation of p53 protein, and the p53-regulated cdk-inhibitor p21<sup>WAF1/CIP1</sup>, were assayed by immunoblotting in a parallel time course after inhibition of

FAS (Fig. 4). p53 and p21 protein levels were unchanged or decreased during the early period of FAS-inhibitor exposure. However, treatment with 10  $\mu\text{g/ml}$  of either cerulenin or c75 induced accumulation of p53 and p21 protein at 16 and 24 h in RKO cells (Fig. 4). Of note, p21 mRNA levels did not show increases of the same magnitude, suggesting a translational and/or posttranslational mechanism(s) regulating p21 accumulation (not shown).

To determine whether significant DNA damage occurred after FAS-inhibitor exposure, alkaline single cell gel electrophoresis (comet assay) was performed on MCF7 breast cancer cells after exposure to concentrations of cerulenin and c75 that resulted in 75% survival (Fig. 5). This assay detects DNA strand breaks and a spectrum of alkali-labile DNA damage at low levels (25, 29). Neither cerulenin nor c75 induced olive tail moments over background values for untreated control cells, indicating that DNA damage was not induced by either agent at doses previously shown to induce inhibition of DNA synthesis and to reduce clonogenic activity (13, 19). This suggests that c75 and cerulenin induced cytotoxic, not genotoxic, damage to cells in an assay that under similar conditions readily detected DNA damage induced by 5 cGy of  $\gamma$  irradiation or 25  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . A similar absence of DNA damage was seen after drug treatment of GM1310B human lymphoblasts (not shown).

**Loss of p53 Function Substantially Increased the Sensitivity of Tumor Cells to FAS Inhibitors.** Inhibition of FAS induced p53 and p21 protein accumulation and  $G_1/G_2$  redistribution in RKO cells, which have an intact p53 pathway (and in other cell lines with wild-type p53, not shown). However, many tumor lines with p53 mutations undergo apoptosis within 24 h of exposure to FAS inhibitors (19). We therefore sought to determine the effect of p53 function on survival after FAS inhibition by comparing two pairs of isogenic cell lines with wild-type and altered p53 function. RKO cells were

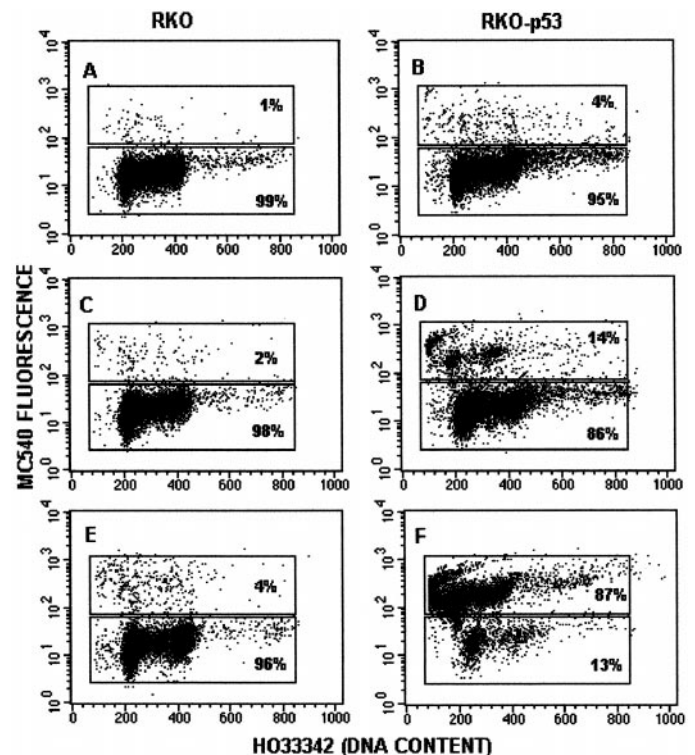


Fig. 6. Loss of p53 function sensitizes carcinoma cells to FAS inhibitor-mediated apoptosis. RKO cells without or with a stably transfected dominant negative mutant p53 gene were subjected to multiparameter flow cytometry after 24 h of exposure to cerulenin. Ungated two-dimensional analysis of DNA content versus MC540 fluorescence is displayed after no drug (A and B), cerulenin (5  $\mu\text{g/ml}$ ; C and D), and cerulenin (10  $\mu\text{g/ml}$ ; E and F). Apoptotic and nonapoptotic cells are in the upper and lower boxes, respectively.

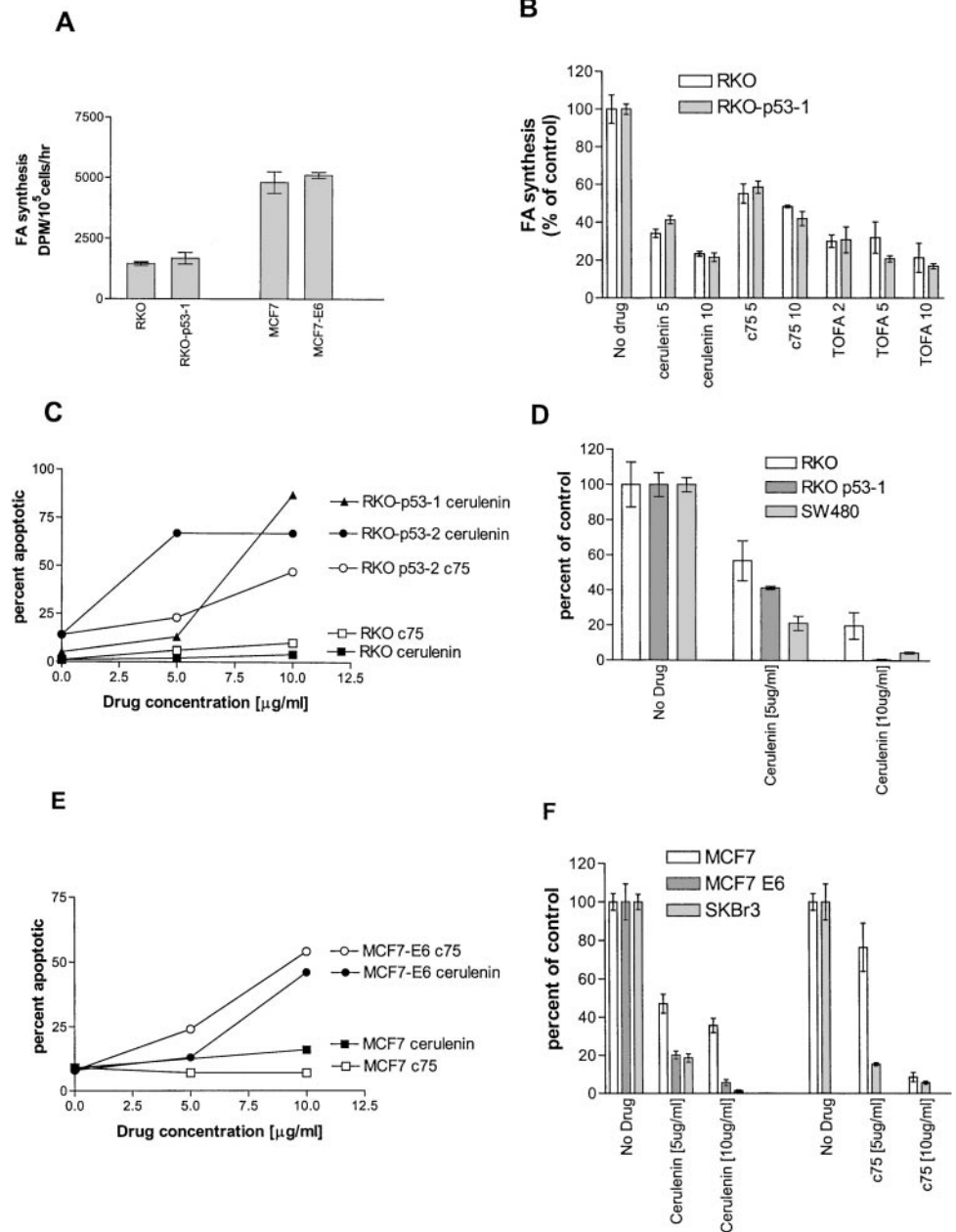
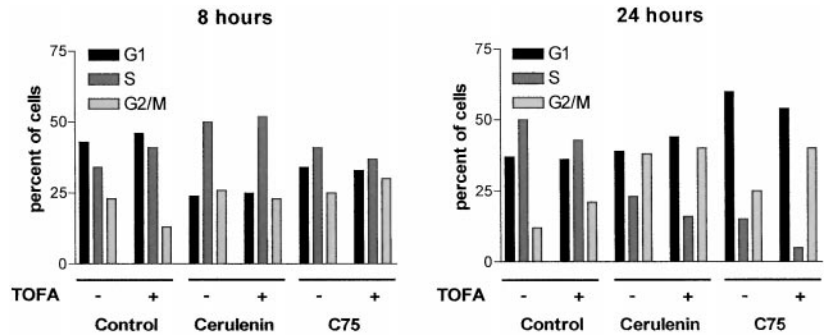


Fig. 7. Loss of p53 function sensitizes colon and breast carcinoma cells to FAS inhibitor cytotoxicity. *A*, constitutive fatty acid synthesis pathway activity of parental and p53-deficient lines are similar. *B*, cerulenin, *c75*, and TOFA inhibit fatty acid synthesis to 60% or less of control levels at the doses used ( $\mu\text{g/ml}$ ). *C* and *E*, the apoptotic fraction of colon and breast carcinoma cells after 24 h exposure to FAS inhibitors, analyzed as in Fig. 6. *D* and *F*, parallel determinations of sensitivity to FAS inhibitors were performed by clonogenic assay after a 6-h drug exposure. SW480 is a colon carcinoma line with a naturally occurring p53 mutation. SKBr3 is a breast carcinoma line with a naturally occurring p53 mutation.

rendered p53-mutant by stable transfection with a dominant-negative mutant *p53* gene (*RKO-p53*); the human breast carcinoma cell line MCF7 was rendered p53-deficient by constitutive expression of the *human papilloma virus 16 E6* gene (*MCF7-E6*; 21). The loss of p53 function sensitized RKO and MCF7 cells to the cytotoxic effect of FAS inhibition. There was a large, dose-dependent increase in apoptosis after cerulenin exposure in RKO-p53 cells compared with the parent RKO line (Fig. 6). The cell cycle distribution of the non-apoptotic (*lower boxes*) and apoptotic (*upper boxes*) subpopulations of RKO cells after 24 h of exposure to 5 or 10  $\mu\text{g/ml}$  cerulenin was determined by multiparameter flow cytometry. Cell cycle position (DNA content) was determined with HO33342 dye, and apoptosis was detected by bright staining with merocyanine 540 (MC540), which detects conformational changes in the plasma membrane that occur early during apoptosis (22–24). The validity of MC540 staining as a measure of entry into apoptosis has been confirmed in our experimental system by evaluation of morphology, change in light scatter

parameters, and TUNEL DNA end-labeling in parallel experiments (Refs. 13 and 19 and data not shown). Entry into apoptosis after FAS inhibition by cerulenin occurred from  $G_1$ , S, and  $G_2$ -M without increased sensitivity in any subpopulation. Apoptosis with lack of cell cycle phase specificity was typical of many experiments with several cell lines (not shown). The fatty acid synthetic pathway activity in these paired lines was very similar, so loss of p53 function had no discernable effect on fatty acid synthesis level (Fig. 7A). And the FAS inhibitors produced comparable reduction of pathway activity in the paired lines (Fig. 7B; Refs. 13 and 19). A similar apoptotic response was seen with three independent RKO-p53 clones and with MCF7-E6, and was seen after exposure to *c75* (Fig. 7, C and E, and data not shown). The cytotoxic effects of the FAS inhibitors on these paired lines were also tested by clonogenic assay (Fig. 7, D and F). Comparison of the two cytotoxicity assays shows that inhibition of FAS causes a reduction in the number of clonable RKO and MCF7 cells that is not detected by the apoptosis assay. The clonogenic assay

Fig. 8. FAS inhibitor-induced growth arrest is independent of malonyl-CoA accumulation. DNA content of RKO cells exposed to cerulenin (10  $\mu\text{g/ml}$ ) or *c75* (10  $\mu\text{g/ml}$ ) for the indicated times, without or with 1 h TOFA (5  $\mu\text{g/ml}$ ) pretreatment to inhibit malonyl-CoA synthesis. Determination of the percentages of cells in G<sub>1</sub>, S, and G<sub>2</sub>-M was done with Multicycle software.



probably detects subpopulations undergoing growth arrest and potentially other growth-inhibitory processes in addition to those undergoing rapid apoptosis. However, it appears that the early apoptosis associated with loss of p53 function illustrated in Fig. 6 reduces further the clonable fraction, resulting in sensitivity to FAS inhibitors that is comparable with that seen with other lines bearing naturally occurring p53 mutations (SW480 colon carcinoma and SKBr3 breast carcinoma cells).

**FAS Inhibitor-induced Growth Arrest Is Independent of Malonyl-CoA Accumulation.** Accumulation of malonyl-CoA, the committed substrate for fatty acid synthesis, is likely to participate in the cytotoxicity of FAS inhibition, inasmuch as down-regulation of malonyl-CoA production alleviated the toxicity of cerulenin and *c75* and substantially reduced the apoptotic fraction at 24 h (13). To determine the role of malonyl-CoA accumulation in delaying cell cycle progression, RKO cells were analyzed by flow cytometry after 8 or 24 h of FAS inhibitor exposure, without or with pretreatment for 1 h with the acetyl-CoA carboxylase inhibitor, TOFA, which blocks the carboxylation of acetyl-CoA to form malonyl-CoA (Fig. 8). Cells pretreated with TOFA, and then with cerulenin or *c75*, showed similar or greater cell cycle delays than cells exposed only to the FAS inhibitors. Of note, however, TOFA pretreatment did rescue FAS inhibitor-mediated apoptosis in RKO-p53 cells, similar to earlier results (Ref. 13 and not shown), indicating that the effects of FAS inhibitors on cell cycle progression are distinct from those mediating apoptotic cell death.

## DISCUSSION

**The Biphasic Stress Response to FAS Inhibition May Result from Lipid Product Depletion.** The kinetics of the response of RKO cells to FAS inhibition illustrated in Figs. 1–4 suggests the rapid onset of a stress response. This response is characterized by a marked reduction in cyclin A- and B-associated kinase activities, an early suppression of DNA replication, and an accumulation of cells in the S and G<sub>2</sub> phases during the first 8 h of drug exposure, and then by enhanced expression of p53 and p21 proteins, and growth arrest in G<sub>1</sub> and G<sub>2</sub> by 16 and 24 h.

Although malonyl-CoA accumulation appears involved in triggering apoptosis after FAS inhibition, the growth arrest stress response produced by FAS inhibition may be attributable to altered lipid production, because acetyl-CoA carboxylase inhibition did not relieve it. Most of the fatty acids produced by tumor cells are incorporated into membrane phospholipids, and phospholipid synthesis is inhibited when fatty acid synthesis is inhibited (6, 7). Phospholipid biosynthesis is greatest during the G<sub>1</sub> and S phases, with doubling of the membrane mass occurring during S phase in preparation for cell division (30). It is possible, therefore, that limitation of phospholipid synthesis during the S phase affects DNA replication, or independently triggers late cell cycle delays similar to the premitotic checkpoints of yeast (31,

32). Notably, two ether lipids that specifically inhibit CTP:phosphocholine cytidyltransferase, an important enzyme in phospholipid synthesis, produce similar G<sub>2</sub>-M delays and are selectively cytotoxic to transformed cells (33). Studies in lower eukaryotes and prokaryotes have shown a requirement for active fatty acid synthesis at the time of cell division, either for simple mitosis or for sporulation (34). The defects in these systems appear related to chromatin configuration or to the transcriptional activation of key genes. In the FAS inhibition system discussed here, however, the specific mechanisms whereby cyclin A- and B-associated kinase activities decrease in RKO cells remain to be studied in detail.

**Role of Tumor Suppressor p53 in the Response to FAS Inhibitors.** The observation that FAS inhibitors induced the accumulation of p53 and p21 proteins suggested that DNA damage might be occurring, either as a direct effect of the drugs on the DNA molecule, or as a downstream effect of FAS inhibition. However, several other observations argue against DNA damage. First, the toxic effect of cerulenin was found to be dependent on its ability to inhibit FAS in yeast, thus ruling out a significant direct effect of cerulenin on DNA (17). Second, toxicity in tumor cells is modulated by alterations in pathway activity and substrate levels (13, 19). Finally, no DNA damage was detected using the single cell gel electrophoresis (comet) screening assay, which has been shown to be very sensitive in detecting low levels of DNA damage. Consistent with these observations, no differences were detected in the sensitivity to FAS inhibitors of cells deficient in ATM (mutated in ataxia telangiectasia) versus controls (not shown).

Whereas the first and most extensively studied function described for the tumor suppressor protein p53 was the induction of growth arrest and apoptosis after DNA damage (20, 35–37), more recently, important roles for p53 have been recognized in the cellular responses to a variety of nongenotoxic metabolic stresses, including hypoxia, acidosis, and perturbations of RNA and protein synthesis (38–43). The current study indicates that perturbation of fatty acid synthesis also belongs on the list of metabolic stresses regulated by p53.

The decision between apoptosis and growth arrest after FAS inhibition clearly is influenced by p53 function. Because constitutive fatty acid synthesis activity and inhibitor effects were similar between the paired parental and p53 deficient cells, it is unlikely that levels of malonyl-CoA accumulation were substantially different. The ability of the cell to survive malonyl-CoA accumulation may be greater in cells with intact p53. The relatively low fatty acid synthesis pathway activity of RKO cells (less malonyl-CoA) combined with intact p53 function may underlie the minimal apoptosis produced by FAS inhibitors in RKO cells and in various nontransformed cells.<sup>4</sup> It is likely that induction of p21 promotes growth arrest and exerts a protective effect after FAS inhibition, as it has been shown to do in a variety of

<sup>4</sup> Authors' observation.



other stress paradigms (44). The triggering of apoptosis after FAS inhibition is very rapid, and probably occurs before p21 induction. FAS inhibitors triggered comparable apoptotic responses in the majority of tumor lines with mutant p53 status that we have studied. The predominant pattern of sensitization by loss of p53 function suggests that endogenous fatty acid synthesis will hold special appeal as an experimental therapeutic target. FAS inhibitors combine the target specificity for cancer cells afforded by both elevated fatty acid synthesis and the loss of p53 function.

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