[CANCER RESEARCH 58, 4611–4615, October 15, 1998]

Advances in Brief

Pharmacological Inhibitors of Mammalian Fatty Acid Synthase Suppress DNA Replication and Induce Apoptosis in Tumor Cell Lines[1]

Ellen S. Pizer,[2] Francis J. Chrest, Joseph A. DiGiuseppe, and Wan Fang Han

Department of Pathology, The Johns Hopkins Medical Institutions, Baltimore, Maryland 21287 [E. S. P., J. A. D., W. F. H.], and Clinical Immunology Section, Gerontology Research Center, National Institute on Aging, Baltimore, Maryland 21224 [F. J. C.]

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 min and DNA synthesis inhibition occurring within 90 min of drug exposure. Meanwhile, apoptotic changes are not detected until 6 h 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 antimetabolite, cerulenin, is a natural product of cephalosporium caerulens. Cerulenin, (2*R*,3*S*)-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, FAS,[3] by binding covalently to the active site cysteine of the β 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.[4]

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 reported previously 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.

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 described previously (9). Cells were screened periodically for *Mycoplasma* contamination (Gen-probe). Cerulenin (Sigma) and c75 were added as stock 5 mg/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 5×10^4/well in 1 ml in 24-well plates and incubated overnight. DNA synthesis was assayed with a 2-h pulse of [^{3}H]thymidine, 1 μCi/well, followed by precipitation in 10% trichloroacetic acid, two washes in 95% ethanol, solubilization in 0.1 N ammonium acetate, and scintillation counting. Fatty acid synthesis was assayed with a 2-h pulse of [U-^{14}C]acetic acid, 1 μCi/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 5 μCi/well of [U-^{14}C]acetic acid to 1.8-ml suspensions of HL60 cells at 3×10^6/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 5 μCi/well of [^{3}H]thymidine to 2.5-ml suspensions of HL60 cells at 3×10^6/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 SE. Calculations and graphing were performed in Prism 2.0 (GraphPad).

Detection of Apoptosis. HL60 cells were exposed to cerulenin (10 μg/ml) or c75 (10 μg/ml) for the indicated intervals, then analyzed by TUNEL labeling of DNA as described previously (11). The TUNEL-positive population was marked by an increase in green fluorescence, collected at 530 ± 20 nm, of 0.5 to 1 log over TUNEL-negative cells. Detection of altered plasma membrane phospholipid packing was by decoration with merocyanine 540 (10 μg/ml) added as a (1 mg/ml) stock in water directly to cells out of culture in medium (Sigma Chemical Co.; Ref. 12). The merocyanine 540-positive population was marked by an increase in red fluorescence, collected at 575 ± 20 nm, of 0.5 to 2 logs over merocyanine 540-negative cells. Cytometry data were acquired on a Becton Dickinson FACSscan and analyzed with LYSYS11, version 1.1.

Results and Discussion

Several lines of evidence suggest linkage between tumor cell proliferation and elevated fatty acid synthesis (9, 13). To determine whether cell cycle progression is altered after inhibition of fatty acid

Received 7/7/98; accepted 9/1/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by R29CA75219. E. S. P. is supported by a Passano Clinician Scientist Award, awarded through the Johns Hopkins University School of Medicine.

[2] To whom requests for reprints should be addressed, at Department of Pathology, AA154C, Johns Hopkins Bayview Medical Center, 4940 Eastern Avenue, Baltimore, MD 21224. Phone: (410) 550-3670; Fax: (410) 550-0075; E-mail: epizer@jhmi.edu.

[3] The abbreviations used are: FAS, fatty acid synthase; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; HPV, human papillomavirus.

[4] F. P. Kuhajda, E. S. Pizer, N. S. Mani, and C. A. Townsend. Synthesis and Anti-Tumor Activity of a Novel Inhibitor of Fatty Acid Synthase, submitted for publication.

synthesis, we measured incorporation of thymidine into DNA after exposure of tumor cells to cerulenin or c75 (Fig 1, *A* and *B*). These experiments demonstrated that fatty acid synthesis inhibitors produce a profound inhibition of DNA replication within 2 h. Because S phase typically requires at least 6 h, this effect is consistent with inhibition of S phase progression rather than inhibition of the G_1-S transition. Fig. 1, *A* and *B*, demonstrates that the dose responses of fatty acid synthesis and DNA synthesis to cerulenin and c75, 2 h after drug exposure, are similar for HCT116 colon carcinoma cells. Cerulenin (10 μg/ml) and c75 (10 μg/ml) both produce inhibition of DNA synthesis to <10% of control levels. Pulse labeling at later time points confirmed that DNA synthesis remained inhibited to <1% of control levels at 8 and 18 h after drug exposure (Fig. 1*C*).

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 min (Fig. 2*A*). Thymidine labeling demonstrated partial inhibition of DNA synthesis during the first 60–90 min after drug exposure, with maximal inhibition occurring after 60 min of exposure to c75 or after 90 min of exposure to cerulenin (Fig. 2*B*). 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 h after drug exposure (11). Similar kinetics of apoptosis induction were observed in HL60 cells using detection of altered plasma membrane phospholipid packing by decoration with merocyanine 540 as the indicator of early entry into apoptosis (data not shown; Ref. 12). The rate at which apoptosis occurs is variable among cell lines, with most carcinoma lines that we have studied requiring more than 6 h after drug exposure before bright staining with merocyanine 540 occurs (data not shown).

We have observed previously 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

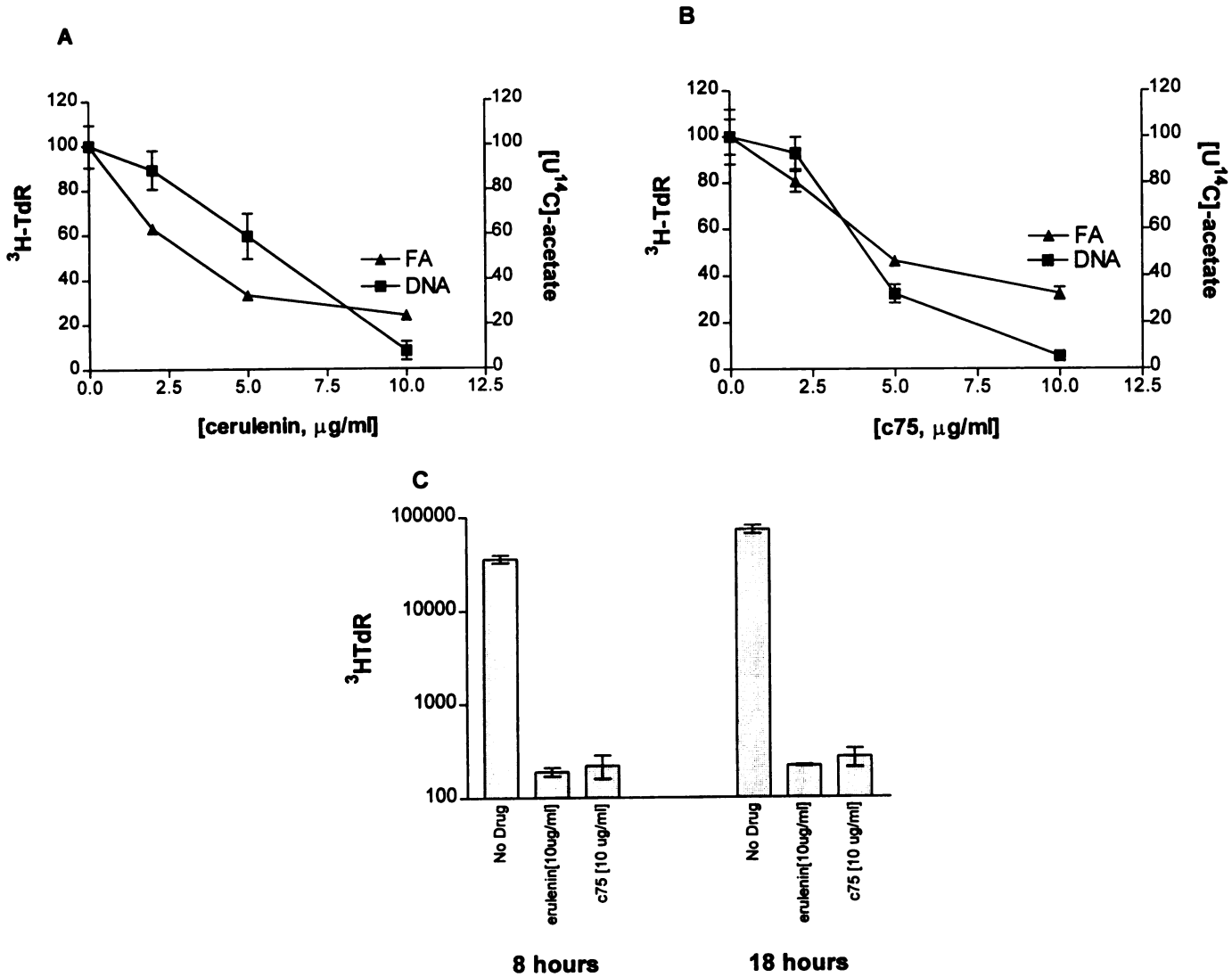
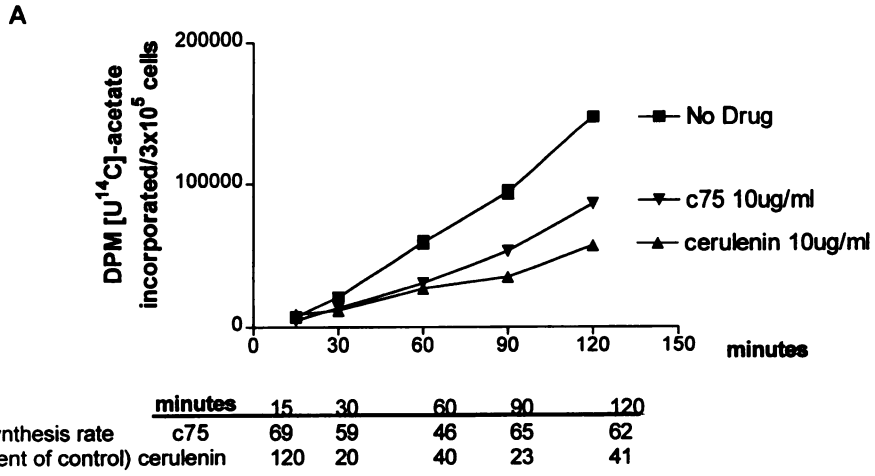
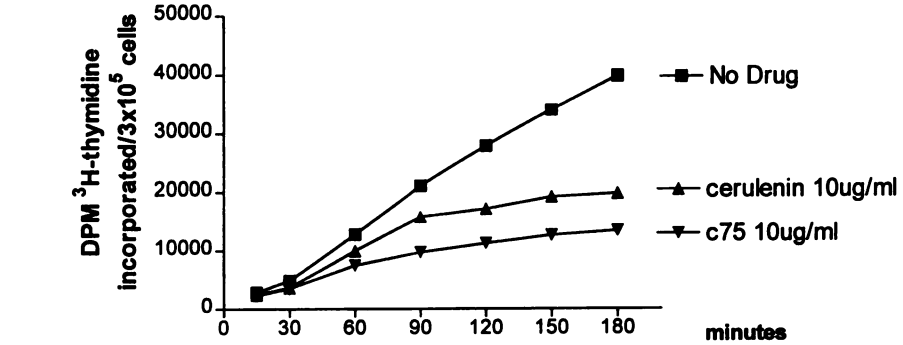


Fig. 1. Inhibition of fatty acid synthesis and of DNA replication by FAS inhibitors have similar dose responses. In *A* and *B*, triplicate cultures of HCT116 colon carcinoma cells were exposed to cerulenin or c75 for 4 h. During the last 2 h, cells were pulse labeled with either [^{3}H]thymidine or [U-^{14}C]acetic acid as described in "Materials and Methods." Label incorporation is shown as a percentage of control. In *C*, similar pulse labeling with [^{3}H]thymidine was performed from 6 to 8 or from 16 to 18 h after drug exposure. Label incorporation is shown as dpm per 5×10^4 cells. *Bars*, SE.



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



	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

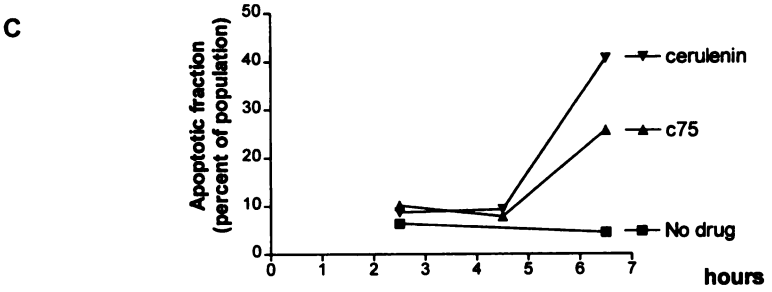


Fig. 2. Inhibition of DNA replication occurs rapidly after inhibition of fatty acid synthesis and in the early interval before cell death. In *A* and *B*, suspension cultures of HL60 promyelocytic leukemia cells in serum-free medium were sampled at intervals during drug treatment and metabolic labeling, as described in "Materials and Methods." *Bars*, SE. 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. In *C*, a time course for induction of apoptotic changes is displayed.

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 ~20-fold difference in overall pathway activity in the two media, both cerulenin and c75 inhibit fatty acid synthesis to approximately one-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 (~20% of control levels *versus* ~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 suggest 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.

Table 1 *Modulation of fatty acid synthesis and DNA replication inhibition by serum concentration*

Serum	10% Fetal bovine serum[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/h) is 20-fold higher in medium without serum than in 10% fetal bovine serum.
[b] Mean percentage of control after maximal inhibition.

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 (14). The early genes of

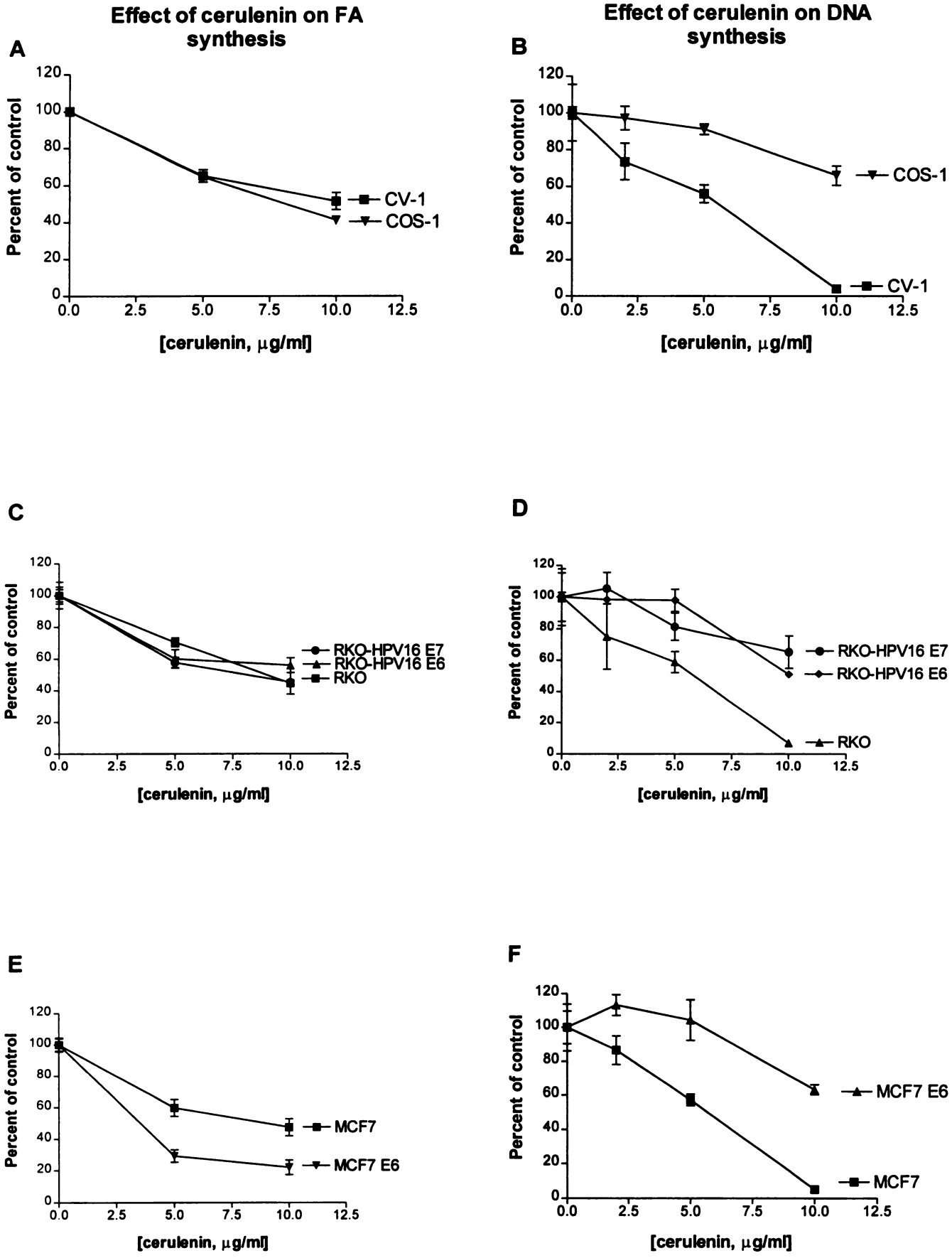


Fig. 3. Expression of certain viral oncogenes abrogates the inhibitory effect of cerulenin on DNA replication. Triplicate cultures of the indicated cells were exposed to cerulenin for 4 h. During the last 2 h, cells were pulse labeled with either [U-^{14}C]acetic acid (*A*, *C*, and *E*) or [^{3}H]thymidine (*B*, *D*, and *F*) as described in "Materials and Methods." Label incorporation is shown as a percentage of control. *Bars*, SE.

certain DNA tumor viruses are able to abrogate higher order S-phase regulation and facilitate passage of infected cells through S phase to accomplish viral replication (15). 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 HPV relieved the inhibitory effect of cerulenin on DNA replication, although fatty acid synthesis inhibition was similar. CV-1, a monkey kidney cell line, is the parent of COS-1, a stably transfected, transformed line that expresses SV40 T antigen (16). The effects of cerulenin on this pair of cell lines were as follows. The dose-dependent inhibition of fatty acid synthesis was similar for CV-1 cells and COS-1 cells, indicating that the drug is able to enter the cells and inactivate FAS comparably (Fig. 3*A*). The dose-dependent inhibition of DNA synthesis in 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 (10 μg/ml; Fig. 3*B*). Similar results were obtained when RKO colon carcinoma cells were compared with stably transfected RKO clones that expressed HPV 16 *E6* or *E7* genes (Fig. 3, *C* and *D*) and when MCF7 breast carcinoma cells were compared with a stably transfected MCF7 clone that expressed the HPV 16 *E6* gene (Fig. 3, *E* and *F*). The RKO-HPV16 E6 clone shown in Fig. 3 is representative of three 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 comodulates 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, because 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, which 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 (17), and similar physiological effects occurred after restriction of phospholipid synthesis (18). The importance of this effect in the ultimate cytotoxic outcome of fatty acid synthesis inhibition in human cancer remains to be determined.

Acknowledgments

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 (NCI, Bethesda, MD). Compound 75 (c75) was supplied by C. Townsend (Johns Hopkins University, Baltimore, MD).

References

1. Omura, S. The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis. Bacteriol. Rev., *40:* 681–697, 1976.
2. Vance, D., Goldberg, I., Mitsuhashi, O., and Bloch, K. Inhibition of fatty acid synthetases by the antibiotic cerulenin. Biochem. Biophys. Res. Commun., *48:* 649–656, 1972.
3. Pizer, E. S., Wood, F. D., Heine, H. S., Romantsev, F. E., Pasternack, G. R., and Kuhajda, F. P. Inhibition of fatty acid synthesis delays disease progression in a xenograft model of ovarian cancer. Cancer Res., *56:* 1189–1193, 1996.
4. Kuhajda, F. P., Jenner, K., Wood, F. D., Hennigar, R. A., Jacobs, L. B., Dick, J. D., and Pasternack, G. R. Fatty acid synthesis: a potential selective target for antineoplastic therapy. Proc. Natl. Acad. Sci. USA, *91:* 6379–6383, 1994.
5. Pizer, E. S., Jackisch, C., Wood, F. D., Pasternack, G. R., Davidson, N. E., and Kuhajda, F. P. Inhibition of fatty acid synthesis induces programmed cell death in human breast cancer cells. Cancer Res., *56:* 2745–2747, 1996.
6. Altenbern, R. Cerulenin-inhibited cells of *Staphylococcus aureus* resume growth when supplemented with either a saturated or an unsaturated fatty acid. Antimicrob. Agents Chemother., *11:* 574–576, 1977.
7. Kuerbitz, S. J., Plunkett, B. S., Walsh, W. V., and Kastan, M. B. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Natl. Acad. Sci. USA, *89:* 7491–7495, 1992.
8. Fan, S., Smith, M. L., Rivet, D. J., II, Duba, D., Zhan, Q., Kohn, K. W., Fornace, A. J., Jr., and O'Connor, P. M., Disruption of p53 function sensitizes breast cancer MCF-7 cells to cisplatin and pentoxifylline. Cancer Res., *55:* 1649–1654, 1995.
9. Pizer, E. S., Wood, F. D., Pasternack, G. R., and Kuhajda, F. P. Fatty acid synthase (FAS): a target for cytotoxic antimetabolites in HL60 promyelocytic leukemia cells. Cancer Res., *56:* 745–751, 1996.
10. Folch, J., Lees, M., and Sloane Stanley, G. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., *226:* 497–509, 1957.
11. Gorczyca, W., Gong, J., and Darzynkiewicz, Z. Detection of DNA strand breaks in individual apoptotic cells by the *in situ* terminal deoxynucleotidyl transferase and nick translation assays. Cancer Res., *53:* 1945–1951, 1993.
12. Mower, D. A., Jr., Peckham, D. W., Illera, V. A., Fishbaugh, J. K., Stunz, L. L., and Ashman, R. F. Decreased membrane phospholipid packing and decreased cell size precede DNA cleavage in mature mouse B cell apoptosis. J. Immunol., *152:* 4832–4842, 1994.
13. Pizer, E., Lax, S., Kuhajda, F., Pasternack, G., and Kurman, R. Fatty acid synthase expression in endometrial carcinoma: correlation with cell proliferation and hormone receptors. Cancer (Phila.), *83:* 528–537, 1998.
14. Pines, J. Cyclins and cyclin-dependent kinases: theme and variations. Adv. Cancer Res., *66:* 181–212, 1995.
15. Jansen-Durr, P. How viral oncogenes make the cell cycle. Trends Genet., *12:* 270–275, 1996.
16. Gluzman, Y. SV40-transformed simian cells support the replication of early SV40 mutants. Cell, *23:* 175–182, 1981.
17. Seyfzadeh, M., Kenner, J., and Nomura, M. spoT-dependent accumulation of guanosine tetraphosphate in response to fatty acid starvation in *Escherichia coli*. Proc. Natl. Acad. Sci. USA, *90:* 11004–11008, 1993.
18. Pizer, L., Merlie, J., and Ponce de Leon, M. Metabolic consequences of limited phospholipid synthesis in *Escherichia coli*. J. Biol. Chem., *294:* 3212–3224, 1974.

Pharmacological Inhibitors of Mammalian Fatty Acid Synthase Suppress DNA Replication and Induce Apoptosis in Tumor Cell Lines

Ellen S. Pizer, Francis J. Chrest, Joseph A. DiGiuseppe, et al.

Cancer Res 1998;58:4611-4615.