

Inhibiting Fatty Acid Synthase for Chemoprevention of Chemically Induced Lung Tumors

Hajime Orita, Jonathan Coulter, Ellen Tully, Francis P. Kuhajda, and Edward Gabrielson

Abstract Purpose: Fatty acid synthase (FAS) is overexpressed in lung cancer, and we have investigated the potential use of FAS inhibitors for chemoprevention of lung cancer.

Experimental Design: Expression of FAS was evaluated in preinvasive human lung lesions (bronchial squamous dysplasia and atypical adenomatous hyperplasia) and in murine models of lung tumorigenesis [4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone – induced and urethane-induced lung tumors in A/J mice]. Then, the ability of pharmacologic inhibitors of FAS to prevent development of the murine tumors was investigated. Finally, the effect of the FAS inhibitor treatment of levels of phosphorylated Akt in the murine tumors was evaluated by immunohistochemistry.

Results: Immunohistochemical studies show that human bronchial dysplasia and atypical adenomatous hyperplasia express high levels of FAS compared with normal lung tissues, suggesting that FAS might be a target for intervention in lung carcinogenesis. FAS is also expressed at high levels in chemically induced murine lung tumors, and the numbers and sizes of those murine tumors are significantly reduced by treating carcinogen-exposed mice with pharmacologic inhibitors of FAS, C75 and C93. C93 treatment is associated with reduced levels of phosphorylated Akt in tumor tissues, suggesting that inhibition of this signal transduction pathway might be involved in the chemopreventive activity of this compound.

Conclusions: We conclude that increased levels of FAS are common in human preinvasive neoplasia of the lung. Based on studies in mouse models, it seems that inhibiting FAS is an effective strategy in preventing and retarding growth of lung tumors that have high expression of this enzyme.

Lung cancer, with a high incidence and 5-year mortality rate of >80%, is the leading cause of cancer deaths in the United States and other industrialized countries (1). Because of the lack of success in treating advanced lung cancer (2, 3), there is an increasing emphasis on preventing this disease through smoking cessation programs. However, although large numbers of individuals have already stopped smoking as a result of the recognized health risks of this habit, these individuals remain at a significantly increased risk of developing lung cancer for the

remainder of their lives (4, 5). Therefore, it is also important to devise chemoprevention strategies to thwart the development of new cancers that are expected to arise as a result of past exposures.

The term “chemoprevention” is commonly used to represent pharmacologic intervention at any preinvasive stage of the neoplastic process (6, 7). Thus, chemoprevention could involve a treatment to block the accumulation of genetic alterations as well as treatment to inhibit or eradicate the preinvasive precursors to invasive cancer. For lung cancer chemoprevention, it is important to consider the treatment of existing preinvasive neoplastic lesions because large numbers of individuals have previous exposure to tobacco carcinogens and because the development of lung cancer is actually quite slow, with a latency period that typically exceeds 30 years from initial carcinogen exposure to clinical presentation of lung cancer (4, 8).

This study explored the possible role of inhibiting fatty acid synthase (FAS) for chemoprevention of lung cancer. FAS is overexpressed in many human cancers and has shown promise as a potential target for cancer therapy in various xenograft models (9, 10). In addition, inhibiting FAS has been shown to decrease incidence and growth of breast cancers in mice with overexpression of the HER2 oncogene (11). Recently, we have shown that a second-generation pharmacologic inhibitor of FAS, C93, can effectively inhibit FAS enzymatic activity in human lung cancer xenografts and also effectively inhibit tumor growth without causing any recognizable toxicity to

Authors' Affiliation: Department of Pathology and Johns Hopkins Cancer Center, Johns Hopkins University School of Medicine, Baltimore, Maryland
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Requests for reprints: Edward Gabrielson, Department of Pathology and Johns Hopkins Cancer Center, Johns Hopkins University School of Medicine, CRB2, Room 304, 1550 East Orleans Street, Baltimore, MD 21231. Phone: 410-502-5250; Fax: 410-550-0075; E-mail: egabriel@jhmi.edu.

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treated animals (12). Based on these observations, we examined the potential use of FAS inhibitors for lung cancer chemoprevention by (a) examining expression of FAS in early preinvasive human lung neoplasia and (b) testing the efficacy of pharmacologic inhibitors of FAS for lung cancer chemoprevention in two mouse lung cancer chemical carcinogenesis models.

Materials and Methods

Preinvasive human lung neoplasia samples. Samples of high-grade bronchial squamous dysplasia/squamous carcinoma *in situ* and atypical adenomatous hyperplasia were obtained from surgical pathology specimens at Johns Hopkins Hospital. For all cases, these preinvasive lesions were recognized in the tissue peripheral to a synchronous lung cancer but were confirmed to be discontinuous to the invasive cancer. All samples used for research were in excess of tissue needed for routine diagnosis, and the use of tissue was approved by the Institutional Review Board.

Mouse models of lung carcinogenesis. We used two murine models of chemically induced lung carcinogenesis for these studies. For induction of tumors by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 8-wk-old female A/J mice (National Cancer Institute, Frederick, MD) were given three i.p. injections of NNK (Toronto Research Chemicals) every other day at a dose of 50 mg/kg (13). For induction of tumors by urethane, a single dose of 1 mg/g urethane (Sigma Chemical Co.) dissolved in 0.9% NaCl (saline) was given by i.p. injection (14) to 8-wk-old female A/J mice.

Treatment with FAS inhibitors and evaluation of tumor growth. C75 and C93 were provided by FASgen, Inc. and dissolved in DMSO for stock solutions. C75 was given as a weekly i.p. injection (20 mg/kg) in DMSO to designated animals beginning 3 wk after exposure to NNK and continuing until the termination of the experiment (up to 20 wk after NNK exposure). The frequency of dosing of C75 was limited to weekly administration by anorexia that is induced by this compound. C93, solubilized in 80% PEG400, 10% Tween 80, and 10% ethanol, was given orally by micropipette to mice every 12 h at doses of 5 mg/kg (total daily dose of 10 mg/kg) or 25 mg/kg (total daily dose of 50 mg/kg). Mice willingly ingested this oral formulation. Oral administration was selected for this compound because of the risk of infection with repeated i.p. injections. Treatments with C93 were also initiated 3 wk after exposure to either NNK or urethane and continued until the termination of the experiment (up to 20 wk after carcinogen exposure).

At the end of each experiment (6 h after final treatment), all mice were euthanized, and lungs were removed and gently inflated with 10% buffered formalin. Tumor nodules on the surfaces of the lungs were then counted as an index of multiplicity. Tumor volume was assessed by processing lungs for histology and examining whole-mount cross-sections of lung tissue (in triplicate for each sample) for percentage of lung tissue occupied by tumor using Photoshop software (Adobe Software) to quantify pixels in the cross-sections. Differences in tumor sizes of treatment and control groups were compared using two-tailed *t* test (assuming unequal variances).

Histology and immunohistochemistry. At the termination of experiments, animals were examined by necropsy and representative tissues (including lungs) were removed for histologic examination. Sections of all tissues (human or mouse) were stained with H&E, and immunohistochemistry was done on specified tumor tissues using antibodies specific for mammalian FAS (clone 6E7; FASgen) at a 1:2,000 dilution and phosphorylated Akt (rabbit monoclonal antibody, Ser⁴⁷³; Cell Signaling) at a 1:50 dilution. Specific staining was visualized using biotinylated secondary antibody followed by peroxidase-labeled streptavidin (LSAB+ System-HRP, DakoCytomation) and incubation with substrate-chromogen solution according to the manufacturer's instructions. Qualitative expression of FAS and phosphorylated Akt was

assessed by comparing levels of these proteins in the tumors with those of surrounding host lung and with human lung cancer tissues stained with identical protocols.

Results

High levels of FAS in human preinvasive pulmonary neoplasia. To explore the potential value of inhibiting FAS for lung cancer chemoprevention, we first evaluated expression of this enzyme in human *in situ* squamous and adenomatous neoplasia. Bronchial dysplasia and carcinoma *in situ* are most commonly seen in lungs of patients with a history of extensive tobacco use and concurrent invasive lung cancers, and all of the eight cases (with one site of *in situ* squamous carcinoma each) were also in lungs that were surgically resected for treatment of invasive lung cancer. Similarly, atypical adenomatous hyperplasia, a noninvasive form of peripheral pulmonary neoplasia, is typically noted in the lungs of patients with synchronous lung cancers. Again, all of the 7 lungs with AAH (total of 22 foci) examined in our study were from such patients.

We examined FAS expression in these small lesions by immunohistochemistry using reagents and conditions identical to those previously used to evaluate FAS expression in invasive lung cancers (12). Typical staining of these lesions is shown in Fig. 1A and B. All 8 of the *in situ* squamous lesions were scored as positive for FAS [4 stained 3+ and 4 stained 4+ using previously defined criteria (12)], and all of the 18 normal bronchi in the same lungs were scored as negative (staining 0 or 1+). Of the 22 atypical adenomatous hyperplasia lesions examined (in lungs from 7 different individuals), 20 were scored as positive for high FAS expression (3 scored 2+, 9 scored 3+, and 8 scored 4+), and all of the 28 bronchi (such as Fig. 1C) in the same lung tissues were scored as negative (χ^2 significance for both types of lesions compared with normal bronchi is $P < 0.0001$).

Chemically induced murine lung tumors express high levels of FAS. Exposing young female A/J mice to NNK or urethane resulted in the appearance of lung tumors as previously reported (13, 14). These tumors grew as expansile and generally noninvasive nodules with a pseudopapillary architecture. To determine whether these murine lung carcinogenesis models would be appropriate for evaluating inhibitors of FAS in lung cancer chemoprevention, we evaluated expression of FAS in tumors using immunohistochemistry. As shown in representative tumors (Fig. 1D and E), these neoplasms uniformly express high levels of FAS protein, which are comparable with the levels that we observed in human lung cancers that express high levels of this protein.

Inhibitors of FAS decrease numbers and volumes of lung tumors in NNK-exposed mice. To test the potential for FAS inhibitors to prevent or retard lung cancer development, we first tested two different pharmacologic agents (C75 and C93) in the NNK exposure murine model of lung carcinogenesis. C75 induces fatty acid oxidation in addition to inhibiting FAS and consequently has a dose-limiting side effect of anorexia (15, 16). Consequently, our dosing of animals for experiments using C75 was limited to a single administration of 20 mg/kg/wk. As shown in Fig. 2A and B, this weekly dosing with C75 was capable of reducing tumor number per animal by ~50% and decreased the total tumor burden in these animals by ~66%.

In contrast to C75, C93 (17) is a highly specific inhibitor of FAS that does not significantly stimulate fatty acid oxidation and does not cause anorexia. Because C93 was not associated with weight loss (or any other recognizable form of toxicity), we tested two dose levels: 10 and 50 mg/kg/d. Both of these dose levels were given as split doses, twice per day. The 10 mg/kg/d dose resulted in a decrease in tumor number per animal and total tumor burden per animal that seems to be slightly less than that seen with the weekly injection of 20 mg/kg C75 (Fig. 2A and B), which could reflect differences in the relative potency of the agents or different efficacy of the dosing schedules. However, C93, given daily at a dose of 50 mg/kg/d (Fig. 2A-C), reduced the number of tumors per animal by ~75% and the total tumor burden per animal by >90%.

Inhibitors of FAS also inhibit lung tumorigenesis in urethane-exposed mice. To determine whether this strategy is effective across various models of lung carcinogenesis, we tested C93 in a chemoprevention experiment using mice that had been exposed to urethane. Again, two dose levels (10 and 50 mg/kg/d, given in split doses) were tested. As shown in Fig. 3, animals receiving low-dose C93 seemed to have a similar number of tumors compared with control animals but a significantly decreased total tumor burden (~80%; $P = 0.04$), whereas the high-dose C93 reduced the number of tumors per animal by ~40% ($P = 0.07$) and the total tumor burden by ~90% ($P = 0.01$). Thus, we observed a dose-dependent inhibition of tumor development in a second chemical pulmonary carcinogenesis model system, indicating that the efficacy of this chemopreventive strategy is not limited to a single animal model system.

Inhibitors of FAS decrease Akt activity in neoplastic cells of chemically exposed mice. Although the mechanisms of cell killing by inhibitors of FAS are not fully understood, one report indicates that inhibiting FAS leads to down-regulation of phosphorylated Akt (18). We therefore used immunohistochemistry to evaluate the effects of C93 treatment on levels of

phosphorylated Akt (an indicator of Akt activation) in lung tumors induced by NNK or urethane in A/J mice. Universally, we observed high levels of phosphorylated Akt in the tumors of untreated mice and markedly reduced levels of phosphorylated Akt in the mice treated with high-dose C93 (Fig. 4). These data suggest that reduction in Akt activity parallels the antineoplastic activity of FAS inhibitors and that these effects on a major signal transduction pathway could be related to the mechanism of FAS inhibitors.

Discussion

Based on lack of success with clinical trials for retinoids in lung cancer chemoprevention (19, 20), there is a widely recognized need to evaluate new agents for this purpose. Addressing this problem, several compounds have been evaluated for lung cancer chemoprevention in preclinical studies, including vitamins, botanical products, minerals, antioxidants, and anti-inflammatory drugs (21–30). The data reported here indicate that inhibitors of FAS rank among the more efficacious agents for preventing lung tumors in the preclinical setting, with C93 treatment resulting in decreased multiplicity of lung tumors as well as decreased growth of tumors in A/J mice exposed to NNK or urethane, major carcinogenic components of tobacco smoke. The potential usefulness of FAS inhibitors in lung cancer chemoprevention is further supported by our observations that high levels of FAS occur commonly in preinvasive human lung neoplasms (as well as in invasive human lung cancers). Finally, the third-generation inhibitor of FAS, C93, does not seem to cause measurable toxicity in the treated mice, and thus, it could potentially be acceptable for testing in individuals without clinical disease.

The experimental models of chemical carcinogenesis used in our investigations are widely used for preclinical lung cancer chemoprevention studies. It is remarkable that the chemical

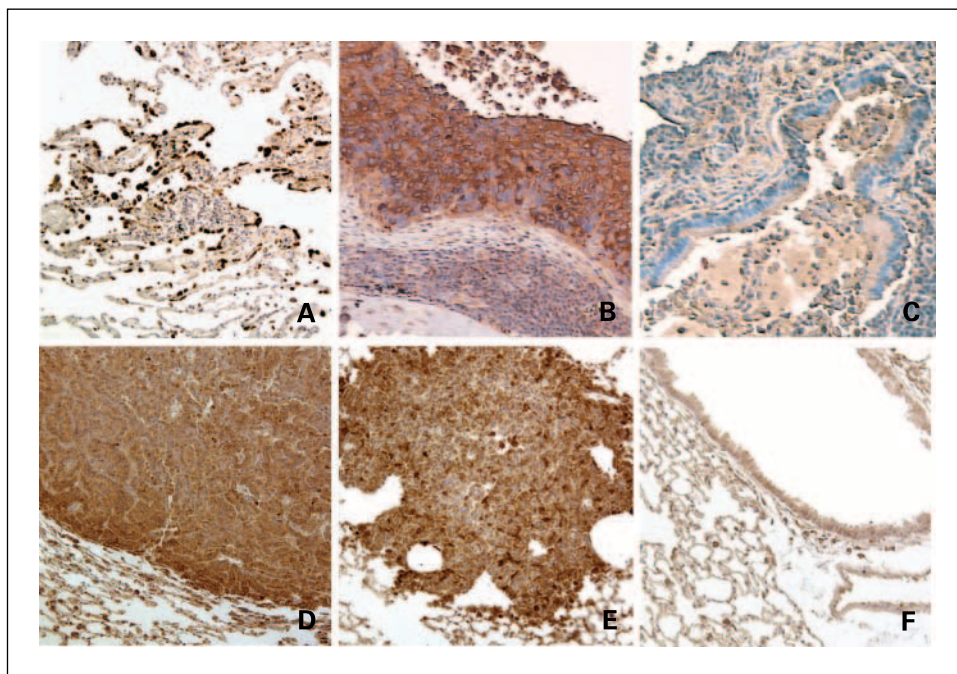


Fig. 1. Expression of FAS in human preinvasive pulmonary neoplasia and in chemically induced murine pulmonary neoplasia. *A*, FAS expression in atypical adenomatous hyperplasia of the lung. *B*, FAS expression in a squamous cell carcinoma *in situ* of bronchial epithelium. *C*, FAS expression in normal human bronchus from the same individual represented in *B*. Samples for *A* and *B* were from patients diagnosed with synchronous invasive lung cancer, but these lesions were anatomically distinct from the invasive cancers. *D* and *E*, FAS expression in lung tumors in A/J mice induced by NNK (*D*) and urethane (*E*). *F*, the relatively increased levels of FAS in these neoplastic tissues can be appreciated by comparing with normal mouse lung tissues. Magnification, $\times 20$.

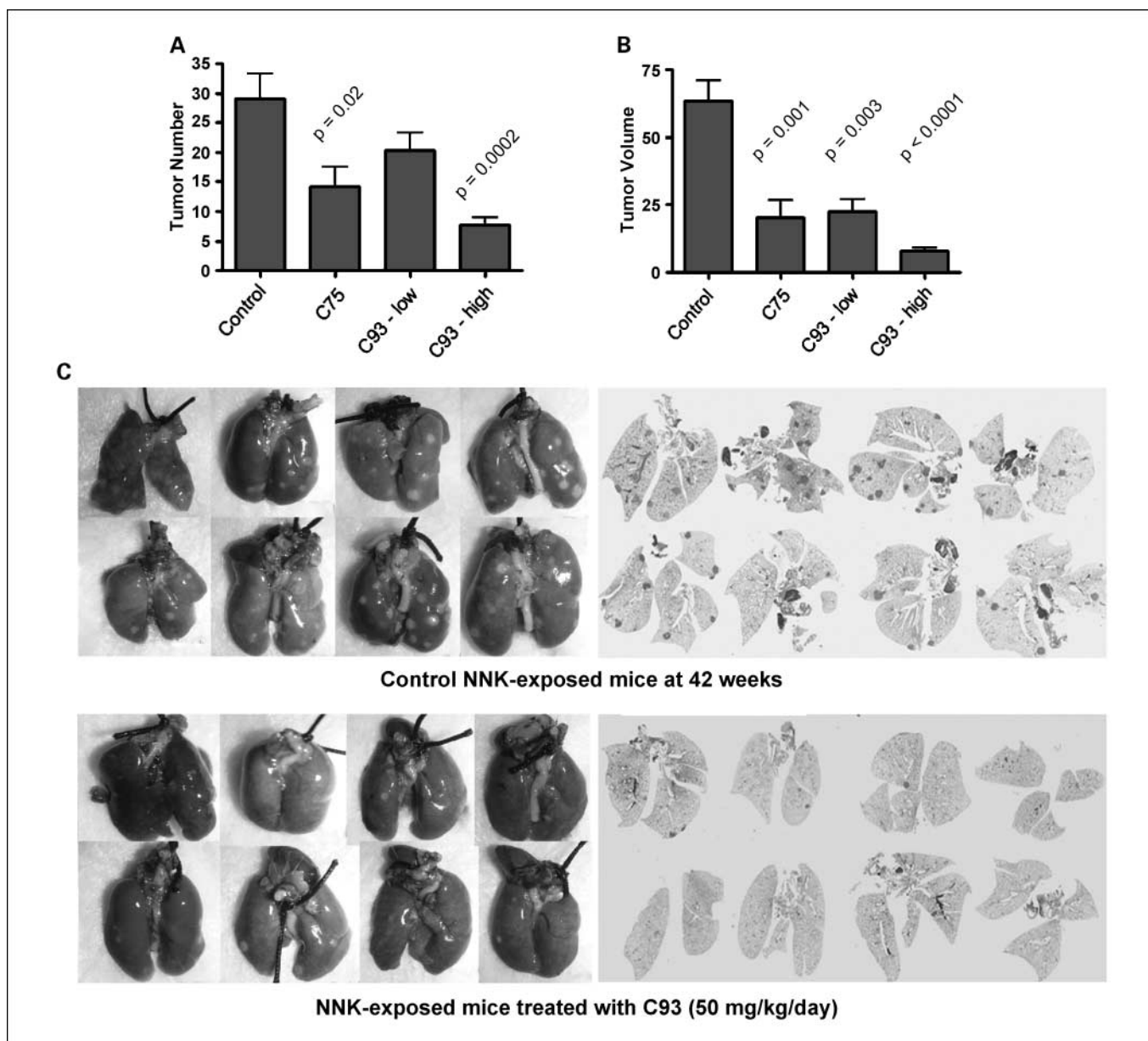


Fig. 2. Inhibitors of FAS decrease numbers and volumes of lung tumors in NNK-exposed mice. **A**, summary of data for tumor multiplicity in NNK-exposed A/J mice treated with inhibitors of FAS compared with control animals ($n = 8$ for each group). Multiplicity was measured by counting tumors on surfaces of lungs. **B**, summary of effects of FAS inhibitors on tumor volumes, determined by measuring cross-sectional area of lung occupied by tumor in three different cross-sections of the lungs of each animal. **A** and **B** columns, means; bars, SE. *P* values were calculated using unpaired *t* test. **C**, images of lung surfaces and cross-sections of lungs (stained with H&E) for control mice and mice treated with high-dose C93 (50 mg/kg/d).

carcinogens used to induce lung tumors in A/J mice for chemoprevention studies (urethane and NNK) are components of cigarette smoke, and the resulting lung tumors contain many of the same molecular alterations found in human adenocarcinomas. For example, ~90% of NNK-induced lung tumors in A/J mice have G→A transitions at the second base of codon 12 in the *KRAS* gene (31), and urethane-induced lung adenomas and adenocarcinomas in the same mouse strain also contain *KRAS*-activating mutations, primarily in codon 61 (AT-GC transition or AT-TA transversion; ref. 32). Furthermore, microarray analysis has shown that the A/J mouse-urethane model reflects the molecular signature of human lung adenocarcinoma, including parallel changes in changes in expression for

glycolytic enzymes, cell cycle proteins, and genes related to angiogenesis and eicosanoid metabolism (33). It is also notable that these tumors express levels of FAS that are similar to those of human lung cancers and preinvasive neoplasms, thus making them suitable for testing FAS inhibitors for chemoprevention in these models.

Several other agents have been evaluated for lung cancer chemoprevention in preclinical studies using A/J mice, including vitamins, minerals, antioxidants, anti-inflammatory agents, and small molecules that target signal transduction pathways (21–28, 34). The more successful among these chemoprevention studies have shown reduction of tumor multiplicity and growth similar to that reported here with C93. Importantly, the

unproven benefits of these agents in human lung cancer chemoprevention and the magnitude of the lung cancer problem provide a rationale for considering multiple compounds for lung cancer chemoprevention. Furthermore, there is a potential for using combinations of agents for lung cancer chemoprevention, particularly if the agents are synergistic in their activity or if they affect nonoverlapping targets.

Interestingly, mechanisms for the antineoplastic effects of FAS inhibitors are not completely understood, but several lines of evidence indicate that the importance of FAS in cancer cells is not simply to meet a requirement for lipids (9). Various studies have suggested that the activity of FAS inhibitors of cancer cells involves increases in PERK-dependent phosphorylation of the translation initiation factor

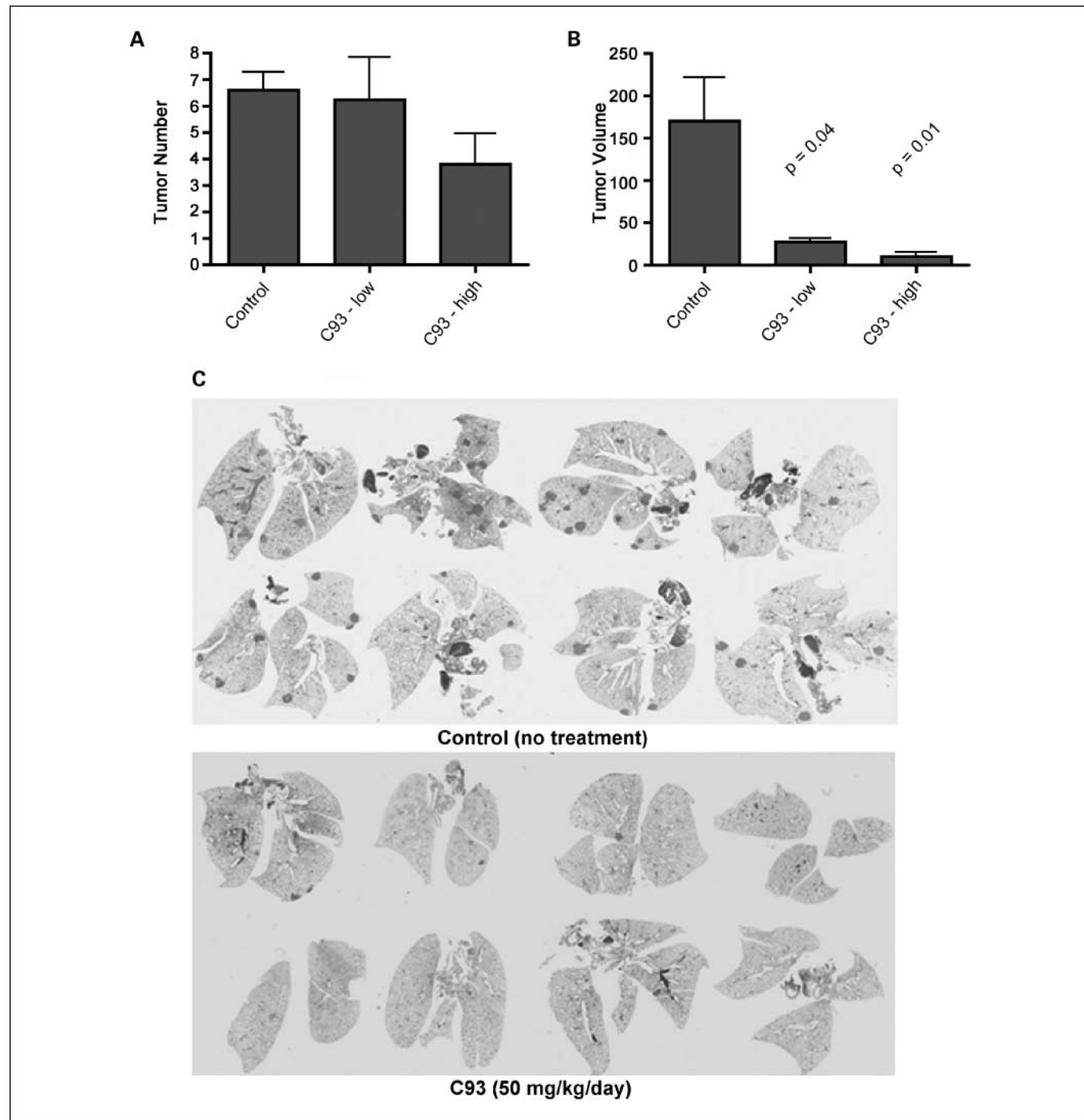
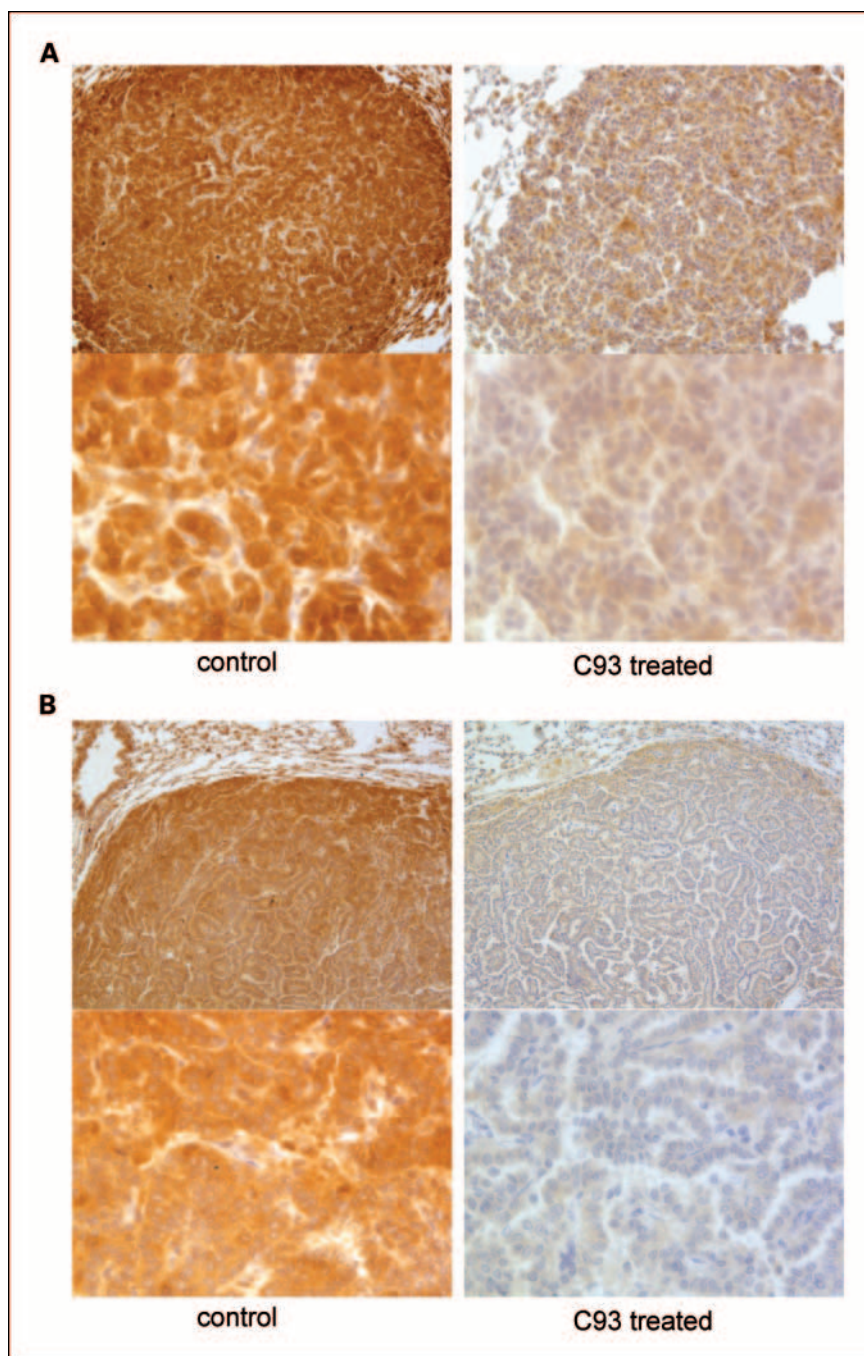


Fig. 3. Inhibitors of FAS decrease numbers and volumes of lung tumors in urethane-exposed mice. *A*, summary of data for tumor multiplicity in urethane-exposed A/J mice treated with C93, an inhibitor of FAS, compared with control animals ($n = 8$ for each group). Multiplicity was measured by counting tumors on surfaces of lungs. *B*, summary of effects of FAS inhibitors on tumor volumes, determined by measuring cross-sectional area of lung occupied by tumor in three different cross-sections of the lungs of each animal. *A* and *B*, columns, means; bars, SE. *P* values were calculated using unpaired *t* test. *C*, images of cross-sections of lungs (stained with H&E) for control mice and mice treated with high-dose C93 (50 mg/kg/d).

Fig. 4. Inhibitors of FAS decrease Akt activity in neoplastic cells of chemically exposed mice. Immunohistochemistry was used to measure levels of phosphorylated Akt in murine tumors. *A*, typical results for tumors induced by NNK, with tumor from untreated animal on left and tumor from animal treated with C93 (50 mg/kg/d) on right. Low-power (*top*) and high-power (*bottom*) images are shown for each tumor. *B*, typical results for tumors induced by urethane, with tumor from untreated animal on left and tumor from animal treated with C93 (50 mg/kg/d) on right. Again, low-power (*top*) and high-power (*bottom*) images are shown for each tumor.



eIF2 α and concomitant inhibition of protein synthesis (35), or activation of AMP-activated protein kinase (36). In studies of ovarian cancer, inhibiting FAS was shown to decrease the level of activated Akt (18), suggesting that the antineoplastic effects of FAS inhibitors could be mediated, at least in part, by inhibiting this important signal transduction pathway. Our experiments also show that treatment of animals with C93, a specific FAS inhibitor, leads to markedly decreased Akt phosphorylation in carcinogen-induced lung tumors. This effect on phosphorylation (and presumably activity) of Akt could be relevant to lung cancer chemoprevention because high levels of phosphorylated Akt have been previously noted

in human preinvasive bronchial neoplasia (37, 38). Furthermore, daily administration of rapamycin, an inhibitor of mammalian target of rapamycin in the Akt signaling pathway, was recently shown to reduce lung tumor multiplicity and size in NNK-exposed A/J mice of a magnitude similar to that observed in the current study (29). These findings, together with our observations of associations between decreased phosphorylated Akt levels and reduced tumor growth in C93-treated mice, suggest that links between Akt activity and FAS activity deserve further consideration as a mechanism for FAS inhibitors in the chemoprevention of carcinogen-induced lung tumors. However, it is notable that we have also

observed antineoplastic activity of FAS inhibitors in lung cancer cell lines (e.g., A549 cells) with relatively low levels of Akt expression (12), suggesting that FAS inhibitors might inhibit tumor development through effects on pathways in addition to those related to Akt-mediated signaling.

An important concern that could limit application of any preventive agent is that of toxicity. To date, no measurable toxicity has been seen in animals treated with C93, but consideration for clinical testing as a chemopreventative agent will obviously require rigorous assessment of toxicity in a therapeutic setting. Other agents that have already been tested in humans, and are now under consideration for lung cancer

chemoprevention, do have some toxicities, and the risk/benefit ratios in the chemopreventative setting are not yet understood. For example, long-term use of cyclooxygenase-2 inhibitors is associated with an increased risk of cardiovascular toxicities, including death (39), and rapamycin is associated with immunosuppression (40). In a manner similar to development of combination chemotherapy, it might be reasonable to consider use of combinations of agents for chemoprevention, particularly if the agents have nonoverlapping toxicities. Based on the lack of detectable toxicity in our preclinical studies, inhibitors of FAS are leading candidates to be evaluated for use in such combinations.

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