Small Molecule Therapeutics

Aerosol Administration of Phospho-Sulindac Inhibits Lung Tumorigenesis

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Abstract
Phospho-sulindac is a sulindac derivative with promising anticancer activity in lung cancer, but its limited metabolic stability presents a major challenge for systemic therapy. We reasoned that inhalation delivery of phospho-sulindac might overcome first-pass metabolism and produce high levels of intact drug in lung tumors. Here, we developed a system for aerosolization of phospho-sulindac and evaluated the antitumor efficacy of inhaled phospho-sulindac in an orthotopic model of human non–small cell lung cancer (A549 cells). We found that administration by inhalation delivered high levels of phospho-sulindac to the lungs and minimized its hydrolysis to less active metabolites. Consequently, inhaled phospho-sulindac (6.5 mg/kg) was highly effective in inhibiting lung tumorigenesis (75%; P < 0.01) and significantly improved the survival of mice bearing orthotopic A549 xenografts. Mechanistically, phospho-sulindac suppressed lung tumorigenesis by (i) inhibiting EGF receptor (EGFR) activation, leading to profound inhibition of Raf/MEK/ERK and PI3K/AKT/mTOR survival cascades; (ii) inducing oxidative stress, which provokes the collapse of mitochondrial membrane potential and mitochondria-dependent cell death; and (iii) inducing autophagic cell death. Our data establish that inhalation delivery of phospho-sulindac is an efficacious approach to the control of lung cancer, which merits further evaluation.

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Introduction
Lung cancer is the leading cause of cancer-related deaths worldwide, with non–small cell lung cancer (NSCLC) accounting for the majority of the cases (80%; ref. 1). Despite advances in chemotherapy and the recent advent of molecularly targeted drugs, patients diagnosed with advanced NSCLC only have a median survival time of 8 to 11 months (2). Given its poor prognosis, novel agents for lung cancer control are urgently needed.

Nonsteroidal anti-inflammatory drugs (NSAID) are promising agents in the control of lung cancer. Epidemiologic data suggest that NSAIDs can decrease the risk of lung cancer among smokers (3); although recent evidence suggests that individual NSAIDs may have contrasting effects on lung cancer risk and survival (4). Sulindac and its sulfone metabolite are efficacious in suppressing lung tumorigenesis in preclinical models (5, 6). Long-term use of NSAIDs, however, is associated with gastrointestinal and renal toxicities (7). Our group has developed phospho-sulindac (Fig. 1A), a sulindac derivative that is safe and effective against colon cancer (8–10). In our continuing effort to evaluate the anticancer activity of phospho-sulindac, the present study examined the activity of phospho-sulindac against human NSCLC in cell culture and in an orthotopic mouse model of NSCLC.

Inhalation administration represents an attractive strategy for the targeted prevention and treatment of lung cancer (11, 12). Aerosol delivery of drugs achieves high local drug levels at the lung epithelium and minimizes systemic exposure (13). A major challenge with effective delivery of phospho-sulindac to the target site(s) is its rapid inactivation by nonspecific esterases (14). We reasoned that delivery of phospho-sulindac via inhalation will bypass the liver and gastrointestinal tract, major sites of esterase expression, and hence will require much lower doses to deliver intact phospho-sulindac to the lungs.

Aberrant activation of the EGF receptor (EGFR) signaling cascades is a hallmark of NSCLC (15). The Raf/MEK/ERK pathway is a key downstream effector of the EGFR that is activated in the majority of NSCLCs (16). PI3K/AKT/mTOR is another downstream survival pathway that is constitutively activated in more than 50% of NSCLCs (17). The pharmacologic inhibition of EGFR-
dependent survival signaling may thus suppress tumor growth, manifested in the induction of apoptosis and/or autophagy (18).

Herein, we show that inhalation delivery of phospho-sulindac results in high levels of intact drug in the lungs and that inhaled phospho-sulindac is highly effective in inhibiting lung tumorigenesis in vivo. Mechanistically, phospho-sulindac inhibits EGFR-dependent survival cascades in NSCLC cells and induces oxidative stress to ultimately induce apoptosis/autophagy, leading to the potent inhibition of tumor growth. The findings of this study suggest that phospho-sulindac is a promising candidate for the development of an effective and safe drug for the control of NSCLC.

Materials and Methods

Reagents

Phospho-sulindac was a gift from Medicon Pharmaceuticals, Inc. All reagents used in the study are of analytic grade. All other chemicals, unless otherwise stated, were from Sigma-Aldrich.

Cell culture

Human NSCLC cell lines A549, H23, and H358 were from American Type Culture collection (ATCC), which characterized these cell lines using cytogenetic analysis. These cell lines were grown as recommended by ATCC. A549-GFP cells were generated by transfection with GFP-lentiviral particles and selection with Geneticin. The
highest 1% GFP-expressing cells sorted by flow cytometry were used for in vivo studies.

**Cytokinetic analyses**

Cell viability was determined by a modified MTT assay (8). Apoptosis and cell proliferation were assessed by Annexin V/propidium iodide (PI) staining (Life Technologies) and the bromodeoxyuridine (BrdUrd) incorporation method (BD Biosciences), respectively (19). Autophagy vacuolization was determined by acridine orange staining (20).

**Analysis of ROS induction**

Reactive oxygen species (ROS) levels were determined by staining with 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA; 10 μmol/L) for 30 minutes; mitochondrial superoxide anion levels were determined by staining with 5 μmol/L MitoSOX Red for 30 minutes at 37°C, and their fluorescence intensity was measured by flow cytometry.

**Mitochondria-depleted cells**

Mitochondria-depleted (p0) derivatives of A549 cells were generated by incubation with 200 ng/mL ethidium bromide, 50 μg/mL uridine, and 1 μmol/L sodium pyruvate for 8 weeks as previously described (21).

**siRNA silencing of EGFR**

A549 cells were transfected with 10 nmol/L EGFR- or control-siRNA (Applied Biosystems) using Lipofectamine 2000 (Life Technologies). The cells were treated with phospho-sulindac 72 hours posttransfection.

**Aerosol drug delivery**

The set up for aerosol drug delivery is shown in Supplementary Fig. S1. Inhalation delivery conditions (50 mg/mL phospho-sulindac in ethanol, 8-minute exposure time) were optimized in pilot studies (Supplementary Fig. S2). The estimated total amount of phospho-sulindac deposited in the lungs of mice in 8 minutes was 15 mg/kg/d and the estimated dose of treatment was 6.5 mg/kg/d.

**Pharmacokinetic analyses**

Mice were exposed to aerosolized phospho-sulindac (50 mg/mL phospho-sulindac in ethanol, 8-minute exposure) and euthanized at designated time points. Their plasma and lungs were collected and the levels of phospho-sulindac and its metabolites were determined by high-performance liquid chromatography (HPLC; ref. 22).

**Efficacy study in an orthotopic lung cancer model**

Seven-week-old BALB/c nude mice (Harlan Inc.) were pretreated for 5 days with aerosol generated from vehicle (ethanol) or 50 mg/mL phospho-sulindac solution (n = 15/group). On day 6, A549-GFP cells (1.5 × 106/mouse) were intrapulmonarily injected into the left lung parenchyma of the mice (23). Inhalation treatment (5 times/week) was resumed 3 days later. After 8 weeks, the mice were euthanized, and blood and lung tissues were collected. Images of the lungs were taken on a fluorescence imaging system (Maestro). All animal experiments were carried out with the approval of the Institutional Animal Care and Use Committee, State University of New York (Stony Brook, NY).

**Analysis of in vivo oxidative stress**

The effect of phospho-sulindac on the redox state of the mice bearing orthotopic lung tumors was assessed by measuring the levels of plasma 8-iso-prostaglandin F2α (8-iso-PGF2α) using an ELISA kit (Enzo Life Sciences).

**Immunohistochemistry of orthotopic lung tumor tissues**

Immunohistochemical staining of paraffin-embedded tumor sections from vehicle- and phospho-sulindac-treated mice were conducted as previously described (9). Proliferating and apoptotic cells were stained using anti-proliferating cell nuclear antigen (PCNA) and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), respectively.

**Statistical analysis**

Differences between experimental groups were calculated using Student t test. P values less than 0.05 were considered significant. Comparison of the survival rate between the control and treatment group was conducted with the Kaplan–Meier method. Z > 1.96 was considered significant.

**Results**

**Phospho-sulindac inhibits the growth of NSCLC cell lines in vitro**

To study the effect of phospho-sulindac (Fig. 1A) on cell growth, we determined its 24-hour IC50 values in NSCLC cell lines (Fig. 1B and Supplementary Table S1). The IC50 values of phospho-sulindac varied little (75–94 μmol/L), considerably more potent in inhibiting NSCLC cells compared with conventional sulindac (IC50 > 1,000 μmol/L), representing a potency enhancement of more than 12-fold. The IC50 values of sulindac metabolites sulindac sulfone (566–1,895 μmol/L) and sulindac sulfide (159–222 μmol/L) in these cell lines were also much higher compared with those of phospho-sulindac. Phospho-sulindac thus has a much more potent effect on lung cancer cells,
although it cannot be ruled out that sulindac may exert its anticaner effect through alternative mechanisms, such as affecting the tumor microenvironment.

We further examined the cytokinetic effect of phosphosulindac in vitro (Fig. 1C–E). Phosphosulindac markedly reduced cell proliferation. At 1×IC_{50}, phosphosulindac inhibited the proliferation of A549, H23, and H358 cells by 94%, 49%, and 80%, respectively. Phosphosulindac blocked cell-cycle progression (G2–M arrest), leading to significant accumulation of cells in the G2–M phase in all 3 NSCLC cell lines. Phosphosulindac significantly induced apoptosis in NSCLC cells. At 1×IC_{50}, apoptosis [Annexin V (+) cells] was induced by 7-fold in A549 cells and 2-fold in H358 cells, whereas at 1.5×IC_{50}, apoptosis was induced by 26-fold in A549 cells and 9-fold in H358 cells. However, phosphosulindac did not appreciably induce apoptosis in H23 cells. Phosphosulindac also induced autophagy, evidenced by the formation of acidic vesicular organelles, widely accepted as a hallmark for autophagy in mammalian cells (ref. 24; Fig. 1E and Supplementary Fig. S3).

To better understand the role of autophagy in the growth inhibitory effect of phosphosulindac, we compared the cytotoxicity of phosphosulindac in A549 cells in the presence and absence of 3-MA, a specific inhibitor of autophagosome formation (25). Blockade of autophagy by 3-MA significantly reduced the cytotoxicity of phosphosulindac as reflected in the 50% increase in its IC_{50} (Fig. 1E). Thus, the induction of autophagy plays an important role in mediating the cytotoxic effect of phosphosulindac. Our results indicate that phosphosulindac inhibits the growth of NSCLC cells in vitro via a potent cytokinetic effect, which includes suppression of cell proliferation and induction of apoptotic and autophagic cell death.

**Inhalation treatment effectively delivers intact phosphosulindac to the lungs**

Given that phosphosulindac is much more potent than its metabolites in inhibiting NSCLC, we reasoned that the efficacy of phosphosulindac could be improved by inhalation administration, which bypasses the major sites of drug metabolism and results in the direct delivery of phosphosulindac to the lungs. We used a nose-only inhalation exposure system, which was optimized for the following parameters: particle size distribution, concentration of drug solution for atomization, and exposure time (Supplementary Fig. S2).

The optimized system (50 mg/mL phosphosulindac, 8-minute exposure) generated promising pharmacokinetic profiles in terms of the delivery of intact phosphosulindac to the lungs (Supplementary Fig. S2D and Supplementary Table S2). The highest concentration of phosphosulindac was 22.2 nmol/g lung tissue right after inhalation treatment (T_{max} = 0 hour), which decreased toward later time points and was undetectable after 24 hours. The area under curve (AUC_{0–24}) was 7.7 nmol/g lung tissue h. Of note, intact phosphosulindac persisted in the lungs even 2 hours after inhalation treatment.

**Inhaled phospho-sulindac inhibits lung cancer growth in vivo**

We next evaluated the efficacy of inhaled phosphosulindac in vivo using an orthotopic lung cancer model in nude mice. In this model, the mice developed lung tumors 2 to 3 weeks after intrapulmonary implantation of A549 cells (26). Compared with control, the phosphosulindac–treated group showed 75% (P < 0.001) reduction in tumor volume assessed by GFP luminosity at sacrifice (Fig. 2A). Lung weight (including cancerous and noncancerous tissues) was also determined to analyze the relative tumor load in the control and treatment groups. The lung weight from another group of mice not implanted with cancer cells or subjected to vehicle or phosphosulindac treatment served as the normal control. The lung weight of the vehicle-treated group was 2.6-fold higher than that of normal mice (Supplementary Fig. S4A). Phosphosulindac inhalation treatment maintained lung weight near the normal levels. Phosphosulindac inhalation treatment also improved the overall survival of mice with orthotopic lung tumors. By week 8, 33% (5 of 15) of the mice in the control group died from the disease, whereas only 6% (1 of 15) in the treatment group died (Supplementary Fig. S4B and S4C).

The potent inhibition of tumorigenesis was likely a result of the delivery of high levels of intact phosphosulindac to the lung tumors. Figure 2B displays the relative levels of phosphosulindac and its metabolites in the tumor tissues. Phosphosulindac was the dominant form identified (>10 nmol/g), followed by sulindac, sulindac sulfone, and sulindac sulfide. It merits emphasis that oral gavage of phosphosulindac (even at much higher doses) could not deliver comparable amounts of intact phosphosulindac to the lungs (Supplementary Fig. S5). Consequently, oral phosphosulindac treatment only moderately inhibited (30%; P < 0.05) the growth of A549 xenografts in nude mice (Supplementary Fig. S6). We have also evaluated the delivery of phosphosulindac to the lungs via intraperitoneal and intravenous administration. In agreement with our expectation, none of these alternative routes delivered significant amounts of intact phosphosulindac to the lungs, which were less than 12% of that achieved with inhalation (Supplementary Fig. S6C). Phosphosulindac exerted a strong cytokinetic effect on the orthotopic lung tumors. Immunohistochemical analyses (Fig. 2C) revealed that phosphosulindac significantly inhibited cell proliferation (P = 0.018) and induced apoptosis (P = 0.024) in the tumor cells in vivo.

**Phosphosulindac suppresses EGFR activation**

Antibody microarray analysis (Kinexus Bioinformatics Corporation) of tumor lysates from vehicle- and phosphosulindac–treated mice revealed significant changes in EGFR signaling pathways. Ablative activation of EGFR signaling has important implications for the pathogenesis of NSCLC (15, 27). We first explored in A549 cells in vitro the effect of phosphosulindac on the level of EGFR phosphorylation and the expression of ADAM10 and
ADAM17, known to induce EGFR activation through ligand cleavage (refs. 28, 29; Fig. 3A). Phospho-sulindac significantly reduced EGFR phosphorylation (Y1068) starting 1 hour posttreatment and the effect became more pronounced toward the later time points. Quantitative analysis of p-EGFR expression by flow cytometry also revealed a significant reduction after 16-hour treatment with phospho-sulindac. In vitro assay showed that phospho-sulindac did not modulate the kinase activity of EGFR (data not shown; EGFR KinEASE FP Fluorescein Green Assay; Upstate Cell Signaling Solutions). Phospho-sulindac moderately decreased the level of ADAM17, starting 8 hours after treatment. Phospho-sulindac, however, did not modulate the levels of ADAM10 or Notch1.

Phospho-sulindac also suppressed EGFR activation in vivo. We compared the levels of ADAM17 in lung xenografts between the vehicle- and phospho-sulindac-treated groups (Fig. 3B). Immunoblots showed that phospho-sulindac significantly reduced the level of ADAM17 by 40%, which presumably led to a significant reduction of EGFR phosphorylation (58%) and activation. Immunohistochemical studies also showed a significant reduction in the expression of p-EGFR (P < 0.038) in the phospho-sulindac-treated group compared with the control (Supplementary Fig. S7A).

Interestingly, the levels of p-EGFR were much lower in responders (small tumors) than those in nonresponders (large tumors). A significant correlation was found between tumor load (GFP luminosity) and p-EGFR level (Western blot, densitometry; Supplementary Fig. S7B). siRNA knockdown of EGFR sensitized A549 cells to phospho-sulindac (Fig. 3C). IC_{50} value (24-hour) of phospho-sulindac in EGFR-knockdown A549 cells (68 μmol/L) was much lower compared with that in mock-siRNA-transfected cells (95 μmol/L). Consistent with this observation, treatment with equimolar phospho-sulindac (95 μmol/L)
resulted in a more than 2-fold higher apoptosis induction in EGFR-knockdown cells versus mock-transfected cells. We observed increased basal level of ROS in EGFR-knockdown A549 cells, which was intensified upon phospho-sulindac treatment (ref. 30; data not shown). This combined effect on ROS levels likely enhanced cell death further leading to decreased IC50 values.

To have a clearer understanding of the effect of phospho-sulindac on the activation of other HER family members in addition to EGFR, we conducted an antibody array assay (RayBiotech. Inc.) on lysates from vehicle- and phospho-sulindac–treated A549 cells. In agreement with previous studies (31), much higher levels of ErbB3 were observed relative to the other HER family members assayed. As shown in Fig. 3D, phospho-sulindac significantly decreased the expression of p-ErbB3 (Y1289).

**Phospho-sulindac inhibits the Raf/MEK/ERK and PI3K/AKT signaling cascades**

In A549 cells, inhibition of EGFR phosphorylation by phospho-sulindac resulted in sequential inactivation of downstream mitogen-activated protein kinases (MAPK) manifested in the decreased expression of p-c-Raf, p-MEK, and p-ERK (Fig. 4A and Supplementary Fig. S8). Of note, p-c-Raf was reduced by more than 80% relative to control 1-hour post-phospho-sulindac treatment and was nearly abrogated toward later time points. A similar dramatic inhibition was observed for p-MEK and p-ERK, but starting at later...
Figure 4. Phospho-sulindac (PS) inhibits Raf/MEK/ERK and PI3K/Akt/mTOR signaling and induces autophagy in vitro and in vivo. The inhibitory effect of phospho-sulindac on: (A) p-Raf/p-MEK/p-ERK, (B) p-ErbB3 and p-PI3K/p-Akt/p-S6K/p-4E-BP1, and (C) p-mTOR (S2448 and S2448/ Rictor/Raptor expression in A549 cells (1.5× IC50) in vitro. Densitometry results are shown as numbers (italics) below the immunoblots. D, phospho-sulindac increased conversion of LC3-I to LC3-II as indicated by the increased LC3-II:LC3-I ratios (italics). E, inhalation phospho-sulindac treatment significantly reduced phosphorylation of signaling components of Raf/MEK/ERK and PI3K/Akt pathways in orthotopic A549 tumors. F, inhalation phospho-sulindac treatment significantly reduced phosphorylation of signaling molecules of the PI3K/Akt pathway revealed substantially decreased expression of p-PI3K, p-Akt, p-mTOR, p-S6K, and p-4E-BP1 in A549 cells (Fig. 4B and C). Of note, as early as 1-hour post-phospho-sulindac treatment, the levels of most of the above signaling molecules were reduced by more than 80% as compared with control. As a consequence, phospho-sulindac exerted a potent inhibitory effect on cell survival and growth in vitro.

It is well established that mTOR negatively regulates autophagy (33). During autophagy, light chain 3 (LC3)-I, the cytosolic soluble form of microtubule-associated protein 1A/1B-LC3, is conjugated to phosphatidyethanolamine to form LC3-phosphatidyethanolamine conjugate (LC3-II) during autophagosome formation (34). Immunoblotting and densitometry analyses showed that phospho-sulindac significantly induced the conversion of LC3-I to LC3-II, an effect sustained throughout the time frame investigated (Fig. 4D).

Analyses of tumor samples confirmed our observations in vitro (Fig. 4E and F). Immunoblotting of tumor lysates showed that phospho-sulindac significantly (P < 0.05) downregulated the phosphorylation of EGFR and its downstream kinases such as mitogen-activated protein/extracellular signal-regulated kinase (MEK; 55%), extracellular signal-regulated kinase (ERK; 72%), AKT (S473, 60%; Th308, 39%), and mTOR (55%). In addition, tumors of the treatment group had significantly higher expression of p-ERK, reflecting a propagating signal.

ErbB3 (HER3) is the principal HER family member, which binds phosphoinositide 3-kinase (PI3K) and drives Akt signaling (32). Immunoblotting of the major signaling molecules of the PI3K/Akt pathway revealed substantially decreased expression of p-PI3K, p-Akt, p-mTOR, p-S6K, and p-4E-BP1 in A549 cells (Fig. 4B and C). Of note, as early as 1-hour post-phospho-sulindac treatment, the levels of most of the above signaling molecules were reduced by more than 80% as compared with control. As a consequence, phospho-sulindac exerted a potent inhibitory effect on cell survival and growth in vitro.

time points (4 hours for p-MEK and 8 hours for p-ERK), reflecting a propagating signal.

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LC3-II/LC3-I ratios. Taken together, phospho-sulindac strongly attenuated the Raf/MEK/ERK and PI3K/AKT/mTOR signaling cascades, which may explain the potent growth inhibition of phospho-sulindac in the orthotopic A549 xenografts.

**Phospho-sulindac induces oxidative stress and mitochondria-dependent cell death**

Induction of oxidative stress from the generation of ROS is a key mediator of cell death of phospho-NSAIDs (8, 9, 35). Using DCFDA, a general ROS probe, we showed that phospho-sulindac increased ROS levels in A549 cells by 1.2-fold at 1 \( \times \) IC\(_{50}\), and by more than 7-fold at 2 \( \times \) IC\(_{50}\). In contrast to the dramatic induction of ROS in A549 cells, by less than 20% at 2 \( \times \) IC\(_{50}\); and it failed to induce ROS in H23 cells (Fig. 5A). The importance of ROS in the anticancer activity of phospho-sulindac was supported by the observation that pretreatment of A549 cells with N-acetylcysteine almost completely abrogated phospho-sulindac’s capability to induce apoptosis (Fig. 5A and Supplementary Fig. S9A). Measurement of F2-isoprostanes is one of the most reliable indicators of oxidative stress in vivo (36). We found that plasma levels of 8-iso-PGF\(_2\)\(_{2}\) were more than 2-fold higher in the phospho-sulindac–treated group than in the control (Supplementary Fig. S9B).

Mitochondria are a major source of ROS (37). We measured the mitochondrial superoxide (O\(_{2}^-\)) levels using the selective probe MitoSOX Red (19). Interestingly, phospho-sulindac (2 \( \times \) IC\(_{50}\)) increased mitochondrial (O\(_{2}^-\)) by 1.6- to 2.5-fold in A549 cells to the cytotoxic effect of phospho-sulindac for 24 hours and cell viability was determined by Trypan blue exclusion. Depletion of mitochondria substantially increased the resistance of A549 cells to the cytotoxic effect of phospho-sulindac. Right, \( \rho^0 \) A549 cells were much more resistant to phospho-sulindac–induced apoptotic cell death, as measured by Annexin V/PI staining and flow cytometry. FITC, fluorescein isothiocyanate.
4-fold in A549, H23, and H358 cells (Fig. 5B), whereas having minimal effect on total cellular ROS in the latter two. These data suggest that oxidative stress induction by phospho-sulindac in these cell lines is likely mitochondria-specific.

Generation of ROS stress led to collapse of mitochondrial membrane potential ($\Delta\psi_{m}$; Fig. 5C and Supplementary Fig. S10A), evidenced by the increased JC-1 fluorescence after phospho-sulindac treatment. Dissipation of $\Delta\psi_{m}$ resulted in activation of the intrinsic apoptotic pathway, showed by the activation of caspase-9 and -3, the downregulation of Bcl-2, and the cleavage of PARP (Supplementary Fig. S10B). There was no cleavage of pro-caspase-8 (data not shown). Hence, phospho-sulindac activated the caspase cascades, culminating in apoptotic cell death.

To verify the involvement of the mitochondria in phospho-sulindac-induced death in NSCLC cells, we generated mitochondria-less ($\rho^0$) A549 cells, confirmed by the absence of mitochondrial protein cytochrome c oxidase subunit IV (COXIV; Fig. 5D and Supplementary Fig. S10C). Compared with their parental cells, the $\rho^0$ cells were significantly more resistant to phospho-sulindac-induced apoptosis (3.6-fold increase over control in parental vs. no induction in $\rho^0$ cells). Accordingly, the $\rho^0$ cells were significantly more resistant to the cytotoxicity of phospho-sulindac (150–300 mg/kg) given via the oral administration, despite its much lower dose (6.5 mg/kg body weight), successfully enhanced the delivery of intact phospho-sulindac to the lungs of mice (43% of phospho-sulindac plus metabolites). Accordingly, inhaled phospho-sulindac was remarkably effective in inhibiting the growth of orthotopically transplanted A549 tumors (75% reduction; $P < 0.001$) in nude mice. Oral administration, however, only delivered minimal levels of intact phospho-sulindac to A549 tumors and resulted in moderate growth inhibition of the tumors.

EGFR, a molecular target of phospho-sulindac, is frequently overexpressed in NSCLCs and plays a crucial role in tumorigenesis (41). Phosphorylation of EGFR is accompanied by engagement of downstream signaling pathways, which promote cell survival, growth, and resistance to apoptosis (41). Although phospho-sulindac did not modulate EGFR kinase activity in vitro (data not shown), it significantly suppressed EGFR phosphorylation in A549 cells in vitro and in orthotopic xenografts, thereby exerting a powerful inhibitory effect on the activation of the downstream pathways that regulate its molecular targets, namely, EGFR and its downstream kinases that mediate cell survival in tumor cells; and (iii) phospho-sulindac induced oxidative stress, as an important mechanism of cell death caused by phospho-sulindac. Increased ROS, an effect more pronounced in mitochondria, is an early event in phospho-sulindac–induced cell death in NSCLC cells. Phospho-sulindac also induced oxidative stress in mice bearing orthotopic lung tumors. In agreement with previous studies (46), a rapid ROS induction is a pivotal event in phospho-NSAIDs’ anticancer effect. phospho-sulindac may also modulate ROS indirectly through its inhibitory effect on Raf/MEK/ERK signaling. Oncogenic $Kras$ was shown to upregulate an Nrf-2–dependent cellular antioxidant program via Raf/MEK/ERK cascade in human lung cancer (47). Hence, the inhibition of Raf/MEK/ERK by phospho-sulindac may contribute to an enhanced oxidative state in A549 cells.

Inhibition of the survival cascades and induction of oxidative stress by phospho-sulindac in NSCLC culminates in apoptotic and autophagic cell death. Phospho-
Figure 6. Proposed mechanism for the anticancer effect of phospho-sulindac in A549 human NSCLC. Phospho-sulindac (PS) inhibits lung tumorigenesis by (i) inhibiting phosphorylation activation of EGFR, leading to attenuated phosphorylation of the downstream signaling molecules (c-Raf, MEK, and ERK); (ii) inhibiting phosphorylation of ErbB3 which presumably contributes to attenuated downstream signaling via PI3K, Akt, mTOR, S6K, and 4E-BP1. Phospho-sulindac also induces autophagic cell death as evidenced by the formation of acidic vesicular organelles and the enhanced conversion of LC3-I to LC3-II. Solid-line box, inhibition; dashed-line box, induction.

In conclusion, inhaled phospho-sulindac is an effective anticancer agent in preclinical models of NSCLC. This anticancer effect is mediated through the inhibition of EGFR survival pathways, in concert with the induction of ROS, culminating in apoptotic and autophagic death of NSCLC cells (Fig. 6). Our data establish phospho-sulindac as a promising drug candidate for NSCLCs, which merits further evaluation.

Disclosure of Potential Conflicts of Interest
B. Rigas has ownership interest (including patents) and equity position in Medicon Pharmaceuticals, Inc. No potential conflicts of interest were disclosed by the other authors.

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