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Inhibition of Fatty Acid Synthase Sensitizes Prostate Cancer Cells to Radiotherapy

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Many common human cancers, including colon, prostate and breast cancer, express high levels of fatty acid synthase compared to normal human tissues. This elevated expression is associated with protection against apoptosis, increased metastasis and poor prognosis. Inhibitors of fatty acid synthase, such as the cerulenin synthetic analog C75, decrease prostate cancer cell proliferation, increase apoptosis and decrease tumor growth in experimental models. Although radiotherapy is widely used in the treatment of prostate cancer patients, the risk of damage to neighboring normal organs limits the radiation dose that can be delivered. In this study, we examined the potential of fatty acid synthase inhibition to sensitize prostate cancer cells to radiotherapy. The efficacy of C75 alone or in combination with X irradiation was examined in monolayers and in multicellular tumor spheroids. Treatment with C75 alone decreased clonogenic survival, an effect that was abrogated by the antioxidant. C75 treatment also delayed spheroid growth in a concentration-dependent manner. The radiosensitizing effect of C75 was indicated by combination index values between 0.65 and 0.71 and the reduced surviving fraction of clonogens, in response to 2 Gy X irradiation, from 0.51 to 0.30 and 0.11 in the presence of 25 and 35 μ M C75, respectively. This increased sensitivity to radiation was reduced by the presence of the antioxidant. The C75 treatment also enhanced the spheroid growth delay induced by X irradiation in a supra-additive manner. The level of radiation-induced apoptosis in prostate cancer cells was increased further by C75, which induced cell cycle arrest in the G₂/M phase, but only at a concentration greater than that required for radiosensitization. Radiation-induced G₂/M blockade was not affected by C75 treatment. These results suggest the potential use of fatty acid synthase inhibition to enhance the efficacy of radiotherapy of prostate carcinoma and that C75-dependent cell cycle arrest is not responsible for its radiosensitizing effect. © 2015

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INTRODUCTION

Prostate cancer is the most commonly diagnosed malignancy in men and the second leading cause of cancer-related deaths in men in industrialized countries. Although radiation therapy is one of the most popular treatment options for clinically localized prostate cancer, resistance is common (1). Therefore, new therapeutic options are urgently required. The use of certain drugs that can enhance the sensitivity of prostate cancer cells to radiotherapy is a favorable strategy whereby the properties of cancer cells can be exploited for therapeutic gain.

Epidemiologic studies have suggested that systemic metabolic disorders, including obesity, metabolic syndrome and diabetes as well as hypercaloric and fat-rich diets, might increase the risk of prostate cancer (2). Increased *de novo* lipid synthesis is an early event in the development of prostate cancer (2) and correlates with unfavorable prognosis and poor survival. Furthermore, the expression and activity of lipogenic enzymes is upregulated by androgens, suggesting a role in androgen-sensitive prostate cancer (3). These observations indicate not only that malignancy is strongly associated with vigorous lipid metabolism but also that therapeutic benefit may be gained by targeting regulatory elements.

Fatty acid synthase (FASN) is the enzyme responsible for endogenous synthesis of saturated long-chain fatty acids from the precursors acetyl-CoA and malonyl-CoA. It plays a crucial role in energy homeostasis by converting excess dietary carbon intake into fatty acids for storage. Because most normal human tissues, except liver and adipose tissue, preferentially use circulating dietary fatty acids, FASN is expressed at low levels in these tissues. However, fatty acid synthesis occurs at very high rates in tumor tissues and FASN expression is elevated in many cancer cells, including both androgen-sensitive and -insensitive prostate adenocarcinoma (4–6). The increased synthesis of fatty acids in cancer cells is required for incorporation into membrane phospholipids and lipid signaling in continuously dividing cells. Increased expression of FASN also appears to protect cells against apoptosis (7) and is associated with

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proliferation and angiogenesis (8, 9), suggesting a role in aggressive, metastatic cancer. Stimulated FASN expression may be caused by increased activities of the phosphatidylinositol-3 kinase (PI3K) and MAP kinase signaling pathways (10), which are associated with prostate cancer progression (11). These signaling pathways can be activated by androgens and progesterone in prostate and breast cancer, respectively (2).

Since noncancerous cells, with the exception of a liver and adipose tissue, do not require FASN activity, FASN inhibitors should be active preferentially in cancer cells in which FASN is upregulated. Inhibition of FASN, either pharmacologically or by siRNA, has previously been demonstrated to decrease viability, proliferation and clonogenic survival and increase apoptosis of cancer cells, but not nonmalignant cells (7, 12–14). Moreover, FASN inhibition reduced the growth rate of prostate cancer in experimental animals (15). There are several reports of the toxicity of the FASN inhibitor cerulenin to cancer cell lines (6, 16–18). However, the promise of cerulenin as a cancer therapeutic is limited by its chemical instability and lack of systemic activity, leading to the development of C75 (α -methylene- β -butyrolactone), a synthetic analog that selectively inhibits FASN (16). Both cerulenin and C75 induce apoptosis in melanoma cells (17) and C75 has been shown to decrease the size of prostate cancer xenografts that overexpress FASN (7). Importantly, no adverse effect, with the exception of weight loss, was observed after treatment of experimental animals with C75 (6, 13, 19, 20). Weight loss was reversible and may have been caused by C75-induced decrease in lipid biosynthesis (21), increase in fatty acid oxidation (22) or appetite suppression (23). This suggests that FASN inhibitors have potential as anti-cancer agents, particularly for the treatment of prostate cancer, where overexpression of FASN is associated with tumor aggressiveness and poor prognosis (2).

It has been suggested that combination therapy of FASN inhibitors with other anti-cancer agents may enhance the chemosensitivity of breast and prostate cancer cells (19, 24, 25). However, the therapeutic application of FASN inhibition in combination with radiation treatment has not been subjected to experimental evaluation. Therefore, we aimed to assess the ability of C75 to sensitize prostate cancer cells to experimental radiotherapy and to determine the role of cell cycle redistribution in radiosensitization.

MATERIALS AND METHODS

Reagents

All cell culture media and supplements were purchased from Life Technologies (Paisley, UK), unless stated otherwise. All other chemicals, including C75, were from Sigma-Aldrich (Dorset, UK). Stock solutions of C75 were prepared in dimethyl sulfoxide (DMSO).

The maximum DMSO concentration in culture media was 0.1% (v/v). Control treatments contained DMSO alone in culture media.

Tissue Culture

Human prostate cancer cell lines, PC3 and LNCaP, were obtained from American Type Culture Collection (ATCC[®], Manassas, VA) and were used in this study for less than 6 months after resuscitation. PC3 cells were maintained in F12K media supplemented with 10% (v/v) fetal bovine serum (Autogen Bioclear, Wiltshire, UK), 2 mM L-glutamine, 0.1 mM sodium pyruvate and 50 μ g/ml gentamicin. LNCaP cells were maintained in RPMI 1640 media supplemented with 10% (v/v) fetal bovine serum (Hyclone, Fisher Scientific, UK), 1% (v/v) HEPES, 1% (v/v) D-glucose, 1 mM sodium pyruvate, 4 mM L-glutamine, 50 μ g/ml gentamicin.

MTT Toxicity Assay

MTT reduction was performed according to the published method of Mosmann (26). Cells were seeded in 96-well plates and incubated for 2 days to allow exponential phase growth. Cells were then washed twice with PBS and drug-containing media was added at the required concentration. After 24 or 48 h incubation, MTT was added to a final concentration of 0.5 mg/ml and cultures were incubated for 2 h. Cells were then solubilized with DMSO before measuring absorbance at 570 nm. Cell growth was also measured, using MTT assay, every 24 h up to 96 h.

Clonogenic Survival Assay

PC3 cells were seeded in 25 cm² flasks at 10⁵ cells/flask. When cultures were in exponential growth phase, media was removed and replaced with fresh drug-containing media. Cells were then incubated for 24 h at 37°C in 5% CO₂. To determine optimal sequencing of therapeutic agents, three different combination treatment schedules were assessed: 1. radiation and C75 administered simultaneously; 2. radiation administered 24 h before C75; and 3. radiation administered 24 h after C75. After treatment, cells were seeded for clonogenic survival assay as previously described elsewhere (27, 28). Cells were incubated at 37°C in 5% CO₂ for 10 days. Colonies were fixed in methanol, stained with crystal violet solution and those colonies of at least 50 cells were counted. To assess the role of reactive oxygen species (ROS) in C75-induced cytotoxicity, cells were co-incubated with the antioxidant N-acetyl-L-cysteine (NAC, 1 mM).

Cells were irradiated using an Xstrahl RS225 X-ray irradiator at a dose rate of 1.6 Gy/min. The cytotoxic interaction of C75 and X radiation *in vitro* was assessed according to the method of Chou and Talalay (29), which is based on the median-effect principle. Briefly, clonogenic assay was performed using a fixed dose ratio of drug to radiation, based on the concentrations required to kill 50% of clonogens (IC₅₀) of each single agent, so that the proportional contribution of each agent in the mixtures was the same at all treatment intensities. The effectiveness of combinations was quantified by calculating a combination index (CI) at various levels of cytotoxicity: CI < 1, CI = 1 and CI > 1 indicate synergism, additivity and antagonism, respectively.

Multicellular Spheroid Growth Assay

Multicellular tumor spheroids consisting of LNCaP cells were obtained using the liquid overlay technique (30). Spheroids were initiated by inoculating cells into a plastic flask coated with 1% (w/v) agar. After 3 days, aliquots of spheroids were transferred to sterile plastic tubes and centrifuged at 12g for 3 min. Thereafter, spheroids were irradiated or resuspended in serum-free culture media containing C75. After treatment, the spheroids were washed twice and those of approximately 100 μ m in diameter were transferred individually into agar-coated wells of 24-well plates, as

previously described (31). Individual spheroid growth was monitored twice weekly for three weeks using an inverted phase-contrast microscope connected to an image acquisition system. Two perpendicular diameters, d_{\max} and d_{\min} , were measured using image analysis software (ImageJ Software, NIH, Bethesda, MD) and the volume, V (μm^3), was calculated using the formula: $V = \pi \times d_{\max} \times d_{\min}^2/6$ (31). The area under the V/V_0 versus time curve (AUC) was calculated for individual spheroids using trapezoidal approximation.

Wound-Healing Assay

The “scratch assay” was used to determine the proliferation and migration of cells after exposure to C75. After serum starvation overnight (0.5% FCS), confluent layers of LNCaP or PC3 cells in 24-well plates were scratched using a sterile pipette (32). After injury, monolayers were gently washed with PBS and incubated in the absence or presence of C75. Quadruplicate wells were photographed using 10 \times magnification every 24 h after induction of injury. Remodeling was measured as diminishing distance across the induced injury (5 measurements per image), normalized to the 0 h control, expressed as a percentage of initial wound size.

Apoptosis Assay

The effect of C75, X radiation and combination treatments on PC3 and LNCaP prostate carcinoma cell lines on induction of apoptosis was assessed using a FITC Annexin V Apoptosis Detection Kit (BD Biosciences, Oxford, UK) according to the manufacturer’s instructions. Briefly, 1×10^6 cells were treated for 24 h, then cells were washed and resuspended in Annexin V binding buffer containing Annexin V-FITC and propidium iodide (PI) in the dark before analysis by flow cytometry using a FACSVerse™ analyzer (BD Biosciences, Oxford, UK). Data were analyzed using FlowJo software. Cells were designated viable (Annexin V⁻, PI⁻), early apoptotic (Annexin V⁺, PI⁻), damaged (Annexin V⁻, PI⁺) and late apoptotic (Annexin V⁺, PI⁺).

Cell Cycle Analysis

After C75 treatment for 3, 6 and 24 h, LNCaP and PC3 cells were trypsinized, then washed twice with PBS. Cells were fixed by treatment with ice-cold 70% (v/v) ethanol; then washed twice with PBS and resuspended in PBS containing propidium iodide (10 $\mu\text{g}/\text{ml}$) and RNase A (200 $\mu\text{g}/\text{ml}$). Cells were stained for 30 min before flow cytometric analysis.

Statistical Analysis

Data are shown as means \pm standard error of the mean (SEM), with the number of independent repetitions provided in the legend to each figure. Statistical significance was determined using Student’s *t* test. A *P* value of <0.05 was considered to be statistically significant and $P < 0.01$ was highly significant. To test for differences in spheroid growth among experimental therapy groups, the Kruskal-Wallis test was used with post hoc testing by the Mann-Whitney U test with Bonferroni correction. Analysis was performed using SPSS® software (IBM Corp., Armonk, New York).

RESULTS

C75 is Toxic to Prostate Cancer Cells

When administered as a single agent at doses greater than 10 μM , C75 reduced the viability of both PC3 and LNCaP cells in a concentration-dependent manner,

according to MTT assay (Fig. 1A and B). Similar susceptibility of both cell lines to C75 treatment was observed. Toxicity was further assessed by clonogenic assay of PC3 cells (LNCaP cells did not form colonies) and spheroid growth assay of LNCaP cells (PC3 cells did not form spheroids). The clonogenic survival of PC3 cells was reduced by treatment with C75 concentrations ≥ 25 μM (Fig. 1C). The IC_{50} value corresponding to 24 h treatment was 35 μM . Treatment with C75 alone, in the dose range 10–50 μM , also reduced the growth of LNCaP spheroids (Fig. 1D) in a concentration-dependent manner. According to the spheroid volume at the completion of the experiment, the IC_{50} value was 50 μM . Spheroids were sterilized by treatment with 100 μM C75.

C75 Reduced Cell Migration

The effect of C75 on growth and migration of LNCaP cells is shown in Fig. 2. Concentrations of C75 ≥ 35 μM reduced both cell proliferation and migration at all time points throughout the 96 h measurement period. However, although lower concentrations of C75 (5 and 25 μM) failed to inhibit growth, these concentrations significantly reduced the closure of scratches in a wound-healing model (Fig. 2B and C), indicating restraint upon cell migration. Inhibition of growth of PC3 cells was demonstrated at a range of concentrations of C75 (Fig. 2D). For PC3 cells, complete wound closure in untreated wells was observed at 24 h. Therefore, the results of C75-induced reduction in migration of PC3 cells are displayed as wound closure at 24 h (Fig. 2E). As was observed after treatment of LNCaP cells with C75, the inhibition of migration of PC3 cells occurred at lower concentrations than those required for growth inhibition.

C75 Enhanced Radiation-Induced Clonogenic Kill

Administration of C75 (35 μM) combined with 2 Gy X irradiation increased the clonogenic kill of PC3 cells induced by either agent alone, regardless of administration schedule (i.e., simultaneous, C75 treatment 24 h before and 24 h after X irradiation). However, the greatest enhancement was observed after simultaneous administration of treatments (Fig. 3A). Representative combination index values for simultaneous treatment of PC3 cells with C75 and X radiation are shown in Table 1. These indicated that supra-additive clonogenic kill (manifest as $\text{CI} < 1$) was observed at all toxicity levels. The decreased clonogenic capacity of PC3 cells resulting from X irradiation was enhanced by treatment with C75 at 25 and 35 μM (Fig. 3B). The IC_{50} values obtained after exposure of PC3 cells to X radiation alone, or in the presence of 25 and 35 μM C75 were 1.93 ± 0.08 , 1.34 ± 0.11 and 1.17 ± 0.10 Gy, respectively. The surviving fractions after 2 Gy irradiation were 0.51 ± 0.01 , 0.30 ± 0.02 and 0.11 ± 0.02 for 0, 25 and 35 μM C75, respectively. The calculated dose enhancement ratios at

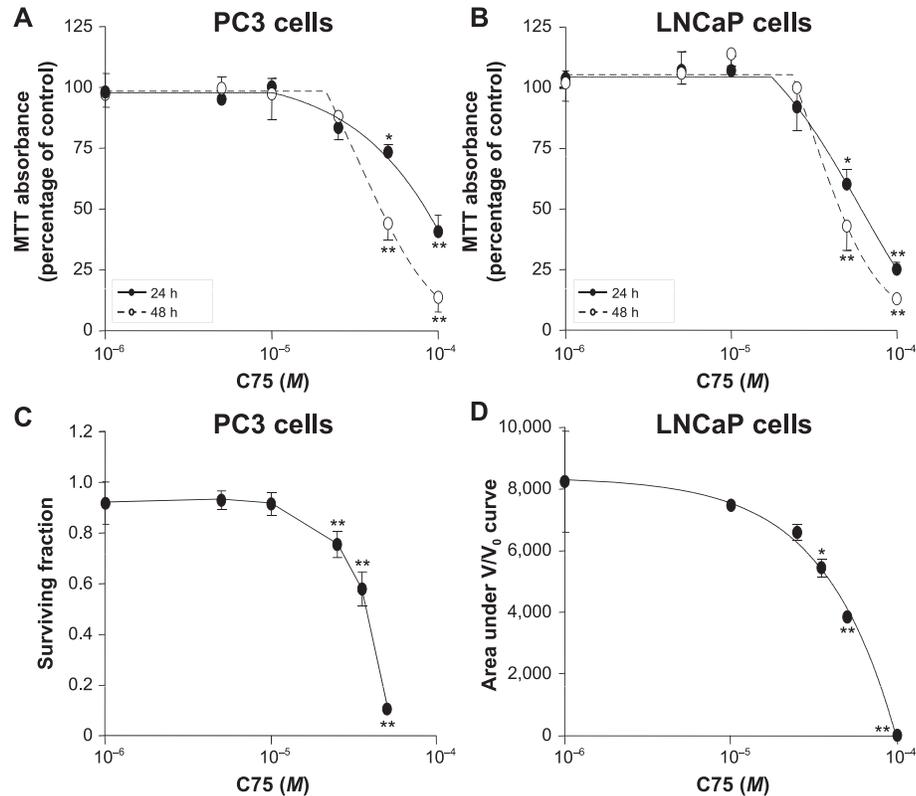


FIG. 1. Toxicity of C75 to prostate cancer cell lines. PC3 (panel A) and LNCaP (panel B) cells were exposed to C75 for 24 and 48 h before cytotoxicity was assessed using MTT assay. Panel C: The ability of C75 to reduce clonogenic survival of PC3 cells was assessed. Panel D: The growth of LNCaP spheroids after 24 h exposure to C75 was determined and is expressed as area under the V/V_0 versus time curve. Data are means \pm SEM, $n = 3$. * $P < 0.05$ and ** $P < 0.01$ compared to untreated controls.

50% clonogenic kill (DER_{50}) were 1.49 ± 0.17 and 2.40 ± 0.28 for 25 and 35 μM C75, respectively. These observations indicate concentration-dependent radiosensitization by C75.

C75 Enhanced the Spheroid Growth Delay Induced by Radiation Exposure

The growth of multicellular spheroids composed of LNCaP cells was delayed in a concentration-dependent manner by exposure to radiation (Fig. 4A). The effect of simultaneous X irradiation and C75 treatment at various concentrations (25, 35 and 50 μM) is shown in Fig. 4B–D. Compared with untreated controls, the rate of increase of spheroid volume was reduced by treatment with C75 as a single agent. Furthermore, the growth inhibitory effect of ionizing radiation was enhanced by a combination of C75 treatment at all concentrations examined.

Growth delay, expressed as the time taken to increase spheroid volume tenfold (τ_{10}) was increased by both C75 and X irradiation as single modalities. However, the growth delay induced by simultaneous combination therapy was significantly greater than the growth inhibitory potency of either agent alone (Table 2). Similarly, AUC values were significantly decreased in a supra-additive manner by

combination treatment (Table 2), reflecting enhanced delay of spheroid growth.

Apoptosis Induction by Radiation Exposure was Enhanced by C75 Treatment

The percentage of apoptotic (early and late) PC3 and LNCaP cells 24 h after 2 Gy X irradiation, 50 μM C75 treatment or a combination of both, is shown in Fig. 5. In both cell lines, apoptosis was increased by 2 Gy exposure or C75 administered as single treatments. Simultaneous exposure to both treatments resulted in an increase in apoptosis to a frequency significantly greater than that induced by either agent alone.

Effect of Antioxidant on Radiosensitizing Ability of C75

The kill of PC3 clonogens treated with C75 was prevented by co-incubation with the antioxidant NAC (Fig. 6), whereas the radiation-induced clonogenic kill was not affected by NAC. The lethality of the combination treatment (C75 + X radiation) was reduced by the addition of NAC. However, the surviving fraction after treatment with radiation, C75 and NAC was significantly lower than that of treatment with radiation alone ($P < 0.05$).

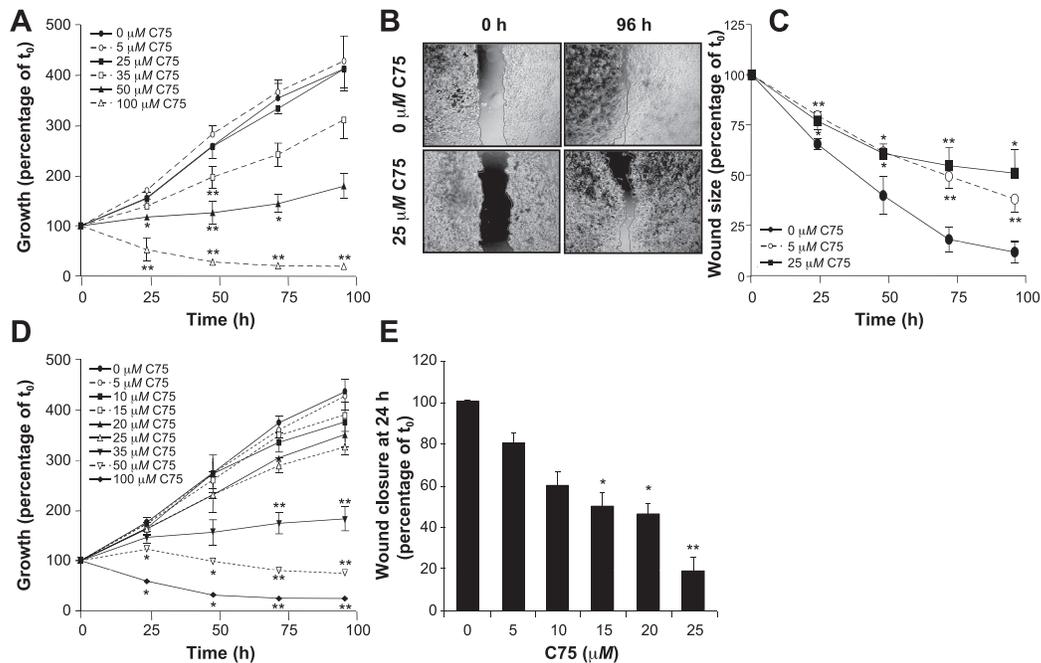


FIG. 2. Panel A: The proliferation of LNCaP cells exposed to C75 for up to 96 h was assessed using MTT assay. Panel B: Representative images of wound-healing assay of LNCaP cells. Wound size was measured in five locations for each photograph. Panel C: The migration of LNCaP cells using wound-healing assay to measure the closure of a scratch in confluent layers of cells after incubation with C75. Panel D: The proliferation of PC3 cells exposed to C75 for up to 96 h was assessed using MTT assay. Panel E: The migration of PC3 cells was assessed using wound-healing assay and results show migration 24 h after incubation with C75. Data are means \pm SEM, n = 3. * P < 0.05 and ** P < 0.01 compared to untreated controls at same time points.

Effect of C75 and Ionizing Radiation on Cell Cycle Progression

The effect of a range of concentrations of C75 and 2 Gy irradiation on the cell cycle distribution of asynchronously growing PC3 and LNCaP cells was determined after

treatment for 3, 6 and 24 h (Fig. 7). Neither C75 nor radiation exposure alone had any effect on cell cycle progression 3 h after the initiation of treatment (data not shown). Cycle progression of neither cell line was significantly affected by C75 treatment for 3 and 6 h. Although C75 caused a significant increase in the G₂/M

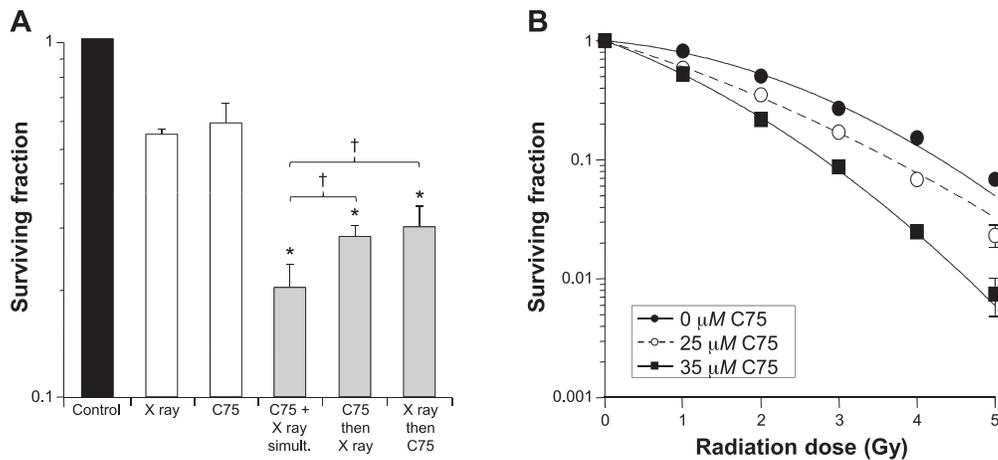


FIG. 3. The effect of C75 on radiation-induced kill. Panel A: The effect of administration schedule of the combination of C75 (35 μ M) and X radiation on the kill of PC3 clonogens was tested using three administration schedules: 1. radiation and drug administered simultaneously; 2. radiation administered 24 h before drug; and 3. radiation administered 24 h after drug. * P < 0.05 compared to single agents, † P < 0.05 compared to simultaneous administration. Panel B: Radiation kill curves of PC3 cells exposed to C75 (25 and 35 μ M) and X radiation at a range of doses, administered simultaneously. Data are means \pm SEM, n = 3.

TABLE 1
The Effect of Treatment of PC3 Cells with C75 and X Radiation on Combination Indices

Effect level	CI value
ED ₃₀	0.71 ± 0.15
ED ₄₀	0.70 ± 0.13
ED ₅₀	0.69 ± 0.12
ED ₇₀	0.67 ± 0.10
ED ₉₀	0.65 ± 0.08

Notes. CI values are mean ± SEM of 3 experiments. ED_x = dose required to kill x% of clonogens.

population of PC3 cells after 24 h, this occurred only after treatment with 50 μM C75 and not with lower concentrations (Fig. 7A). In contrast, increased accumulation in G₁ phase was observed after 24 h exposure of LNCaP cells to C75 (Fig. 7A).

In both cell lines, radiation treatment alone (1–4 Gy) caused a rise in the G₂/M population 6 h after irradiation (Fig. 7B). However, 24 h after irradiation of PC3 cells the G₂/M population returned to control levels for all radiation

doses except 4 Gy, where arrest of the G₂/M phase persisted. In the case of LNCaP cells, radiation doses <4 Gy caused an increase in the G₁ phase and a decrease in G₂/M phase 24 h after irradiation (Fig. 7B). When administered concurrently with radiation, C75 did not affect the radiation-induced increase in G₂/M observed at 6 h (Fig. 7C and D). Similarly, 24 h after administration, the combination treatment did not significantly alter the effect on the cell cycle of each agent alone (data not shown).

DISCUSSION

A characteristic of cancer cells is increased glucose consumption and lactate production, even in the presence of oxygen (aerobic glycolysis or the Warburg effect). However, glycolytic metabolism is less prevalent in prostate cancer than in the majority of other solid cancers (2). Furthermore, increased *de novo* lipid synthesis and stimulation of the activity of lipogenic enzymes is a feature of prostate cancer (2, 33). This upregulation is controlled by androgens and, crucially, persists or re-emerges with development of

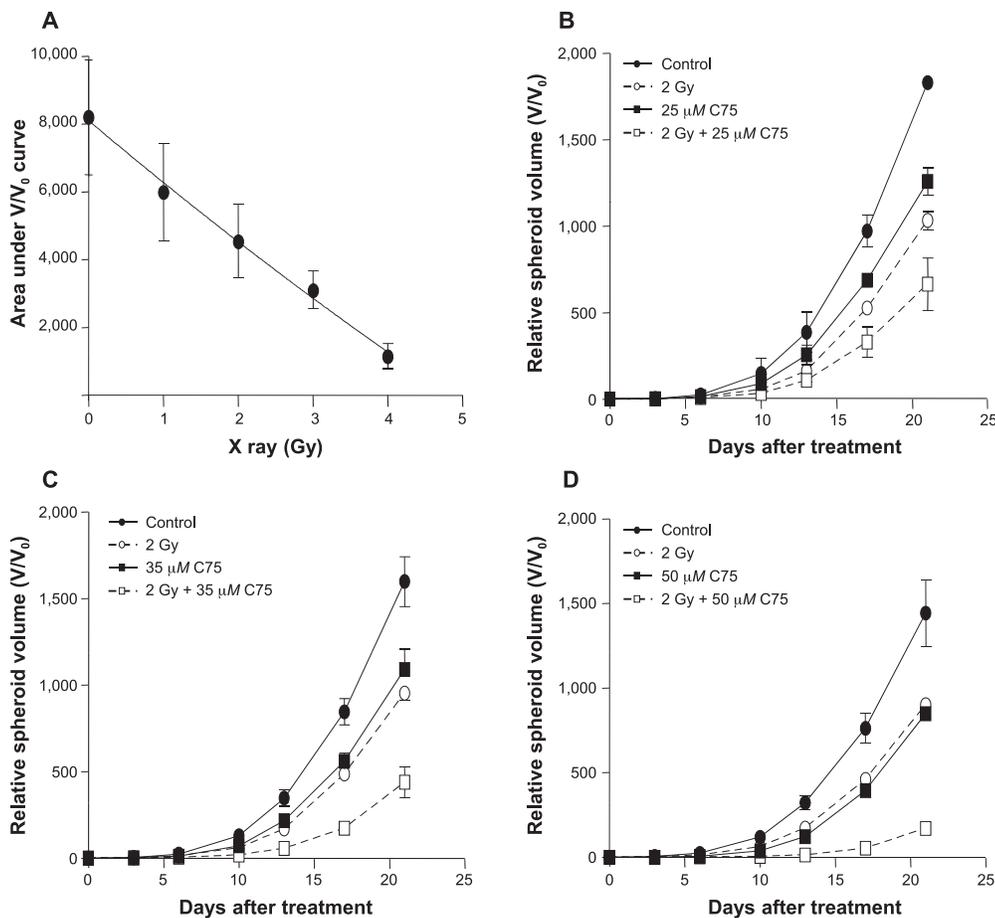


FIG. 4. The effect of radiation and C75, alone or in combination, on the growth of LNCaP spheroids. Spheroids composed of LNCaP cells were exposed to X radiation (panel A) or combinations of X radiation and C75 (panels B–D) and 24 h later were transferred to agar-coated plates. Each spheroid was then photographed twice per week and change in spheroid volume (V/V_0) was measured up to 21 days. Data are means ± SEM, $n = 3$.

TABLE 2
Comparison of the Effect of Single Agent Treatment
with Combination Treatment on the Growth of
LNCaP Spheroids

Treatment	τ_{10} (days)	AUC
Control	5.1 \pm 0.3	8491 \pm 58
2 Gy X irradiation	5.7 \pm 0.5	4523 \pm 243
25 μ M C75	5.5 \pm 0.8	6599 \pm 246
2 Gy X irradiation + 25 μ M C75	6.6 \pm 0.5	3224 \pm 838**
35 μ M C75	6.4 \pm 0.3	5453 \pm 297
2 Gy X irradiation + 35 μ M C75	8.4 \pm 0.6 [†]	1869 \pm 511****
50 μ M C75	7.3 \pm 0.2	3854 \pm 140
2 Gy X irradiation + 50 μ M C75	11.3 \pm 0.5****	637 \pm 81****

Notes. Data are expressed as the time taken to increase spheroid volume tenfold (τ_{10}) and the area under the volume-time curve (AUC). Values are means \pm SEM of three separate experiments. *C75 compared with X radiation plus C75. [†]X radiation compared with X radiation plus C75. */[†] $P < 0.05$, **/^{††} $P < 0.01$.

androgen-independent cancer (34), hence drugs inhibiting the dysregulated lipogenesis observed in prostate cancer are currently being assessed (33).

Changes in fatty acid metabolism in tumor cells can be driven by dysregulated intracellular signaling in response to growth factors (10). The resultant elevated expression and activity of FASN, and increased biosynthesis of fatty acids, are characteristic of several cancers, particularly those of prostate and breast (35, 36), whereas low levels of FASN are observed in most normal tissues. Increased levels of endogenously synthesized fatty acids enable membrane biosynthesis during proliferation and activate intracellular, autocrine and paracrine signaling pathways associated with growth and survival. Consequences of elevated FASN may

be survival advantage due to resistance to apoptosis, tumor aggressiveness, increased metastasis and poor prognosis (7). It is possible that these effects can be overcome by pharmacological inhibitors of FASN. Therapeutic selectivity is expected due to the low activity of FASN in most noncancerous cells.

The results of the current investigation indicate that two prostate cancer cell lines (LNCaP and PC3), which express high levels of FASN (17), succumbed to treatment with the FASN inhibitor C75. This was manifest by reduced proliferation and migration, decreased clonogenic capacity and delayed growth of multicellular tumor spheroids in a concentration-dependent manner. Previously, the efficacy of C75, as a single agent, has been indicated *in vitro* (13, 15–18, 37, 38) and *in vivo* (6, 13, 19, 20, 39). FASN activity in both PC3 and LNCaP cells was previously shown to be decreased by 30% with 30 μ M C75 treatment (40). Moreover, 30 μ M C75 has been reported to decrease proliferation in these cell lines (15, 18, 37). Protein levels of FASN protein levels were, however, unaffected by C75 (37). Although FASN expression and activity in prostate tumors are elevated by androgens, they are also increased during the development of androgen-independent malignant disease (6), indicating that FASN inhibition may be clinically useful even during progression of hormone-refractory prostate cancer. This notion is supported by the cytotoxicity of C75 to both LNCaP and PC3 cells, which are androgen-dependent and -independent, respectively.

Migita *et al.* (7) demonstrated that FASN is overexpressed in prostate intraepithelial neoplasia compared with adjacent normal tissue, indicating that it plays a role in the initial phases of prostate tumorigenesis, and in metastatic

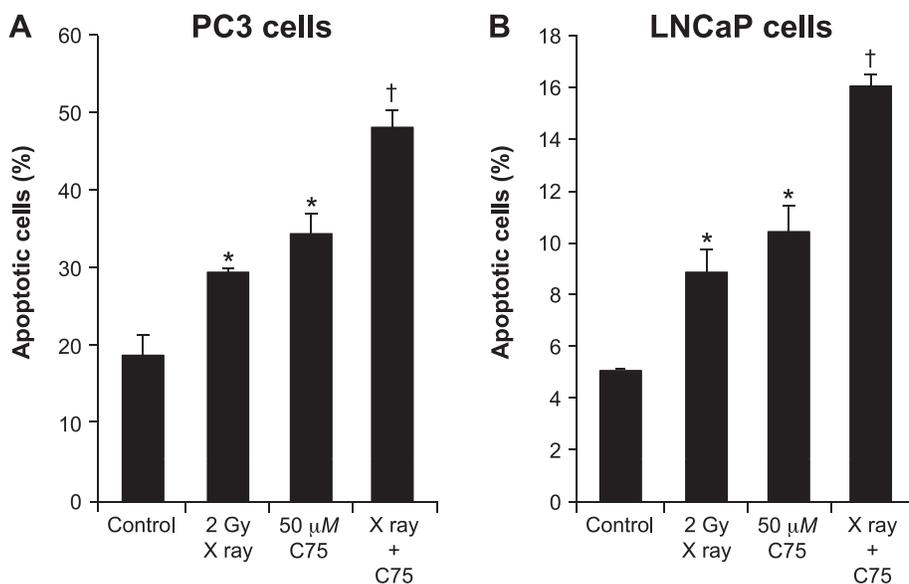


FIG. 5. Induction of apoptosis by exposure to radiation and C75. PC3 (panel A) and LNCaP (panel B) cells were 2 Gy X irradiated and treated with 50 μ M C75 or a combination of both for 24 h, and apoptosis was analyzed by co-staining with Annexin V-FITC and propidium iodide. Graphs show early (Annexin V-positive and PI-negative) and late (Annexin V-positive and PI-positive) apoptotic cells. Data are means \pm SEM, n = 3. * $P < 0.05$ compared to untreated controls; [†] $P < 0.05$ compared to radiation exposure alone and C75 alone.

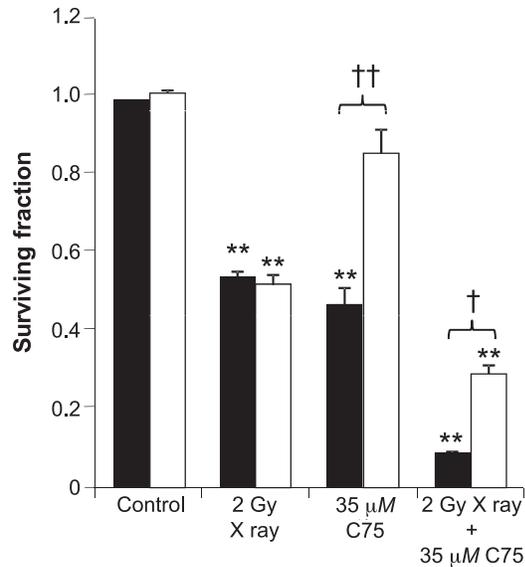


FIG. 6. Clonogenic assay of PC3 cells performed in the absence (black bars) and presence (white bars) of the antioxidant NAC (1 mM), which was co-incubated with treated cells for 24 h prior to clonogenic assay. Data are means \pm SEM, $n = 3$. ** $P < 0.01$ compared to untreated controls; † $P < 0.05$ and †† $P < 0.01$ compared to no NAC.

prostate cancer, where it is associated with aggressiveness. It has been suggested that inhibition of FASN may prevent or inhibit metastasis in colorectal cancer (41) and the FASN inhibitor orlistat was shown to decrease angiogenesis and metastases in experimental models (42, 43). We tested the ability of C75 to inhibit migration of prostate cancer cells. Using concentrations that elicited no anti-proliferative effect, the ability of cells to repair a scratch injury was significantly reduced, indicating that C75 impaired cellular migration. C75-induced decrease in migration may be one mechanism whereby this drug reduces the metastatic capacity of prostate cancer cells. As metastasis is a hallmark of advanced prostate cancer, this suggests that C75 may reduce aggressiveness and the associated poor clinical outcome.

In agreement with previous reports (17, 19, 38, 44), we observed that C75 induced apoptosis in cancer cells. The accumulation of the pro-apoptotic FASN substrate malonyl Co-A is thought to be responsible for the cytotoxic effect of C75 (19), whereas growth inhibition may be caused by insufficiency of lipid products of FASN, such as phospholipids required for incorporation into cellular membranes

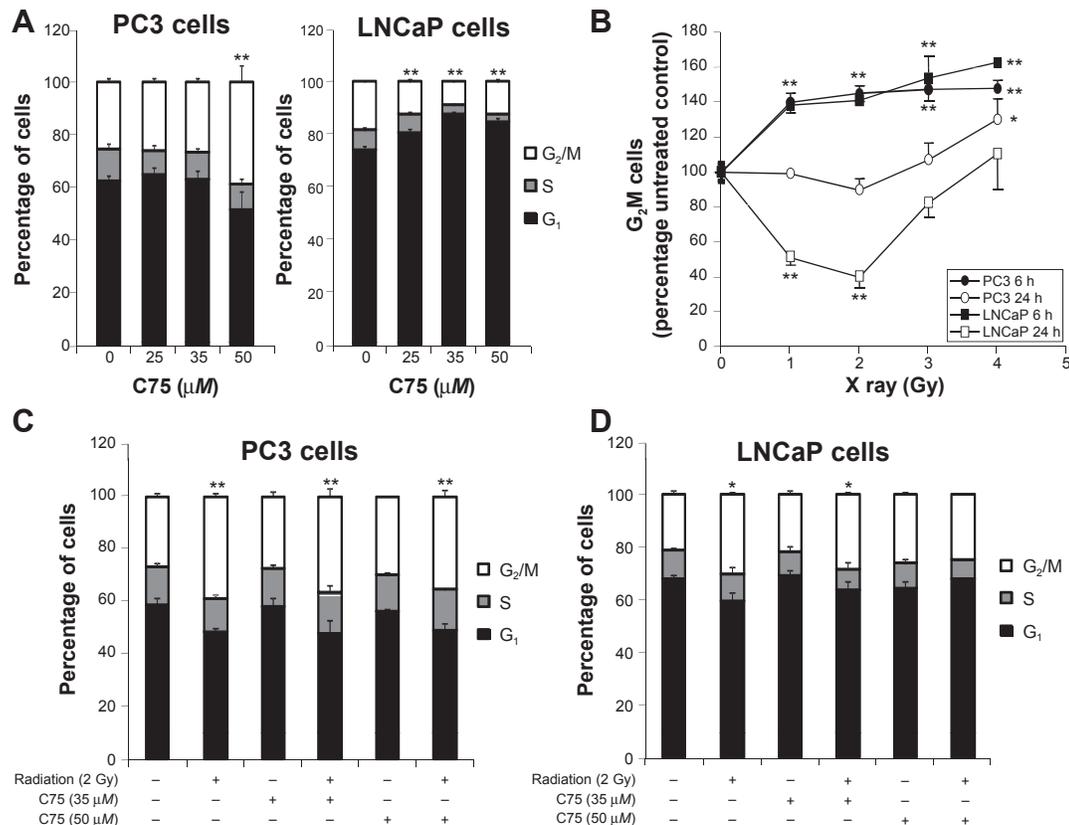


FIG. 7. Cell cycle analysis after treatment of cells with radiation and C75, alone or in combination. Propidium iodide-stained cells were analyzed using flow cytometry to determine cell cycle distribution. Panel A: Cell cycle distribution of PC3 and LNCaP cells treated with C75 for 24 h before fixing and staining. Panel B: PC3 and LNCaP cells in G₂/M phase of cell cycle are shown 6 and 24 h after exposure to X radiation. Cell cycle distribution in PC3 (panel C) and LNCaP (panel D) cells 6 h after simultaneous treatment with radiation and C75. Data are means \pm SEM, $n = 3$. * $P < 0.05$ and ** $P < 0.01$ compared to untreated controls at the same time points.

during proliferation (44, 45). Inhibition of FASN by C75 treatment has also been reported to induce, in tumor cells but not in normal cells, an endoplasmic reticulum stress response, manifest as increased expression of endoplasmic reticulum stress-regulated genes, leading to cell death (46). Endoplasmic reticulum stress also triggers oxidative stress through generation of ROS. The abrogation of C75-induced clonogenic kill in the presence of antioxidant NAC in this study indicated that elevated ROS levels may be partly responsible for the cytotoxicity of C75. It has also been recently reported that the apoptotic effect of C75 in liver carcinoma cells is caused by increased ROS generation (47), suggesting that the activation of apoptotic pathways by C75 may occur via ROS-induced oxidative stress in addition to accumulation of malonyl Co-A.

FASN inhibitors have been shown to sensitize cancer cells to other chemotherapeutic agents (18, 24, 25). However, the potential of FASN inhibitors to radiosensitize tumors has not been evaluated. Radiotherapy is an important element in the clinical management of prostate cancer and its efficacy is expected to be enhanced when combined with radiosensitizing agents (48). We demonstrated that the radiation-induced decrease in the surviving fraction of PC3 clonogens was enhanced when C75 was administered in combination with X irradiation. Although all schedules of administration of combined therapeutic modalities (simultaneous and 24 h apart) were more effective than single agent treatments, the greatest enhancement of clonogenic kill was observed when simultaneous administration of ionizing radiation and C75 was applied. Therefore, this schedule was utilized in subsequent experiments.

It has been suggested that the FASN inhibitor cerulenin did not increase the sensitivity of glioma cells to radiation (14). However, in the previous study, the MTT assay was used to measure cell viability. This procedure does not allow one to distinguish between clonogens and cells that have sustained sufficient injury to restrict their ability to undergo cell division. In contrast, in the current study, clonogenic survival was assayed, enabling the determination of the toxicity of experimental therapy to cells with unlimited capacity for proliferation. Clonogenic assay is considered the gold standard for *in vitro* preclinical studies of radiosensitizers (49, 50). Clonogenic assay data were used to calculate combination indices and dose enhancement ratios as recommended by Alcorn *et al.* (48). Combination index values less than 1 indicate synergism; in this study, values were ≤ 0.71 at all levels of toxicity examined. Dose enhancement ratios observed were ≥ 1.49 , indicating sensitization. Furthermore, the spheroid growth delay assay used in this study provided a more reliable estimate of the response of prevascular metastases to experimental therapy than two-dimensional cell culture models (31). Using this methodology, C75 was observed to sensitize prostate cancer cells to radiation. C75 also

increased the pro-apoptotic effect of X radiation in the two prostate cancer cell lines.

The observed radiosensitizing activity may be partially explained by the generation of ROS, since antioxidant treatment was able to overcome the cytotoxicity of C75. However, we observed that the C75-induced generation of ROS was not entirely responsible for its radiosensitizing effect, exemplified by partial abrogation of sensitization induced by the antioxidant NAC. As NAC primarily scavenges hydrogen peroxide, it does not preclude other ROS contributing to the sensitization. This suggests the involvement of alternative modes of action, possibly involving disruption of the cell cycle, thereby influencing sensitivity to radiation (51).

FASN inhibition by C75 *in vitro* does not result directly in DNA damage (52). However, C75 has been shown to alter cell cycle distribution of tumor cells. For example, there are reports of C75-induced G₁-phase arrest in LNCaP prostate cancer cells (15) and Hep3B hepatocarcinoma cells (53), S-phase arrest in MCF-7 breast cancer cells (36), G₂/M-phase arrest in A375 melanoma cells (17), HepG2 and SMMC7721 hepatocarcinoma cells (53). We demonstrate here that C75 was able to induce an increase in the G₂/M population of PC3 cells. This may be caused by upregulation of p21 and p38 MAPK activation (17, 53). However, G₂/M arrest was observed only in PC3 cells treated with the highest experimental concentration of C75 (50 μ M). Lower concentrations of C75 (25 and 35 μ M) had no effect on the cell cycle distribution of PC3 cells, despite their being sufficient to sensitize to radiation.

In LNCaP cells, G₁ arrest was observed 24 h after C75 treatment, as also reported by Chen *et al.* (15), using similar concentrations and incubation times. Radiation exposure also caused an increase in the G₁ phase of the cell cycle 24 h after exposure of LNCaP cells, whereas no increase in G₁ was observed in PC3 cells. The observed differences in cell cycle redistribution among cell types in response to radiation exposure and C75 may be due to their respective p53 status. C75-induced accumulation of p53 in LNCaP cells [characterized by wild-type p53 (54)] may be expected to induce cell cycle arrest at G₁, whereas PC3 cells [harboring nonfunctional p53 (54)] arrest in G₂/M, which can be modulated by p38 MAPK (53). The inconsistent role of p53 in C75-induced cell cycle arrest is further suggested by reports of p53 accumulation in one study (52) but not in another (53), despite both cell lines' expressing wt p53. This may be a result of cell-specific alternative cell cycle regulatory pathways.

Cells in the G₂/M phase of the cell cycle are more sensitive to radiation than during other phases (51), suggesting that cell cycle redistribution induced by C75 treatment could contribute to the enhanced efficacy of radiation in PC3 cells. However, C75 did not induce G₂/M block in LNCaP cells, but nevertheless radiosensitized these cells. In contrast, induction of S-phase arrest by cerulenin may account for its lack of radiosensitizing effect (14) as

cells in S phase are relatively radioresistant (51). C75-induced G₂/M block in PC3 cells indicated that the scheduling of administration of the C75/radiation combination would influence efficacy. While it may be anticipated that C75 would be more likely to radiosensitize when administered 24 h before radiation, this was not observed. In contrast, simultaneous administration had a greater effect than pre-exposure to C75, indicating that cell cycle arrest is not necessary for the sensitizing effect of C75.

Compared with clonogenic cell kill in monolayer cultures, a higher concentration of C75 was required to attain 50% inhibition of spheroid growth. The IC₅₀ values were 35 and 50 μ M C75 in clonogenic assay and spheroid growth assay, respectively. Although cell line-dependent effects may play a role in the observed differences, these are difficult to confirm here due to the lack of colony-forming ability of LNCaP cells and spheroid-forming ability of PC3 cells. Similar concentration dependency was, however, observed in MTT assay of the two cell lines. The relative resistance of multicellular spheroids is commonly observed and is most likely due to differences in drug penetration and the microenvironment of the various layers within the spheroid (55). However, we have demonstrated that the same concentration of C75 (35 μ M) was able to induce radiosensitivity in both two- and three-dimensional models and that G₂/M cell cycle arrest was not observed at this concentration, indicating that cell cycle alteration by C75 is not the major mechanism of radiosensitization.

Alternative mediators of C75-induced radiosensitization may include intracellular signaling pathways. These include the PI3K/Akt signaling pathway, which may contribute to radioresistance (56). Downregulation of this pathway by C75 resulted in apoptosis (21). Alternatively, AMP-activated protein kinase (AMPK) may be activated by C75, resulting in inhibition of lipogenic pathways, including FASN, and decreased proliferation of cancer cells (37). Activators of this pathway may also lead to radiosensitization (57).

As demonstrated here and in previous studies, the fatty acid synthase inhibitor C75 has potential as an anti-cancer drug. We have demonstrated that, in both androgen-dependent and -independent prostate cancer cell lines, C75 is able to act as a single agent to decrease cell proliferation and migration as well as to induce apoptosis, clonogenic kill and tumor growth delay. Moreover, C75 is able to enhance the sensitivity of prostate cancer cells to experimental radiotherapy and this is unlikely to be caused by cell cycle redistribution. Although it has not been assessed in this study, FASN concentration is considered to be very low in noncancerous cells, and C75 has previously been shown to have little or no toxic effect on noncancerous tissue (12, 13). Therefore, C75 would not be expected to enhance radiation-induced kill. Nonetheless it is recommended that C75 treatment in combination with radiotherapy be assessed in experimental animals before proceeding to clinical evaluation. It may also be possible to increase the

specificity of radiotherapy by using radiopharmaceuticals that selectively target upregulated cell surface molecules, such as prostate-specific membrane antigen (PSMA), which is an attractive target for prostate cancer imaging and therapy (58). This strategy has already been utilized in preclinical studies (59) and in a small-scale clinical trial (60), with encouraging results, and warrants further investigation in combination with radiosensitizers such as C75.

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