Identification of a Highly Effective Rapamycin Schedule that Markedly Reduces the Size, Multiplicity, and Phenotypic Progression of Tobacco Carcinogen – Induced Murine Lung Tumors

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Abstract

Purpose: Human and murine preneoplastic lung lesions induced by tobacco exposure are characterized by increased activation of the Akt/mammalian target of rapamycin (mTOR) pathway, suggesting a role for this pathway in lung cancer development. To test this, we did studies with rapamycin, an inhibitor of mTOR, in A/J mice that had been exposed to the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).

Experimental Design: Tumorigenesis was induced by i.p. injection of NNK, and rapamycin was administered 1 or 26 weeks after NNK administration. Biomarkers associated with mTOR inhibition were assessed in lung and/or surrogate tissues using immunohistochemistry and immunoblotting. Rapamycin levels were measured using mass spectroscopy.

Results: Rapamycin was administered on a daily (5 of 7 days) regimen beginning 26 weeks after NNK decreased tumor size, proliferative rate, and mTOR activity. Multiplicity was not affected. Comparing this regimen with an every-other-day (qod) regimen revealed that rapamycin levels were better maintained with qod administration, reaching a nadir of 16.4 ng/mL, a level relevant in humans. When begun 1 week after NNK, this regimen was well tolerated and decreased tumor multiplicity by 90%. Tumors that did develop showed decreased phenotypic progression and a 74% decrease in size that correlated with decreased proliferation and inhibition of mTOR.

Conclusions: Tobacco carcinogen – induced lung tumors in A/J mice are dependent upon mTOR activity because rapamycin markedly reduced the development and growth of tumors. Combined with the Food and Drug Administration approval of rapamycin and broad clinical experience, these studies provide a rationale to assess rapamycin in trials with smokers at high risk to develop lung cancer.

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The death toll from lung cancer in the United States is the highest of all cancers, estimated to kill more than 172,000 Americans in 2006 (1). Presently, there are over 90,000,000 current or former smokers in the United States at permanent increased risk for the development of lung cancer. Moreover, tobacco use accounts for 30% of overall cancer mortality (2). Given the large population at increased risk of developing lung cancer, the development of approaches to prevent smoking-related lung cancers would have great clinical benefit.

Several studies have linked activation of the Akt/mammalian target of rapamycin (mTOR) pathway and lung tumorigenesis. Preclinical studies have shown that physiologically relevant concentrations of nicotine and its metabolite, the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), rapidly stimulate activation of Akt and mTOR in primary human bronchial and small airway epithelial cells, thereby promoting a partially transformed phenotype (3, 4). Phenotypic progression of NNK-transformed human bronchial epithelial cells and NNK-induced murine lung lesions is characterized by increased activation of Akt and mTOR (5). Clinical studies have shown that Akt is activated in

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the majority of human dysplastic bronchial epithelial lesions (6), and that activation of Akt is a poor prognostic factor for non–small cell lung cancer patients, particularly for those with stage I cancer or small lung tumors (7). These studies suggest that the Akt/mTOR pathway is an important mediator of the formation and maintenance of tobacco carcinogen–induced lung tumors, and that targeting this pathway might be a viable approach for lung cancer chemoprevention.

Numerous inhibitors of components of the Akt/mTOR pathway are being developed for cancer therapy (8), but most of these are still in preclinical stages of development. The best-characterized and most developed inhibitors of the pathway target mTOR. Rapamycin, the prototypic mTOR inhibitor, was discovered in 1975 as a potent antifungicide and is produced naturally by *Steptomyces hygroscopicus* (9, 10). Rapamycin is currently Food and Drug Administration approved to prevent renal allograft rejection and to coat cardiac stents, but its potential to inhibit the proliferation of cancer cell lines was shown over 20 years ago (11–13). Rapamycin inhibits mTOR by binding to the FK506-binding protein FKBP-12. This complex binds mTOR and prevents its ability to phosphorylate substrates such as the eukaryotic translation initiation factor 4E-binding protein 1 and S6 kinase 1. S6 kinase 1 phosphorylates and activates the small ribosomal protein S6, and loss of S6 phosphorylation has been used as a biomarker for mTOR inhibition *in vivo*. More recently, rapamycin analogues, such as CCI-779, RAD-001, and AP23573, have been explicitly designed for development as cancer drugs. Promising preliminary data from over 600 patients in a phase III trial using CCI-779 in patients with advanced renal cell cancer show that CCI-779 improved overall survival compared with those who received IFN-α (14).

Although the antiproliferative and antitumor effects of rapamycin and its analogues have been shown, a requirement for mTOR activity in the formation and maintenance of tobacco carcinogen–induced lung tumors has not. We did studies in a mouse model system of tobacco carcinogenesis to determine the effects of mTOR inhibition on established tumors and on new tumor formation. We report that treatment with pharmacologically relevant doses of rapamycin reduces the size of established tumors and, when given before tumor development, profoundly reduces lung tumor multiplicity, size, and phenotypic progression. These studies show that mTOR plays a critical role in the development of tobacco carcinogen–induced lung tumorigenesis.

Materials and Methods

Animals. Female A/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 5 weeks of age and housed according to the guidelines of the Animal Care and Use Committee of the NIH. Mice were fed AIN-93G/M chow from Dyets (Bethlehem, PA) and autoclaved water *ad libitum* and were weighed weekly. NIH animal facilities are accredited by the Association for Assessment of Laboratory Animal Care. All experiments were carried out under an NIH-approved animal study protocol.

Carcinogen and drug treatment. For the study assessing the effect of rapamycin on established tumors, 25 mice per group were given a single dose of NNK (Toronto Research Chemicals, North York, ON) at 6 weeks of age. For the prevention of tumor development study, NNK was obtained from EaglePicher Pharmaceuticals (Lenexa, KS), and 15 mice per group were treated with three doses of NNK over 3 weeks beginning at 6 weeks of age. The purity of both NNK samples was verified by high-performance liquid chromatography analysis. NNK was prepared in 0.9% NaCl solution, and 5 μL solution per gram of mouse were delivered by i.p. injection to achieve a dose of 100 mg/kg. Rapamycin was obtained from LC Laboratories (Woburn, MA), prepared as a 25× solution in DMSO, and stored at −20°C in aliquots along with aliquots of DMSO until use. For injection, aliquots were thawed and diluted in a vehicle of 5% Tween 80 and 5% polyethylene glycol in 0.9% NaCl to yield a final concentration of 4% DMSO. Mice were given 5 μL rapamycin or vehicle control per gram of mouse to achieve a final dose of 1.5 mg/kg. For the mice treated for 12 weeks with rapamycin every other day, a loading dose of 4.5 mg/kg was used on the first day of treatment to facilitate achievement of steady-state blood concentrations more rapidly (15). All chemicals were from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Determination of sirolimus (rapamycin) in murine whole blood. Samples were prepared according to the method of Taylor and Johnson with modification (16). Briefly, to 50 μL of whole blood were added 50 μL of 100 ng/mL 32-desmethoxyrapamycin (Supelco, Bellefonte, PA) in methanol and 250 μL of 0.1 mol/L zinc sulfate solution followed by vortex mixing for 1 min. Acetonitrile (500 μL) was added followed by vortex mixing for 1 min to efficiently denature and precipitate the protein complement. The final solution was centrifuged at 12,000 × *g* for 5 min to pellet proteins and cell debris. The supernatant was removed and diluted with the addition of 2 mL of DDI water. The diluted extract was applied to a preconditioned Oasis HLB (Waters, Milford, MA) 30 mg solid-phase extraction cartridge and washed sequentially with 2 mL of water, 50% methanol/water, and hexane. Rapamycin (LC Laboratories) and 32-desmethoxyrapamycin were eluted with 1.5 mL of 50% isopropanol-hexane. The eluate was evaporated under vacuum centrifugation, and the residue was dissolved in 50 μL of 50% acetonitrile/water for analysis.

Analysis was carried out in a manner similar to that of Pieri et al. (17) using an Agilent Technologies series 1100 Capillary LC/MSD-Trap equipped with a binary pump, online degasser, column oven, thermostated micro autosampler, and ion-trap mass spectrometer (17). Separations were conducted by reverse-phase chromatography using a 150 mm × 0.5 mm inner diameter micro-column packed with 5 m Luna C8(2) stationary phase obtained from Phenomemex (Torrance, CA) operated at 60°C. The gradient mobile phase used was composed of water and acetonitrile delivered at a flow rate of 20 mL/min, 50% acetonitrile for 3 min, increased to 75% in 7 min then to 90% in 2 min for a total runtime of 15 min. Autosampler variables included an injection volume of 5 μL and a sample tray temperature of 4°C. The mass spectrometer was operated in positive-ion mode using electrospray ionization with ion current acquired over the range of *m/z* 900 to 940.

Analytes were identified by comparing the retention time of the standard compound to that of the sample in the extracted ion chromatograms from the MS signal output corresponding to the [M + Na] ion of rapamycin (*m/z* 936.5) and 32-desmethoxyrapamycin (*m/z* 906.5). Quantification was accomplished using the internal standard method by applying the peak area ratio obtained from the sample chromatogram to a calibration curve generated using standard solutions from 10 to 2,000 ng/mL. The limit of quantification was 10 ng/mL, and the limit of detection was 2 ng/mL.

Tissue preparation. At the completion of the study, mice were euthanized, and blood was collected from five mice in each group by retro-orbital bleeding into BD vacutainer vials containing EDTA (Franklin Lakes, NJ). Spleen and liver were also collected from five mice in each group, flash frozen, and stored at −80°C. Lungs were insufflated with freshly prepared neutral buffered formalin (4% paraformaldehyde in phosphate buffer, pH 7.2), removed from the pleural cavity, fixed overnight in neutral buffered formalin, and transferred to 80% ethanol. Individual lung lobes were analyzed for tumors under a dissecting microscope. Tumor diameter was measured

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using a micrometer, and tumor volume was calculated using the formula $\pi d^3/6$. Whole lungs were sent to HistoServ (Gaithersburg, MD) for processing and H&E staining.

Immunoblotting. Lysates from spleen and liver were prepared by allowing thin frozen slices of tissue to thaw in radioimmunoprecipitation assay buffer [1% NP40, 1% deoxycholic acid sodium salt, 1% SDS, 0.15 mol/L NaCl, 0.01 mol/L Na_3PO_4 (pH 7.2), 2 mmol/L EDTA, 50 mmol/L NaF, 0.2 mol/L Na_3VO_4, and protease inhibitor cocktail from Roche (Indianapolis, IN)] on ice for 30 min. Lysates were sonicated, cleared by centrifugation at 10,000 rpm for 10 min, and stored at −80°C. Following SDS-PAGE, protein was transferred to nitrocellulose, blocked for 1 h at room temperature with 5% milk in wash buffer (1× TBS with 0.1% Tween 20), and incubated with primary antibody overnight with rocking at 4°C. Primary antibodies were obtained from Cell Signaling Technology (Danvers, MA) unless otherwise indicated. Labeling was detected using horseradish peroxidase – labeled secondary antibodies using enhanced chemiluminescence detection reagent from Amersham (Piscataway, NJ).

Immunohistochemistry. Sections of lung tissue on poly-L-lysine – coated slides were obtained from HistoServ and analyzed for protein expression or phosphorylation for five mice per group. Unless otherwise noted, antigen retrieval was carried out using preheated target retrieval solution (pH 6.0) from DakoCytomation (Carpinteria, CA) for 30 min in a boiling rice cooker. For erbB3, no antigen retrieval was required and, for F4-80, slides were warmed in a 37°C ultra-pure water bath for 30 min followed by treatment with 0.025% trypsin at 37°C for 10 min. Vectastain Elite ABC kits from Vector Laboratories (Burlingame, CA) were used according to manufacturer's instructions for blocking, dilution of primary antibody, and labeling. The antibody directed to F4-80 was from Abcam (Cambridge, MA); erbB3 was from Neomarkers (Freemont, CA); Ki-67 was from Vision Biosystems (Norwell, MA); and all other primary antibodies were obtained from Cell Signaling Technology. For Ki-67, the primary antibody was incubated with sections for 1 h at room temperature. All other antibodies were incubated with sections for 16 h at 4°C. 3,3-Diaminobenzidine was prepared fresh from tablets (Sigma-Aldrich). Terminal deoxynucleotidyl transferase – mediated nick-end labeling staining was carried out with a kit from Chemicon (Temecula, CA) according to manufacturer's instructions. Specificity of staining was assessed by comparison with samples stained in the absence of primary antibody.

All slides were blinded to the investigators (C.A.G., P.A.D., and I.L.) before scoring, and in all cases, tumors and/or normal lung were assessed for five mice per group. The proliferation index was achieved by dividing the number of Ki-67 – positive cells by the number of ×40 fields occupied by the tumor. For phosphorylated Akt, phosphorylated S6, and erbB3, staining intensity was scored for the whole of the tumor as absent (0), mild (1), moderate (2), or strong (3). For phosphorylated extracellular signal-regulated kinase, a staining index was achieved by summing the products of the fraction of cells staining with intensity times the intensity, as just described. The number of lung-infiltrating macrophages was assessed by counting the number of F4-80 – positive cells in 10 randomly selected fields viewed with a 40× objective. The number of tumor-associated macrophages was assessed by counting the number of macrophages per lesion and dividing by the number of ×40 fields occupied by the lesion.

Statistical analyses. A Mann-Whitney test was used to determine statistical differences in tumor multiplicity and size, and Student's *t* test was done for all other measures.

Results

Effect of rapamycin on tobacco carcinogen – induced tumors. To determine the effects of mTOR inhibition on established tumors, tumors were initiated with a single i.p. dose of NNK (100 mg/kg). Twenty-six weeks after tumors had been allowed to form, mice were treated for a 6-week interval with rapamycin administered by i.p. injection (1.5 mg/kg/d every 5 of 7 days; Fig. 1A). Mice were weighed weekly, and no significant differences in weight gain were observed during rapamycin treatment compared with untreated controls, suggesting that rapamycin was well tolerated (data not shown). Rapamycin did not affect the multiplicity of spontaneous or NNK-induced tumors (Fig. 1B). Tumor multiplicity for control versus rapamycin treatment was 1.67 versus 1.50 and 3.05 versus 2.74 tumors per mouse for spontaneous and NNK-induced tumors, respectively. In contrast, rapamycin treatment decreased the size of NNK-induced tumors from 1.14 to 0.61 mm^3 (P = 0.0046; Fig. 1C). Similar results were obtained with treatment of spontaneous tumors in that tumor size was decreased from 0.84 to 0.26 mm^3. However, this reduction was not statistically significant perhaps due to the small number of spontaneous tumors available for analysis.

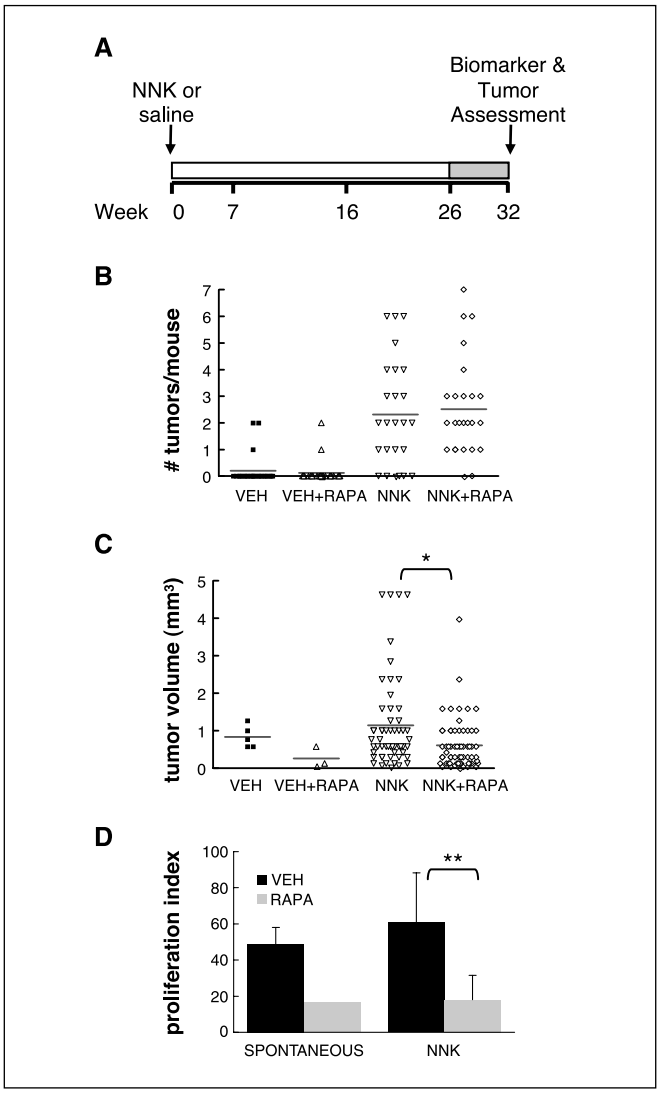

Fig. 1. Effect of rapamycin on established lung tumors. *A,* experimental schema. Shaded area indicates rapamycin treatment. *B,* tumor incidence and multiplicity. *C,* tumor size. *, P = 0.0046. *D,* cellular proliferation, as assessed by Ki-67 staining in vehicle- and rapamycin-treated tumors. **, P = 0.003.

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To establish if decreased size was due to a decrease in tumor cell proliferation and/or an increase in tumor cell apoptosis, Ki-67 and terminal deoxynucleotidyl transferase–mediated nick-end labeling staining were done. NNK-induced tumors that were treated with rapamycin had a lower proliferative index than vehicle-treated tumors (P = 0.003; Fig. 1D). Although the average proliferative index for spontaneous tumors was also lower for spontaneous tumors treated with rapamycin, the difference was not statistically significant, again perhaps due to the small number of tumors available for analysis following rapamycin treatment. Analysis of tumors for apoptotic cells showed no difference in terminal deoxynucleotidyl transferase–mediated nick-end labeling staining for tumors treated with rapamycin compared with those treated with vehicle (data not shown), which suggests that induction of apoptosis by rapamycin did not contribute to inhibition of tumor growth in this study.

Decreased size of NNK-induced lung tumors correlated with mTOR inhibition because rapamycin decreased phosphorylation of S6 in normal bronchial epithelium and lung tumors from treated animals (Fig. 2A). In addition, decreased phosphorylation of S6 was observed in hepatic lysates from rapamycin-treated animals (Fig. 2B). Thus, systemic administration of rapamycin inhibited mTOR in multiple tissues. Because inhibition of mTOR by rapamycin can be associated with feedback activation of Akt (18), we investigated the effect of rapamycin on Akt phosphorylation in our model. Decreased phosphorylation of Akt at S473 (P = 0.05) but not T308 was observed (Fig. 2C). We also investigated changes in phosphorylation of extracellular signal-regulated kinase, which also regulates proliferation (19). No differences in activation of extracellular signal-regulated kinase with rapamycin treatment were observed (Fig. 2D).

These results show that mTOR activity is required for the maintenance of established NNK-induced tumors and show that rapamycin slows tumor growth by inhibiting cell proliferation. Moreover, they suggest that feedback activation of Akt and/or compensatory activation of the extracellular signal-regulated kinase pathway may not be relevant in this model.

Optimization of rapamycin dosing regimen. Because rapamycin was given 5 days of every 7 days in the first study, and because the response to rapamycin was modest, we assessed the dosing of rapamycin. A daily (5 of 7 days) regimen includes a 72-h washout period, raising the possibility that mTOR activity could recover during this time. Therefore, we compared an every-other-day (qod) regimen with the daily (5 of 7 days) regimen. A/J mice were treated over 1 week with rapamycin given every day or qod for 5 days (Fig. 3A). mTOR inhibition in lungs and surrogate tissues as well as blood levels of rapamycin were determined 4 h after the last dose on day 5 and at the end of each rest period that would correspond to the drug nadir. For the daily (5 of 7 days) regimen, this corresponds to 72 h after the last dose of rapamycin, and for the qod regimen, this corresponds to 48 h after the last dose of rapamycin.

Immunohistochemical analysis of phosphorylated S6 in large and small airways revealed that mTOR was inhibited by either regimen 4 h after rapamycin administration (Fig. 3B, *4-6* and *10-12*). Inhibition of mTOR correlated with average rapamycin plasma levels of 1,488 and 777 ng/mL for the every day versus qod regimen, respectively. The fact that inhibition was comparable despite rapamycin levels that differed by ~2-fold

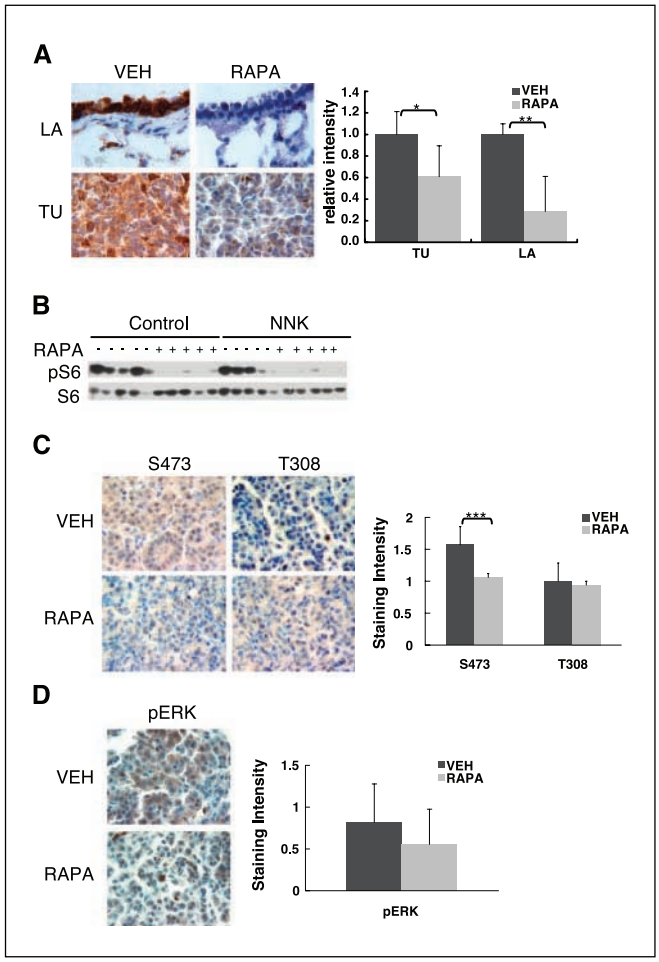

Fig. 2. Biomarker modulation after treatment with rapamycin given on a daily (5 of 7 d) regimen. *A,* representative images of phosphorylated S6 in NNK-induced lung tumors (*TU*) and large airways (*LA*) in the absence (*VEH*) or presence of rapamycin (*RAPA*). The relative intensity of staining for S6 phosphorylation in large airways and adenomas was averaged for five mice per group. *, $P < 0.01$; **, $P = 0.005$. *B,* immunoblotting of phosphorylated S6 (*pS6*) and total S6 in hepatic lysates. Each lane represents lysate from one mouse. *C* and *D,* activation of Akt and extracellular signal-regulated kinase (*ERK*) in NNK-induced lung tumors. Representative images of phosphorylation of Akt at S473 orT308 (*C*) or extracellular signal-regulated kinase (*D*) in lung tumors in the absence (*VEH*) or presence of rapamycin treatment (*RAPA*). The relative intensity of staining for Akt or extracellular signal-regulated kinase phosphorylation (*pERK*) in adenomas was averaged for five mice per group. ***, $P = 0.05$. Staining for Akt and extracellular signal-regulated kinase was predominantly cytoplasmic.

suggests that exceeding the cumulative weekly dose of 4.5 mg/kg in the qod group does not result in greater mTOR inhibition. In spite of similar inhibition on day 5, recovery of mTOR activity was different with each regimen. Phosphorylated S6 recovered to control levels 72 h after the last dose of daily rapamycin (Fig. 3B, *7-9*). This corresponded to rapamycin levels that were undetectable. On the other hand, 48 h after the last dose of qod rapamycin, recovery of S6 phosphorylation was incomplete. It had completely recovered in one mouse but had only partially recovered in the remaining two mice (Fig. 3B, *13-15*). Rapamycin levels in this group were 16.4 ng/mL, indicating that the drug nadir was higher, and that mTOR inhibition was better sustained after the washout period in the qod schedule.

These results were confirmed by immunoblot analysis of phosphorylated S6 and 4E-binding protein 1 in hepatic lysates

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(Fig. 3C). Phosphorylation of S6 was inhibited by either regimen of rapamycin administration 4 h after the last dose on day 5, but recovery was different. S6 phosphorylation recovered less with the qod schedule. Interestingly, phosphorylation of 4E-binding protein 1 did not recover during the washout period with either regimen. The basis for these differences in the recovery of phosphorylation of S6 and 4E-binding protein 1 is unclear, but it suggests that S6 phosphorylation might more accurately reflect rapamycin levels than phosphorylation of 4E-binding protein 1. These data indicate that qod dosing of rapamycin maintains mTOR inhibition in multiple tissues better than daily (5 of 7 days) dosing. Therefore, a study to assess the efficacy of qod rapamycin administration was done.

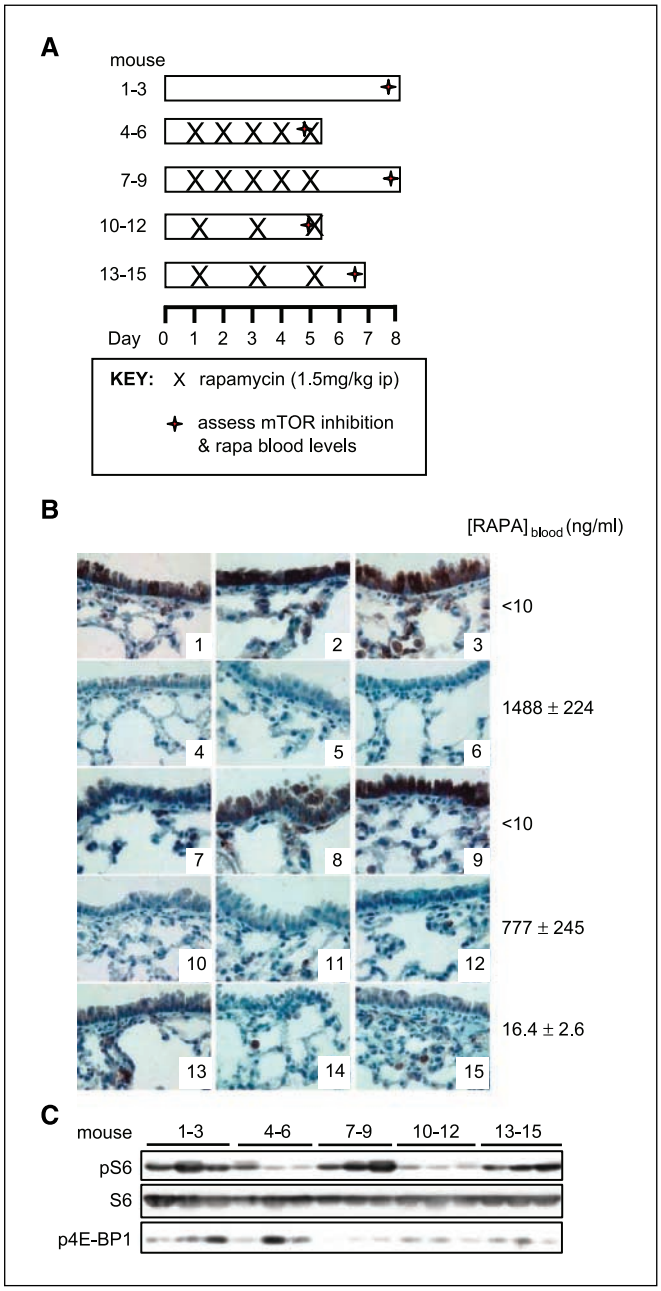

Fig. 3. Comparison of two dosing schedules of rapamycin. *A,* experimental schema. Mice were treated with rapamycin by i.p. injection 5 of 7 d (*mice 4-9*) or qod (*mice 10-15*). *B,* phosphorylation of S6 was assessed by immunohistochemistry in untreated controls (*1-3*), 4 h after treatment (*4-6* and *10-12*), or following the washout period of 72 h (*7-9*) or 48 h (*13-15*). Mean rapamycin blood levels (ng/mL) for each group (*right*). *C,* immunoblotting for levels of total S6 and phosphorylated S6 and 4E-binding protein 1 (*p4E-BP1*) in hepatic lysates. Mouse designation is as above.

Continuous treatment with rapamycin prevents NNK-induced tumor development. To increase tumorigenesis and increase the likelihood of seeing differences in tumor multiplicity, A/J mice were exposed to three doses of NNK (100 mg/kg) or vehicle given once a week for 3 weeks (Fig. 4A). Beginning 1 week after NNK administration, mice were treated with rapamycin qod for 12 weeks, when the study was terminated.

Rapamycin decreased the number of NNK-induced tumors by 90% (Fig. 4B). Mice that received NNK without rapamycin had an average of 20.8 tumors per mouse, whereas those that were treated with rapamycin developed an average of 2.2 tumors per mouse. In addition to reducing tumor multiplicity, tumors arising in the presence of rapamycin were 74% smaller than their untreated counterparts (Fig. 4C). Histologic analysis of NNK-induced lung lesions revealed that rapamycin treatment decreased the proportion of hyperplastic lesions by 45% and the proportion of adenomas by 82%, indicating that rapamycin reduced the phenotypic progression of NNK-induced lung lesions (Fig. 4D). Similar to results obtained with rapamycin treatment of established tumors, staining for the nuclear-specific antigen Ki-67 revealed a significantly lower rate of proliferation in rapamycin-treated tumors compared with their untreated counterparts (Fig. 4E). Moreover, terminal deoxynucleotidyl transferase – mediated nick-end labeling staining showed no difference in the rate of apoptosis between tumors arising in the presence and absence of rapamycin (data not shown).

Rapamycin exposure was associated with lower levels of phosphorylated S6 in tumors and surrogate tissues. Tumors arising in the presence of rapamycin had less intense staining for phosphorylated S6 ($P < 0.0001$; Fig. 5A). In addition, decreased activity of mTOR was observed in hepatic lysates (Fig. 5B). Unlike the first study, no differences in S473 phosphorylation of Akt were observed in tumors arising in the presence or absence of rapamycin (Fig. 5C). Because lesions in the first study were tumors, and because the lesions arising in this study included tumors as well as hyperplastic lesions, we analyzed S473 phosphorylation in these two populations (Fig. 5C). No difference in S473 phosphorylation was observed in either population, suggesting that tumors that develop in the presence of rapamycin are able to maintain S473 phosphorylation, unlike established tumors. Similar to the first study, decreased proliferation was not accompanied by decreased extracellular signal-regulated kinase phosphorylation (Fig. 5D). Because decreased lung tumorigenesis can be related to decreases in infiltrating macrophages (20), we also did immunohistochemistry with an antibody that assesses a macrophage marker (F4-80) to assess the number of infiltrating macrophages in lung tumors and surrounding lung tissues. In neither compartment did rapamycin decrease the number of infiltrating macrophages (Fig. 5E). These results show that rapamycin given on a qod schedule can prevent the formation and growth of NNK-induced tumors. Taken together with results from the first tumorigenesis study, these data indicate that NNK-induced tumors are dependent on mTOR for tumor development and maintenance.

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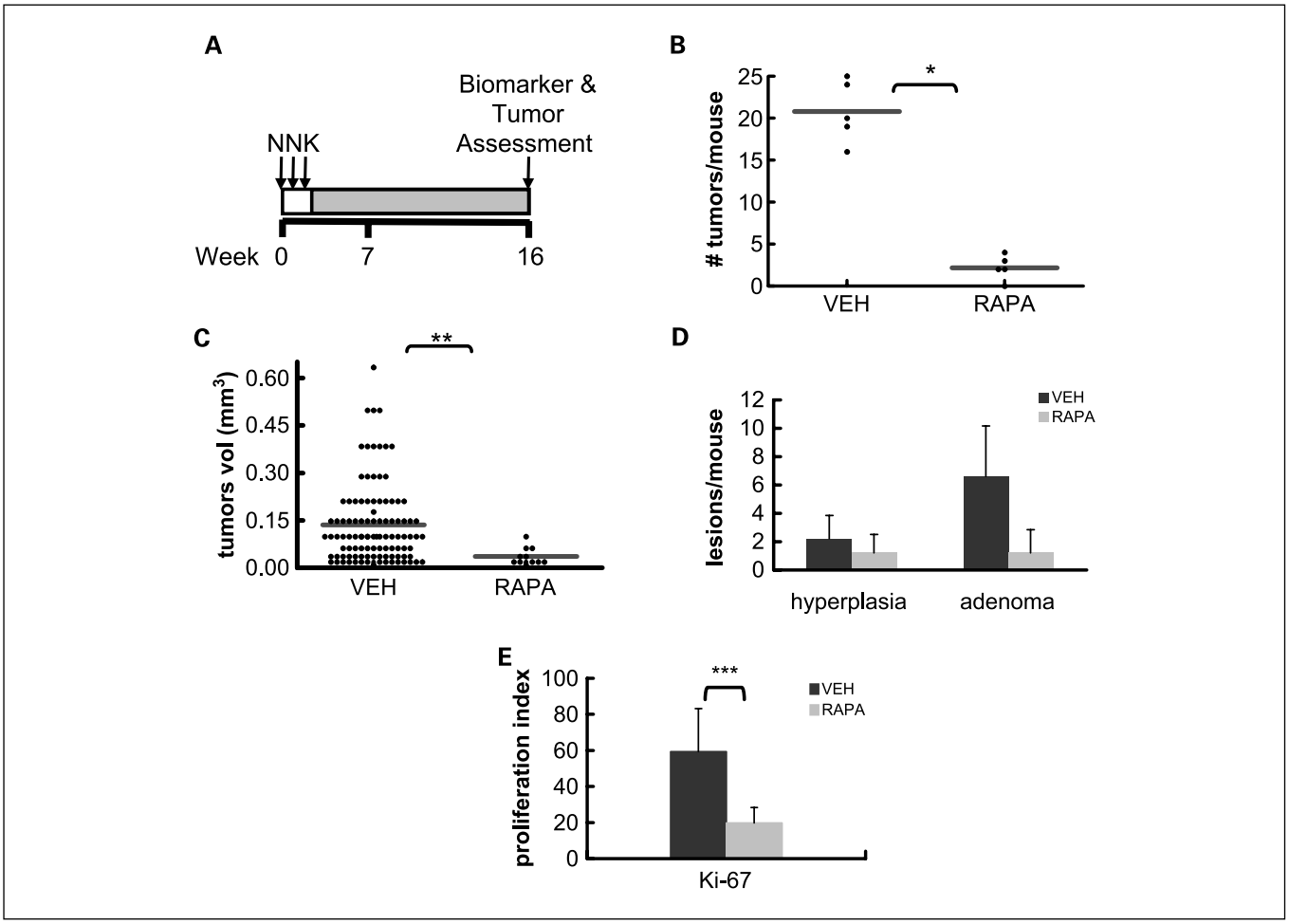

Fig. 4. Continuous treatment with rapamycin prevents the development of NNK-induced tumors. *A,* experimental schema. *B,* tumor incidence and multiplicity. *, $P < 0.0001$. *C,* tumor size. **, $P < 0.0001$. *D,* phenotypic progression. *E,* cellular proliferation index (number of Ki-67 positive cells per tumor). ***, $P < 0.0001$.

Discussion

These studies show that mTOR is required in the earliest stages of NNK-induced tumor development and suggest that mTOR inhibition is a possible strategy to prevent the development and progression of tobacco carcinogen–induced lung tumors. Continuous treatment with rapamycin given before the development of observable tumors prevented the development of 90% of NNK-induced tumors. Continuous treatment is likely necessary because rapamycin that was administered for only 6 weeks, beginning 1 week after NNK, followed by 12 weeks of observation did not decrease tumor size or multiplicity, despite mTOR inhibition at the end of the treatment period (data not shown). This observation suggests that activation of mTOR is a critical early event in NNK-induced tumorigenesis, and that continuous treatment can prevent or delay tobacco carcinogen–induced tumor development. In addition to preventing tumorigenesis, our data show that administration of rapamycin to established NNK-induced tumors decreases tumor size but does not affect tumor multiplicity. This is consistent with a study by Yan et al. who showed that treatment with a higher dose of rapamycin (2.0 mg/kg) on a daily (5 of 7 days) schedule for 14 weeks, beginning 12 weeks after administration of a polycyclic aromatic hydrocarbon [benzo(*a*)pyrene], reduced lung tumor load by 84% (21). Similar to our results, Yan et al. did not observe a reduction in tumor multiplicity. However, they did not assess mTOR inhibition. Thus, our data closely link inhibition of mTOR signaling during the earliest stages of NNK-induced tumorigenesis with decreased tumor multiplicity.

One critical issue in the development of mTOR inhibitors as preventive or therapeutic agents is elucidating a biomarker for response. Phosphorylation of S6 is the most commonly evaluated biomarker for mTOR inhibition by rapamycin or rapamycin analogues such as CCI-779 or RAD001 (22). In some studies, responsiveness to mTOR inhibition has been associated with changes in other biomarkers such as hypoxia-inducible factor-1α, erbB3, or Akt (23, 24). In our studies, there were no differences in circulating levels of a hypoxia-inducible factor-1α target, vascular endothelial growth factor, or in expression of erbB3 (data not shown). Assessment of Akt phosphorylation revealed that there was no change in phosphorylation of Akt at T308 with rapamycin. However, the status of Akt phosphorylation at S473 was different if rapamycin was used to treat established tumors or prevent tumor development. In established tumors, S473 phosphorylation was decreased by rapamycin treatment, suggesting that

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long-term rapamycin treatment may result in inhibition of mTOR/rictor (TORC2) complexes that can phosphorylate Akt at S473 (25). On the other hand, there was no difference in the intensity of staining for S473 phosphorylation in tumors that developed during 12 weeks of rapamycin treatment. There are potential explanations for these observations. In the first study, established tumors were treated with rapamycin, whereas in the second study, tumors arose in the presence or absence of

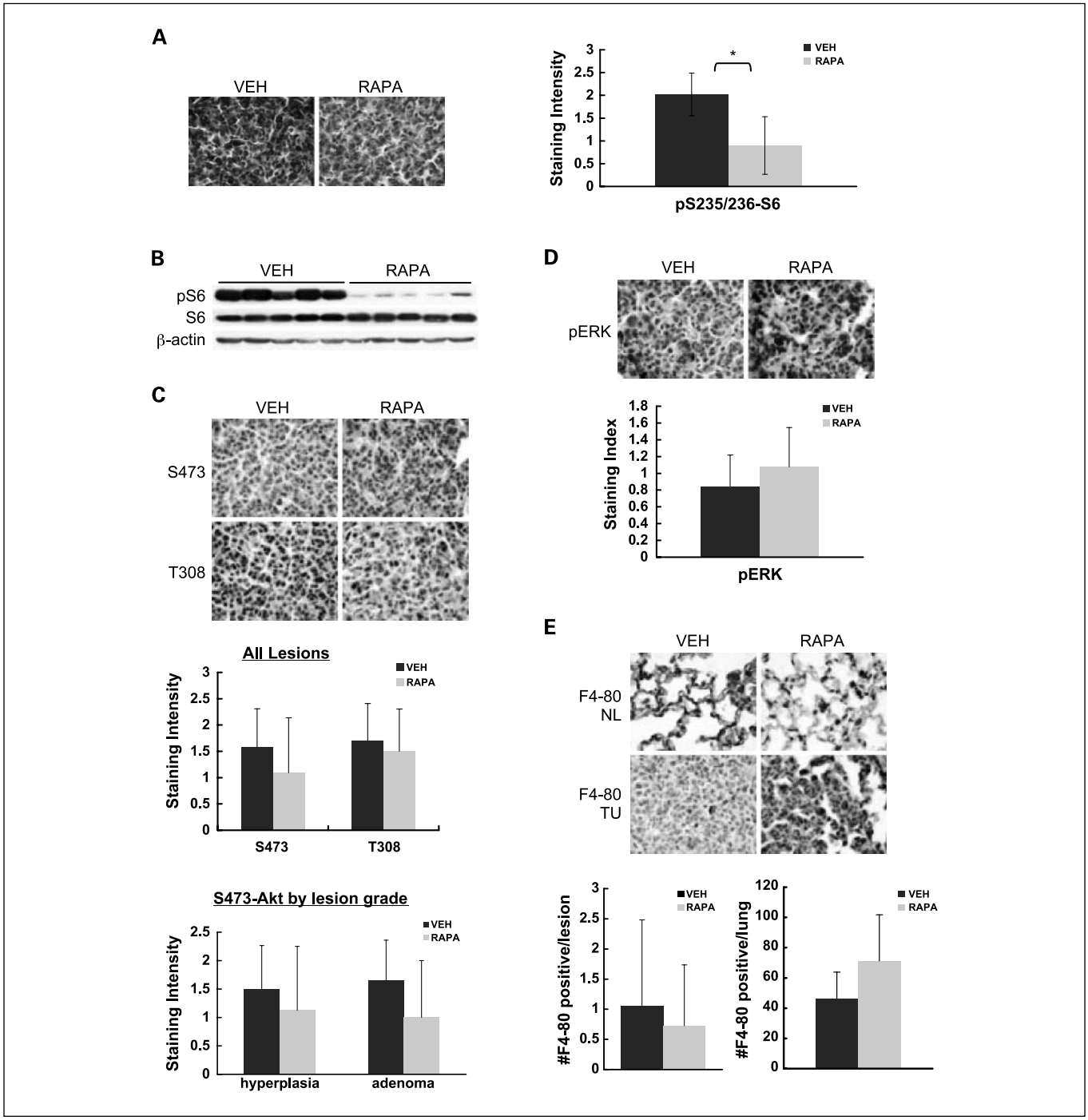

Fig. 5. Biomarker modulation after treatment with rapamycin given on a qod regimen. *A,* representative images of phosphorylated S6 in lung tumors arising in the absence (*VEH*) or presence of rapamycin treatment (*RAPA*). The percentage of tumor cells with S6 phosphorylation intensity of ≥2 was quantified for tumors from five mice per group, *, $P < 0.0001$. *B,* immunoblotting of phosphorylated and total S6 in hepatic lysates. Each lane represents lysate from one mouse. *C,* activation of Akt. Representative images of phosphorylated Akt at S473 and T308 in NNK-induced lung tumors in the absence or presence of rapamycin. Staining for phosphorylation of Akt revealed cytoplasmic, homogeneous staining throughout the tumors. The relative intensity of staining in all lesions was averaged for five mice per group, and for S473 phosphorylation, staining intensity was analyzed in hyperplasic lesions and tumors. *D,* activation of extracellular signal-regulated kinase. Representative images of phosphorylated ERK in NNK-induced lung tumors in the absence or presence of rapamycin. Staining for extracellular signal-regulated kinase phosphorylation was predominantly cytoplasmic but was more intense near the periphery of tumors. Thus, a staining index was used to account for intensity as well as distribution of staining. *E,* tumor-infiltrating macrophages. Representative images of staining for the macrophage antigen F4-80 in normal lung (*NL*) and tumors (*TU*) arising in the absence (*VEH*) or presence of rapamycin treatment (*RAPA*). The number of macrophages was quantified for surrounding lung tissues and for all NNK-induced lesions.

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rapamycin. Thus, rapamycin-resistant tumors in the second study may develop through a different mechanism that increases S473 phosphorylation in an mTOR-independent manner. Additionally, because lesions arising in the presence of rapamycin were primarily preneoplastic and not tumors, feedback regulation of the Akt/mTOR pathway could vary during different stages of tumor development.

In a transgenic model driven by somatic activation of K-Ras (26), Wislez et al. showed that treatment with CCI-779 decreased the number and size of lung tumors that was associated with decreased S6 phosphorylation and macrophage infiltration (20). Despite similar decreases in S6 phosphorylation in this study and our studies, rapamycin did not decrease macrophage infiltration in our studies. This suggests that although K-Ras mutations are characteristic of the tumors induced in each model system, differences in infiltrating macrophages could be related to possible K-Ras mutations in the macrophages in the Wizlez et al. study, different tumorigenic events in each system, strain background, or the timing of assessment of macrophage infiltration. Overall, S6 phosphorylation was the best biomarker in our studies, as levels of phosphorylated S6 correlated well with plasma rapamycin levels.

Combined with the results obtained by Wislez et al. (20) and Yan et al. (21), our data provide a strong rationale to consider testing rapamycin in smokers who are at high risk to develop lung cancer. What are the issues that limit the application of these findings to a clinical trial? Perhaps the most practical issue is whether doses of rapamycin that achieve an antitumor effect in mice are achievable in humans. Our pharmacokinetic study of two dosing regimens showed that although peak concentrations of 777 to 1,488 ng/mL were achieved, mTOR inhibition was maintained at trough rapamycin levels of 16.4 ng/mL with qod dosing. This concentration is achievable in humans at doses used for immunosuppression (27), but these levels were attained in conjunction with other immunosuppressive agents with the intent to maximize immunosuppression, raising the issue of whether or not doses of rapamycin that have antitumor efficacy also cause immune suppression.

A second issue that could limit application of any preventive agent is toxicity. In addition to immunosuppression, other toxicities associated with administration of rapamycin or its analogues include vomiting, diarrhea, thrombocytopenia, rash, stomatitis, and hyperlipidemia (28). These toxicities differ from those associated with other preventive agents. For example, selective estrogen receptor modulators, such as tamoxifen, can reduce the incidence of breast cancer, but tamoxifen is associated with increased risk of endometrial cancer and thromboembolic events (29), thus limiting its use despite its effective chemopreventive activity. Raloxifene, another selective estrogen receptor modulator, has similar chemopreventive activity that is associated with reduced toxicities, raising the possibility that improved toxicity profiles might be achieved with modified selective estrogen receptor modulators or other classes of drugs such as retinoids. The retinoid fenretinide reduces the risk of developing a second breast cancer in premenopausal women (30). Its use is associated with decreased dark adaptation, dermatologic abnormalities and, to a lesser extent, gastrointestinal dysfunction and visual toxicities. Related rexinoid compounds, such as bexarotene, have an improved toxicity profile and therefore may have promise as chemopreventive agents for many types of cancer, including lung cancer (31). Perhaps the most sobering lesson regarding toxicity of preventive agents was learned through the experience with inhibitors of cyclooxygenase-2. Although cyclooxygenase-2 inhibitors can prevent the formation of adenomatous colonic polyps in populations at risk (32, 33), long-term use of cyclooxygenase-2 inhibitors is associated with an increased risk of cardiovascular toxicities, including death (34). Thus, risk/benefit ratios must continually be assessed for patients receiving chemopreventive agents.

Of the toxicities associated with rapamycin, the immunosuppressive effects of rapamycin are most important. Rapamycin (sirolimus) has a "black-box warning" from the Food and Drug Administration stating that its use is associated with increased risk of lymphoma development.[5] However, the studies that provided the basis for that warning were done in combination with other immunosuppressive agents such as cyclosporine, making it difficult to attribute the relative contribution of rapamycin alone to the development of lymphoma. Although cyclosporine has been associated with tumor promotion (35, 36), the use of mTOR inhibitors in transplant patients may be associated with tumor inhibition. For example, a recent analysis of cancer incidence in over 30,000 kidney allograft recipients reported a 3-fold lower incidence of all *de novo* malignancies in patients that were maintained on rapamycin as part of their therapy than those that did not receive rapamycin (37). This observation raises the possibility that rapamycin administered as a single agent may not be associated with an increased risk of lymphoma or other cancers. Still, the potential for harm suggests that only those smokers at highest risk should be initially enrolled in prevention trials with rapamycin. The highest-risk groups could include those that bear polymorphisms in genes that control DNA repair and/or the metabolic activation and detoxification of tobacco carcinogens (38), those who have an inherited susceptibility to lung cancer (39), and those that have the greatest exposure to tobacco carcinogens, which can be measured in the urine and blood of smokers, and those exposed to environmental tobacco smoke (40, 41). Interestingly, for people with an inherited susceptibility to lung cancer linked to chromosome 6q (39), the dose-response relationship with tobacco exposure and lung cancer is absent, suggesting that there is no amount of exposure that is safe in this population (42). Successful delineation of the immunosuppressive effects of rapamycin from its effects on tumorigenesis would help establish a favorable risk/benefit ratio and would facilitate testing rapamycin in patients with a history of tobacco exposure at increased risk to develop lung cancer. Given the disappointing results of prior clinical lung cancer prevention trials (43, 44), safe and effective approaches are needed for this patient population.

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[5] http://www.fda.gov/ohrms/dockets/ac/02/briefing/3832b1_04_FDA-Rapamune-Backgrounder.htm

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Identification of a Highly Effective Rapamycin Schedule that Markedly Reduces the Size, Multiplicity, and Phenotypic Progression of Tobacco Carcinogen–Induced Murine Lung Tumors

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