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Advances in Brief

Inhibition of Fatty Acid Synthesis Delays Disease Progression in a Xenograft Model of Ovarian Cancer[1]

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Abstract

One of the key limiting factors in the treatment of advanced stage human epithelial malignancies is the lack of selective molecular targets for antineoplastic therapy. A substantial subset of human ovarian, endometrial, breast, colorectal, and prostatic cancers exhibit increased endogenous fatty acid biosynthesis and overexpress certain enzymes in the pathway. Cell lines derived from these tumors use endogenously synthesized fatty acids for cellular functions, whereas normal cells and tissues appear to utilize dietary lipids preferentially. We have previously shown that the difference in fatty acid biosynthesis between cancer and normal cells is an exploitable target for metabolic inhibitors *in vitro*. Here, we report observations *in vivo* using the i.p. model of the multiply drug-resistant OVCAR-3 human ovarian carcinoma in nude mice which demonstrate that: (*a*) fatty acid synthase overexpression in OVCAR-3 is comparable to levels in primary human tumors assessed by immunohistochemistry; (*b*) fatty acid synthetic activity of OVCAR-3 is comparably elevated *in vitro* and *in vivo* and is 4 to >20-fold higher than normal murine tissues; (*c*) treatment with the specific fatty acid synthase inhibitor, cerulenin, markedly reduces tumor cell fatty acid biosynthesis *in vivo*; (*d*) fatty acid synthase inhibition produces regression of established ascites tumor; and (*e*) treatment with cerulenin causes reduction in ascites incidence, delay in onset of ascites, and significantly increased survival ($P < 0.04$).

Introduction

The treatment of advanced stage human epithelial malignancies has progressed little in the last decade despite great effort. Drug resistance and limiting toxic side effects of therapy contribute to the ultimate failure of treatment for the majority of advanced stage cancer patients. One of the key factors holding back therapeutic advances is the lack of selective molecular targets for antineoplastic therapy. Meanwhile, disordered intermediary metabolism in cancer cells has been known for the better part of this century (1); however, little attention has been paid to fatty acid metabolism. One early study showed elevated levels of fatty acid synthesis in tumor tissues, although the significance of the observations was not appreciated (1, 2). Our work indicates that differences in fatty acid metabolism between normal and cancer cells can be exploited to achieve selective destruction of tumor cells. A number of studies have recently shown that some clinical human ovarian, endometrial, breast, colorectal, and prostatic cancers and cell lines derived from these tumors express unexpectedly high levels of the enzymes required for endogenous fatty acid biosynthesis (Refs. 3–7 and Fig. 1).[3] Tumor cells which express high levels of fatty acid-synthesizing enzymes use endogenously synthesized fatty acids for membrane biosynthesis and also export large amounts of lipid.[4] In contrast, it appears that normal cells and tissues preferentially utilize dietary lipids (8).[4] We have previously shown that these biochemical differences provide a selective target for metabolic inhibitors in the *in vitro* setting. Observations *in vitro* demonstrate that: (*a*) inhibition of the β-ketoacyl synthase site of fatty acid synthase is selectively cytotoxic to cancer cells with increased fatty acid biosynthesis but not to normal cells; and (*b*) addition of palmitate, the product of fatty acid synthase, reverses the cytotoxic effects of its inhibition, demonstrating mechanistic specificity (9, 10). Greater predictive value for therapeutic utility is obtained when novel drugs are tested *in vivo* in an animal model of disease as well as in tissue culture. Adaptation of the human ovarian carcinoma cell line OVCAR-3 to i.p. growth in nude mice has produced a xenograft model of ovarian cancer that closely resembles advanced stage human disease (11). Our experimental treatment of this tumor is closely analogous to the actual clinical treatment of ovarian cancer, which frequently involves direct i.p. infusion of chemotherapeutic agents (12). Here, we describe the i.p. xenograft of the multiply drug-resistant OVCAR-3 human ovarian carcinoma in nude mice as a model for treatment of advanced stage ovarian carcinoma by inhibition of fatty acid synthesis.

Materials and Methods

Determination of Fatty Acid Synthetic Activity in Carcinoma Lines. Cell lines were maintained in culture media supplemented with fetal bovine serum as recommended by the American Type Culture Collection. Triplicate samples of 2×10^5 cells were pulse labeled for 2 h with [U-^{14}C]acetate (Amersham). After washing, total lipids were extracted from cells (9) and counted.

OVCAR-3 Xenograft. The care and experimental manipulation of mice described in this report was in accordance with guidelines of the Johns Hopkins University for the ethical treatment of animals. Athymic nude mice (Harlan) were given injections of 0.05 ml (5.0×10^6) or 0.1 ml (1.0×10^7) packed OVCAR-3 cells on day 0. Because the ascites tumor grows in papillary tissue fragments, determination of cell number was achieved in parallel by counting tumor nuclei after lysis of cells in 1% NP40 (Pierce). Mice were weighed daily, and the results were normalized to original body weight. Cerulenin (Sigma) was administered in daily injections of 80 mg/kg/day i.p. in 0.2 ml 25% DMSO for 7 days. Controls received vehicle only. Metabolic effects were measured by maintenance of mice in metabolic cages with daily quantitation of urine and stool output.

Immunohistochemical Detection of Fatty Acid Synthase in OVCAR-3 Xenograft. Staphylococcal protein A-fractionated rabbit antihuman fatty acid synthase antiserum, which is monospecific by Western blot, was applied to sections of formalin-fixed, paraffin-embedded murine tissues. Immunohistochemical localization of fatty acid synthase in deparaffinized 8-μm sections

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[3] E. S. Pizer, R. J. Kurman, G. R. Pasternack, and F. P. Kuhajda. Expression of fatty acid synthase is closely linked to proliferation and stromal decidualization in cycling endometrium, manuscript in press.

[4] H. S. Heine, F. P. Kuhajda, F. D. Wood, A. Kayler, and G. R. Pasternack. Increased rates of fatty acid synthesis in breast cancer and identification of unique products, manuscript in preparation.

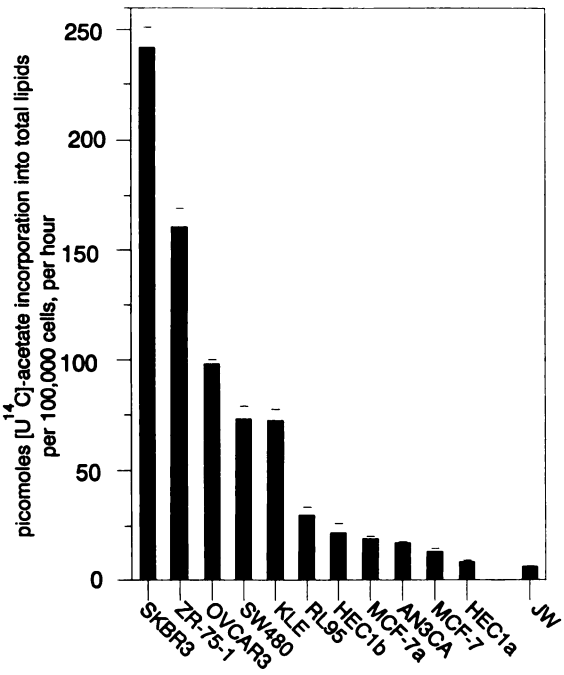

Fig. 1. Comparison of fatty acid synthetic activity in a panel of human carcinoma lines. Fatty acid synthesis is expressed as pmol [U-^{14}C]acetate incorporation into lipids/10^5 cells/h. RL95–2, AN3Ca, KLE, HEC1a, and HEC1b are endometrial carcinoma lines. OVCAR-3 is an ovarian carcinoma line. ZR-75-1, SKBR3, MCF-7, and MCF-7a are breast carcinoma lines. SW480 is a colorectal carcinoma line. JW is a nontransformed human skin fibroblast line.

was performed on the BioTek 1000 228 automated immunostainer using the BioTek 1000 kit. Reactivity was detected with biotin-antirabbit immunoglobulin raised in goat, avidin-horseradish peroxidase (Vector), and 3,3′-diaminobenzidine as the chromagen with hematoxylin counterstain. The IgG fraction of preimmune serum was nonreactive on a serial section of the same tissues.

Determination of Lipid Synthetic Activity in OVCAR-3 Ascites Tumor and Normal Murine Tissues. Xenografted nude mice were pulse labeled *in vivo* for 2 h with 0.5 mCi [U-^{3}H]acetate each; total lipids were extracted from tissues (13) and counted. Distribution of labeled precursors between ascites and blood plasma occurred within 1 h, and <2% of available label was incorporated into lipids. For determination of lipid synthetic activity after cerulenin treatment, three to four xenografted nude mice in each group were left untreated, or treated with injection vehicle only (25% DMSO), cerulenin (80 mg/kg), or cerulenin (240 mg/kg) for 6 h with pulse labeling during the last 2 h. Since no differences were detected between untreated and vehicle-treated mice or between low- and high-dose cerulenin treatment, the groups were pooled for analysis.

Results and Discussion

Preliminary biochemical and functional studies of OVCAR-3 cells and other human carcinoma lines of various histological types show a range of elevation of fatty acid synthesis in the carcinoma lines, with OVCAR-3 levels elevated approximately 10-fold above levels in control skin fibroblasts (Fig. 1). The fungal metabolite, cerulenin, [(2*S*,3*R*)2,3-epoxy-4-oxo-7E,10E-dodecadienamide], a specific, noncompetitive inhibitor of fatty acid synthase, binds covalently to the condensing enzyme site on fatty acid synthase, causing complete inactivation (14, 15). Of importance, the condensing enzyme site on fatty acid synthase utilizes a biochemically distinctive catalytic mechanism which is not shared by other vital cellular enzymes (16) and is thus well suited to be a therapeutic target. Cerulenin is growth inhibitory *in vitro* to OVCAR-3 and all other carcinoma lines tested with ID_{50} of <5 μg/ml (22.4 mM) at 72 h compared to control skin fibroblasts which are not growth inhibited at this drug concentration (data not shown). The various carcinoma lines display similar sensitivity to cerulenin, which appears to be independent of the magnitude of elevation of fatty acid synthesis. Of importance, clonogenic (limiting dilution) assays performed with cerulenin on human breast cancer cell lines show that cerulenin is indeed cytotoxic to cancer cells (data not shown).

The i.p. xenograft of OVCAR-3 recapitulates the typical behavior of ovarian cancer; it grows as solid tumor nodules involving the pelvic organs, peritoneal surfaces, and omentum and produces abundant cellular ascites (Fig. 2*A*). Moreover, OVCAR-3 expresses fatty acid synthase immunoreactivity at a level comparable to primary ovarian, endometrial, breast, and other carcinomas with elevated fatty acid synthase. Extraabdominal spread occurs late; as with the human disease, death results from bowel obstruction, lung metastases, and other complications. Fatty acid synthetic activity in xenografted OVCAR-3 tumor was assessed by *in vivo* labeling tumor-bearing mice with [U-^{3}H]acetate, lipid extraction of organs and tissues, and quantitation of lipid products (13). OVCAR-3 ascites tumor uniformly demonstrates marked elevations of fatty acid synthesis *in vivo*. Expression in tumor was greater than 4-fold higher than mean levels and 7-fold higher than basal levels in liver, the major physiological site of fatty acid synthesis after a low-fat meal, and was more than 20-fold higher than levels in spleen, skeletal muscle, or brain (Fig. 2*B*). Hepatic fatty acid synthesis was not repressed by dietary lipid since all mice received a standard laboratory diet containing only 12% of calories from fat. This demonstration of correspondingly high levels of fatty acid synthase expression and fatty acid synthesis in tumor cells compared to normal tissues in the xenografted mouse suggests that clinical human cancers with high levels of fatty acid synthase expression will likely exhibit a high therapeutic index and selective sensitivity to fatty acid synthesis inhibition.

When metabolic labeling *in vivo* with [U-^{3}H]acetate, as described above, was performed after i.p. administration of cerulenin, ascites tumor fatty acid synthesis was reduced by 43%, demonstrating that cerulenin inhibits fatty acid synthesis *in vivo* as well as *in vitro*. No augmentation of inhibition was achieved with high-dose cerulenin (240 mg/kg) as compared to the standard dose (80 mg/kg), indicating that drug activity was probably maximum. There was no effect on the mean hepatic fatty acid synthesis after administration of cerulenin (Fig. 2*C*), probably because the drug was entirely absorbed locally by tumor cells in ascites as described below. Consistent with this, in parallel experiments using mice without tumors, inhibition of fatty acid synthesis in the range of 50% was observed in liver following cerulenin therapy (data not shown). Attempts to quantify fatty acid synthesis in solid tumor nodules were confounded by variable amounts of contaminating components of necrotic tumor and benign fibroadipose tissue.

An analysis of the fate of cerulenin after i.p. injection was performed to determine why cerulenin therapy of tumor-bearing mice did not affect hepatic fatty acid synthesis. Cerulenin is hydrophobic, with low solubility in aqueous solution (17). Nine tumor-bearing mice were sacrificed at various times after i.p. injection with 2 mg cerulenin. Total ascites (between 1.5 and 3 ml/mouse; mean, 2.07) was removed and ascites fluid was tested for cerulenin in a cytotoxicity assay using HL60 leukemia cells in serum-free/fatty acid-free medium (10). Cytotoxic activity was below measurable levels in all samples, including three taken within 10 min of drug administration. Ascites fluid alone had a mild growth stimulatory effect, and had no effect on cerulenin added separately to HL60 cells. When cerulenin was mixed with whole ascites, ascites cells, or ascites fluid *ex vivo*, all cytotoxic activity was lost from samples containing whole ascites or ascites cells. In contrast, all of the cerulenin activity was recovered from cell-free ascites fluid (data not shown). Thus, it appears that tumor cells in ascites rapidly and completely absorbed the cerulenin after i.p.

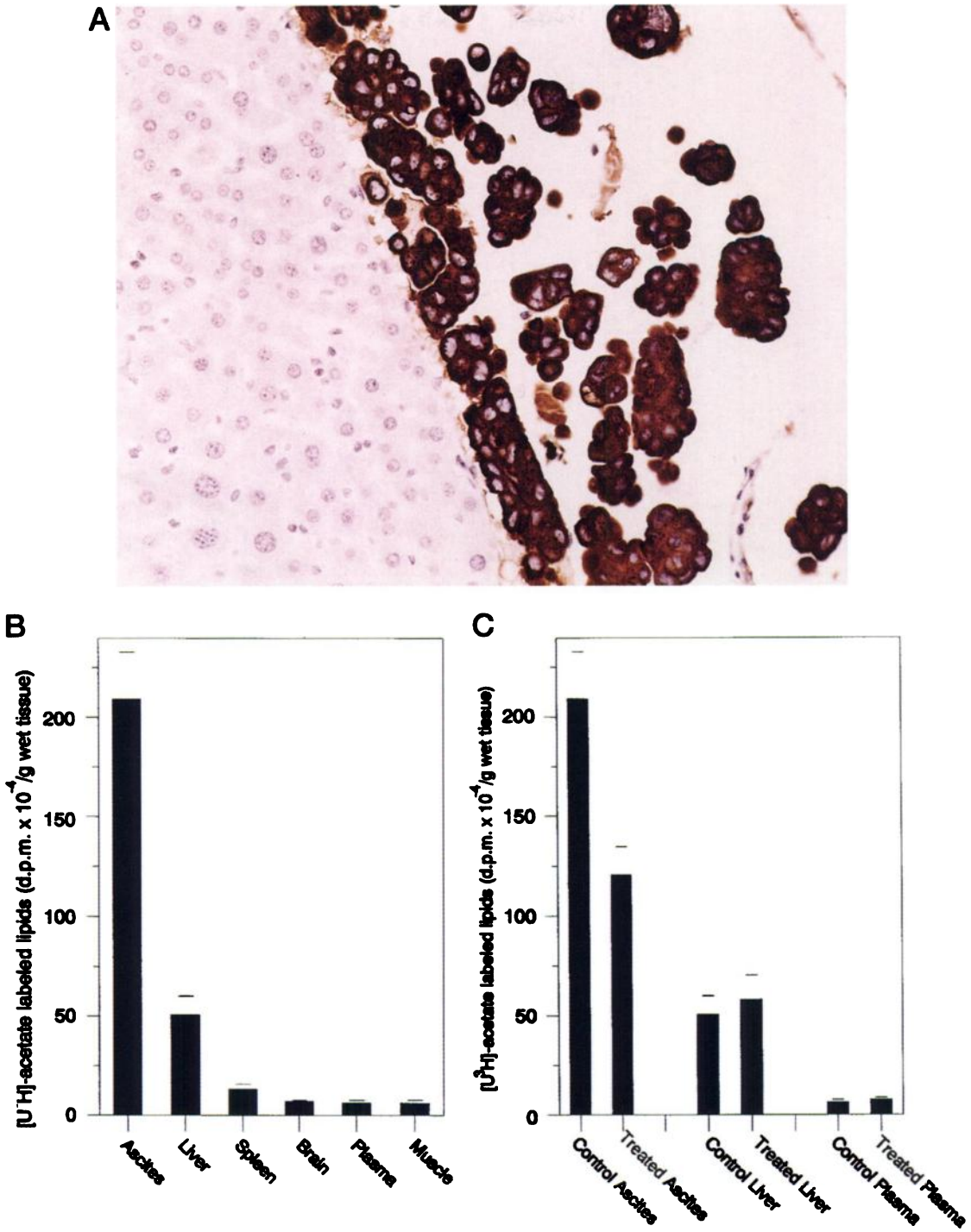

Fig. 2. *A*, immunohistochemical detection of fatty acid synthase in an OVCAR-3 xenograft. Papillary tumor fragments stud liver surface, giving rise to ascites tumor. Fatty acid synthase immunoreactivity (brown pigment) is high in tumor cells and absent in liver. *B*, relative lipid synthetic activity in OVCAR-3 ascites tumor and normal murine tissues. Mean values of activity levels for each tissue from seven xenografted nude mice are plotted. *Bars*, SD. *C*, lipid synthesis in OVCAR-3 ascites tumor and in liver with and without cerulenin treatment. Control mice were left untreated or were treated with injection vehicle only (25% DMSO). Treated mice received cerulenin (80 mg/kg) or cerulenin (240 mg/kg). Since no differences were detected between untreated and vehicle-treated mice or between low- and high-dose cerulenin treatment, the groups were pooled for analysis. Pulse labeling and determinations of lipid synthetic activity were as in *B*. Mean values of activity levels for each tissue from six or seven xenografted nude mice are plotted. *Bars*, SD. Labeling of plasma reflects lipid synthetic activity of the liver.

injection. Taken together, these data indicate that i.p. treatment of tumor-bearing mice with our standard cerulenin dose (80 mg/kg) produces effective fatty acid synthesis inhibition in the ascites tumor, and that the drug activity is limited to the ascites tumor in this setting.

As a measure of ascites accumulation, similar tumor-bearing mice were treated with daily cerulenin injections for 7 days while body weight was monitored (Fig. 3). Cerulenin treatment not only caused cessation of tumor-associated weight gain due to tumor ascites, but caused a statistically significant regression, although tumor regrowth occurred after withdrawal of therapy. In a related experiment, three mice per group received cerulenin or vehicle only for 4 days. Mice sacrificed 24 h after withdrawal of cerulenin treatment had 37% smaller ascites volumes than controls (data not shown). The treatment-associated loss of body mass corresponded to the difference in

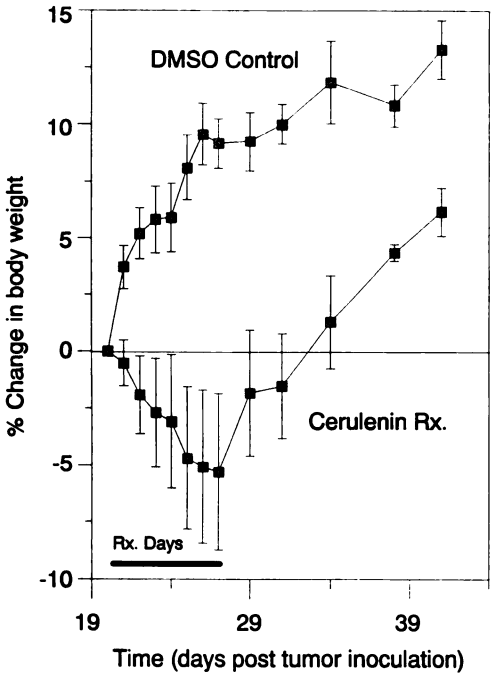

Fig. 3. Effect of cerulenin treatment on tumor-associated weight gain in the OVCAR-3 xenograft. Twelve nude mice were given injections of 0.1 ml (1.0×10^7) packed OVCAR-3 cells on day 0. Commencing on day 19, six mice received cerulenin, 80 mg/kg/day i.p. in 0.2 ml 25% DMSO for 7 days, or vehicle only. Mice were weighed daily, and the results were normalized to original body weight. *Bars*, SD. The *dark bar* on the *X*-axis indicates the treatment period. Cerulenin treatment causes significant regression in tumor ascites-associated weight gain.

mean ascites mass after 4 days of treatment. This effect was transient, however, because mice sacrificed 96 h after withdrawal of treatment had equivalent ascites volumes to controls. The reduction in ascites mass was due to a reduction in tumor cell mass rather than in cell-free fluid volume, since the mean ascites packed cell volume was between 67 and 73% in all groups; the SD was between 2.5 and 6.9%. There was no difference in solid tumor mass between the groups at either time point.

A significant delay in disease progression was achieved when cohorts of mice were treated with cerulenin early after inoculation with OVCAR-3 cells (analogous to treatment of ovarian cancer after optimal surgical debulking, Fig. 4). In the absence of significant ascites tumor burden, cerulenin produces systemic effects manifested as reduction in total body mass. The ultimate treatment effect was again greatest on the ascites tumor with solid tumor growth and distant metastasis relatively unaffected. Cerulenin-treated mice experienced a reversible mean weight loss of 25% of original body mass during the treatment period as the only observed side effect. In a parallel experiment using tumor-free nude mice, cerulenin treatment produced a 26% weight loss associated with 42% decreased fecal output (probably secondary to decreased food intake) and 36% reduction in fluid intake (data not shown). Urinalysis for glucose and ketones failed to demonstrate any diabetogenic effect of cerulenin. Mice regained inception body mass within 10 days following treatment. Cerulenin treatment resulted in a significant survival increase as demonstrated by life table analysis (Fig. 4*B*), Wilcoxon test ($P = 0.036$), and as a reduction in the relative risk of death, expressed as a Cox hazard ratio of 2.007, $P = 0.054$ (95% confidence interval, 0.97–4.11; Stata statistical software, Version 4.1). In addition, cerulenin treatment resulted in both a reduction in the relative risk of developing ascites (70% of controls *versus* 26% of treated mice, $P = 0.0161$, χ^2) and a delay in onset in those mice that did develop

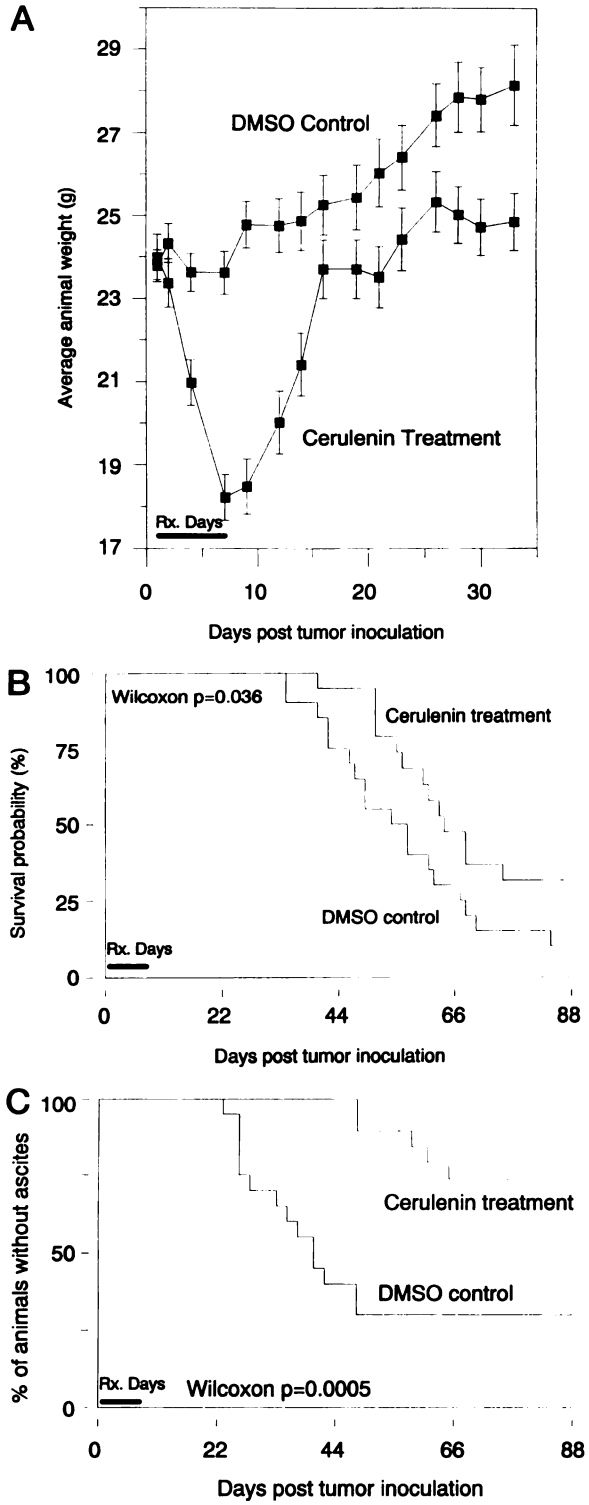

Fig. 4. *A*, effect of cerulenin treatment on disease progression in the OVCAR-3 xenograft. Groups of 20 mice received either 0.1 ml or 0.05 ml packed OVCAR-3 cells i.p. on day 0; since the difference in dose yielded no significant differences in subsequent growth, the groups were pooled for subsequent analysis. Beginning on day 1, animals were weighed, and 19 animals (one injection death) were treated with cerulenin, 80 mg/kg/day i.p. in 0.2 ml 25% DMSO, for 7 days *versus* vehicle only. Daily mean body weights were determined for treatment and control groups. Early weight loss in the treatment group reflects systemic toxicity of cerulenin. Late rapid weight gain in the control group reflects accumulation of malignant ascites. Graph is terminated at first death from disease. *B*, effect of cerulenin treatment on survival of the OVCAR-3 xenograft. Kaplan-Meier life table analysis. The experiment shows a highly significant delay in disease progression with cerulenin therapy ($P = 0.036$, Wilcoxon test). *C*, Kaplan-Meier analysis of ascites development following cerulenin treatment. Ascites development is defined as increase in body mass to 1.24 of original mass. Cerulenin treatment prevents the development of malignant ascites and delays its onset ($P = 0.0005$, Wilcoxon test).

ascites [Fig. 4*C*; Wilcoxon test, $P = 0.0005$ by life table analysis, Cox hazard ratio of 4.79, $P = 0.0013$ (95% confidence interval, 1.70–13.48)].

The data provide proof in principle that the metabolic pathway leading to *de novo* fatty acid synthesis is a selective target for antimetabolic chemotherapy of cancer. Selectivity is conferred by two major factors: (*a*) OVCAR-3 cells, like certain other human cancers, appear to be dependent on *de novo* fatty acid biosynthesis and therefore sensitive to fatty acid synthesis inhibition; and (*b*) normal tissues seem predominantly to utilize dietary lipids and therefore to be resistant to the cytotoxic effects of fatty acid synthesis inhibition (8).

Cerulenin is not a candidate drug in its current formulation due to bioavailability limitations; however, in this model, it demonstrates the feasibility of fatty acid synthesis inhibition as a selective anticancer therapy since it dramatically reduces disease locally. Furthermore, cerulenin inhibition of liver fatty acid synthesis occurred in tumor-free mice, therefore, systemic activity is readily achievable. Reformulation of cerulenin to improve its pharmacokinetics may increase its therapeutic index; however, new inhibitors of fatty acid synthesis developed with cerulenin as a structural guide for rational drug design may be required to achieve the maximum potential of this treatment strategy.

The ability to measure fatty acid synthase may afford additional benefits in a therapeutic setting. Since fatty acid synthase levels correspond to *de novo* fatty acid synthetic activity, diagnostic assessment of elevated fatty acid synthase expression may predict the sensitivity of an individual tumor to fatty acid synthesis inhibition, providing a coupled diagnostic and therapeutic system.

The fatty acid synthesis pathway may yield other potential therapeutic targets in addition to fatty acid synthase. For example, acetyl-CoA carboxylase, the rate-limiting enzyme of the pathway, is subject to complex regulation at the level of transcription, allosteric inhibition, and phosphorylation (18). As the biochemistry of fatty acid synthesis is further explored in cancer cells, acetyl-CoA carboxylase and its regulatory pathway may also be exploited therapeutically. Furthermore, as the functional roles of endogenously synthesized fatty acids in tumors are further clarified, additional targets may emerge.

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