A novel sulindac derivative inhibits lung adenocarcinoma cell growth through suppression of Akt/mTOR signaling and induction of autophagy

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Abbreviations: eGFP, enhanced green fluorescent protein; mRFP, monomeric red fluorescent protein; tf-LC3, tandem fluorescent-tagged LC3.
Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as sulindac sulfide (SS) have shown promising antineoplastic activity in multiple tumor types, but toxicities resulting from cyclooxygenase (COX) inhibition limit their use in cancer therapy. We recently described a N,N-dimethylethyl amine derivative of SS, sulindac sulfide amide (SSA), that does not inhibit COX-1 or -2, yet displays potent tumor cell growth inhibitory activity. Here, we studied the basis for the growth inhibitory effects of SSA on human lung adenocarcinoma cell lines. SSA potently inhibited the growth of lung tumor cells with IC$_{50}$ values of 2-5 µM compared with 44-52 µM for SS. SSA also suppressed DNA synthesis and caused a G$_0$/G$_1$ cell cycle arrest. SSA-induced cell death was associated with characteristics of autophagy, but significant caspase activation or PARP cleavage were not observed after treatment at its IC$_{50}$ value. siRNA knockdown of Atg7 attenuated SSA-induced autophagy and cell death, while pan-caspase inhibitor ZVAD was not able to rescue viability. SSA treatment also inhibited Akt/mTOR signaling and the expression of downstream proteins that are regulated by this pathway. Overexpression of a constitutively active form of Akt was able to reduce autophagy markers and confer resistance to SSA-induced cell death. Our findings provide evidence that SSA inhibits lung tumor cell growth by a mechanism involving autophagy induction through the suppression of Akt/mTOR signaling. This unique mechanism of action along with its increased potency and lack of cyclooxygenase inhibition support the development of SSA or related analogs for the prevention and/or treatment of lung cancer.
Introduction

Lung cancer is the leading cause of cancer death in patients over age forty, accounting for 226,160 new cancers and 160,340 deaths annually in the United States (1). Lung cancer is usually aggressive and characterized by early progression and metastases, as approximately 60% of patients with small cell lung cancer (SCLC) and up to 40% of patients with non-small cell lung cancer (NSCLC) will present with Stage IV disease at the time of diagnosis. The 5-year survival rate for lung cancer patients is only 15.9%, with most dying from metastasis (2). Early detection of lung cancer can improve outcome as evident from the National Lung Screening Trial (NLST, ACRIN A6654) that showed mortality can be reduced by 20% from screening high-risk patients with low dose helical computed tomography compared with chest x-ray (3). Similarly, data from the International Early Lung Cancer Action Program (I-ELCAP) showed that Stage I lung cancer detected by annual low dose computed tomography scans improved survival with early treatment (4). With the potential for early detection of lung cancer in high-risk populations, it is important to develop effective new therapeutic strategies.

Epidemiological studies indicate that long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with a significant decrease in cancer incidence and delayed progression in patients with colorectal, breast, lung, and other cancers (5, 6). The use of NSAIDs is also associated with reduced risk from cancer-related mortality and distant metastasis (7-9). With regard to lung cancer, NSAIDs have been shown to reduce the incidence of lung carcinoma by 21%. The reduction in risk is increased to 32% when adjusted for smoking, and the efficacy is greatest in former smokers
with a risk reduction of as much as 42% (10). Rodent studies support these observations by demonstrating that NSAIDs inhibit tumor formation in carcinogen-induced models of lung tumorigenesis (11-13). Unfortunately, the long-term use of NSAIDs and COX-2 inhibitors is not recommended because of potentially fatal gastrointestinal, renal, and cardiovascular toxicities associated with the depletion of physiologically important prostaglandins resulting from their COX-1 and -2 inhibitory activity (14, 15).

Because smoking contributes to chronic pulmonary inflammation, it is logical that NSAIDs may inhibit lung cancer development through suppressing inflammatory mediators such as prostaglandins. However, some investigators have implicated alternative mechanisms by which NSAIDs may exert their antineoplastic activity, suggesting that it may be feasible to develop derivatives that do not inhibit COX-1 or COX-2 and have reduced toxicity (16-19). As support for a COX-independent mechanism, the sulfone metabolite of sulindac, which does not inhibit COX-1 and -2, has been reported to inhibit tumorigenesis in colon, mammary, lung and bladder models (20-22). In a mouse model of tobacco carcinogen-induced lung tumorigenesis, sulindac sulfone strongly inhibited both tumor incidence and multiplicity (13). Sulindac sulfone also inhibited tumor growth and metastasis that led to increased survival of rats with orthotopically implanted human lung tumors alone or in combination with docetaxel (23-25). Although sulindac sulfone did not receive FDA approval for human use because of hepatotoxicity, these studies support the development of other non-COX-inhibitory derivatives of sulindac.
We recently described a N,N-dimethylethyl amine derivative of sulindac sulfide (SS) referred to as sulindac sulfide amide (SSA) that does not inhibit COX-1 or COX-2, yet inhibits colon tumor cell growth \textit{in vitro} and \textit{in vivo} (26). Because of the strong efficacy of sulindac sulfone in lung cancer models, we conducted additional studies with SSA in human lung cancer cell lines to determine their level of sensitivity and to investigate the underlying mechanism of action. Here we describe a novel component of SSA-induced cytotoxicity which involves autophagy induction via suppression of Akt/mTOR signaling.

**Materials and Methods**

**Drugs and Reagents**

Sulindac sulfide amide (SSA) was synthesized and characterized as described previously (26). Lipofectamine LTX and PLUS transfection reagents were purchased from Invitrogen. LC3 antibody was purchased from Novus Biologicals. Akt1/2/3 (pan-Akt) antibody was from Santa Cruz Biotechnology, MDM2 antibody was from EMD Biosciences and p62 antibody was from Abgent. All other antibodies were purchased from Cell Signaling Technology. pEGFP-LC3 and ptfLC3 plasmids were provided by Dr. John Shacka (University of Alabama at Birmingham, Birmingham, AL). Constitutively-active Akt (Myr-Akt1, Addgene plasmid 9008) and empty vector (pcDNA3, Addgene plasmid 10792) plasmids were purchased from Addgene. Z-VAD-FMK was purchased from EMD Chemicals. All other drugs and reagents were purchased from Sigma-Aldrich unless otherwise stated. All compounds were dissolved in DMSO and the maximum final concentration of DMSO was 0.1% in all experiments.
Cell Culture

The human lung adenocarcinoma cell lines A549, H1299 and HOP-62 were obtained from the American Type Culture Collection and grown under standard cell culture conditions in RPMI 1640 containing 5% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO₂. All ATCC cell lines were expanded upon delivery, and numerous vials of low passage cells were preserved in liquid N2. No vial of cells was passaged for more than 2 months. Cell line characterization is performed by ATCC through STR profiling and re-authentication was not performed.

Cell Viability Assay

Tissue culture microtiter 96-well plates were seeded at a density of 5,000 cells per well and incubated for 18 to 24 h before being treated with the specified compound or vehicle control. The inhibition of cell growth caused by treatment was determined as described previously (27).

Apoptosis Assays

Cell death was quantified by using the Annexin V Alexa Fluor 488 & Propidium Iodide (PI) Dead Cell Apoptosis kit from Invitrogen. In brief, 2 × 10⁵ to 3 × 10⁵ cells were exposed to SSA or vehicle control in 6-well plates and incubated for 24 hours before analysis. The cells were then harvested and analyzed with a Becton Dickinson FACSCalibur instrument (excitation, 488 nm; emission, 530 nm) according to manufacturer's instructions. The cells that were positive for Annexin V alone, and Annexin V & PI were counted. Activity of caspases 3 and 7 was measured using the
Caspase-Glo 3/7 Assay (Promega) as previously described (27). PARP cleavage was measured by western blotting.

**Cell Proliferation Assay**

Cell proliferation was determined by using the Click-iT EdU Alexa Fluor 488 Proliferation Assay (Invitrogen). Cells were seeded at a density of 1x10^6 cells per 10-cm tissue culture dish and incubated with SSA, SS or vehicle control. 6 hours after initial dosing, 5-ethynyl-2'-deoxyuridine (EdU, 10 μM) was added into the cell culture media and cells were incubated for an additional 18 hours. Cells were harvested and analyzed according to the manufacturer’s instructions. The percentage of proliferating cells was quantified by using a Becton Dickinson FACSCalibur instrument.

**Cell Cycle Measurements**

Cells (2 x 10^5 to 3 x 10^5) were exposed to SSA, SS or vehicle control in 6-well plates and incubated for 24 h before analysis. The cells were trypsinized, washed with PBS, and fixed in 1 mL of 70% ethanol at 4°C overnight, followed by incubation with RNase (1 mg/ml) and staining with propidium iodide (40 μg/ml). DNA content was determined by flow cytometry using a Becton Dickinson FACSCalibur instrument.

**Visualization of Intracellular Vacuoles**

Acidic compartments were visualized by labeling the cells with Lyso-ID Green detection reagent (Enzo) according to the manufacturer’s instructions. Nuclei were counterstained with DRAQ5. Images were obtained using an Evotec Opera confocal microscope with a 20x objective lens. For histological analysis, cells were harvested
by CellStripper (CellGro), resuspended in PBS and smeared on microscope slides. Cells were then immediately fixed in 10% neutral buffered formalin before being stained with hematoxylin and eosin.

**Transmission Electron Microscopy**

Cells were seeded at a density of 1x10^6 cells per 10-cm cell culture dish and treated with SSA or vehicle control for 24 hours. Cells were harvested using CellStripper (CellGro), washed once in PBS and then fixed with 2% glutaraldehyde in 0.2 M HEPES, pH 7.4, at room temperature for 2 hours. Additional sample processing and transmission electron microscopy were performed as described previously by Shacka et al. (28).

**Transfections**

All plasmid transfections were performed in OptiMEM reduced-serum medium (Invitrogen) containing 0.3% Lipofectamine LTX transfection reagent, 0.1% PLUS reagent and 1 µg/ml DNA. Cells were incubated for 24 hours before treatment. For RNA interference experiments, A549 cells (75% confluent) were transfected by using the Amaxa Nucleofector II device protocol (Lonza). Briefly, 1x10^6 cells were pelleted and resuspended in 100 µL of Nucleofactor reagent followed by addition of either control or ATG7 siRNA (200 nmol/L). Transfer of the reaction mixture was completed by electroporation in the Amaxa Nucleofector II device X-001 Nucleofector program was used. After transfection, cells were transferred to culture plates for 18 hours before experiments were set up. siRNAs corresponding to the human cDNA
sequence for ATG7 and the non-silencing negative control siRNA were from Dharmaco Research.

**Autophagic Imaging and Flux Assays**

Cells seeded on coverslips at 50% confluency were transiently transfected with either the eGFP-LC3 or the tfLC3 plasmid. 24 hours after transfection, cell culture media was replaced with fresh and cells were treated with 5 μM SSA or vehicle control. After 24 hours of treatment, cells were fixed in 10% neutral buffered formalin for 10 minutes, then rinsed with tris buffer and counterstained with DAPI. Slides were mounted using Fluoromount (Sigma). Imaging was performed on a Zeiss Axio Imager.M2 fluorescence microscope connected to a Zeiss AxioCam MRm camera. Autophagy induction was quantified by counting the number of cells with eGFP-LC3 translocation into dots (a minimum of 700 cells/sample) after treatment. Image processing was performed by using the NIH ImageJ software.

**Western Blot Analysis**

Immunoblotting was performed as described previously (27). For p-mTOR, a 5% polyacrylamide gel was used in order to achieve proper separation.

**Experimental Design and Data Analysis**

Drug effects on cell growth and IC50 values were determined as described previously (27). All experiments were repeated a minimum of three times to determine the reproducibility of the results. All values represent a comparison between drug treatment at the specified concentration and vehicle-treated controls. All error bars
represent standard error of the mean (SE). Statistical analysis was performed using Student's t-test with $p < 0.05$ deemed as statistically significant.

Results

**In vitro tumor cell growth inhibitory activity of SSA**

Previous molecular modeling studies revealed the importance of the carboxylic acid group on SS for inhibition of COX-1 and COX-2 (26). As depicted in Figure 1A, SSA was designed by substituting the carboxylic acid on SS for a N,N-dimethylethyl amine moiety to block COX binding. Despite lacking COX inhibitory activity, SSA was found to potently inhibit the growth of human lung adenocarcinoma cell lines, A549, H1299, and HOP62 with IC$_{50}$ values ranging from 2-5 μM (Figure 1B). By comparison, the non-selective COX-1/2 inhibitor, SS, was approximately 10-20x less potent with IC$_{50}$ values ranging from 44-52 μM. Next, we examined whether the growth inhibitory effects of SSA involved apoptosis induction and/or inhibition of proliferation. To assess the induction of apoptosis, cells were treated with SSA for 24 hours and the activation of effector caspases 3 and 7, which are specific biochemical markers of apoptotic cell death, was measured. As shown in Figure 1C, SSA treatment was not able to induce significant caspase activation at its IC$_{50}$ value in either cell type. However, caspase activation by SSA was dose-dependent and higher concentrations were able to lead to significant levels of apoptosis compared to vehicle control. This activation, nonetheless, was low compared with that induced by SS and the apoptosis-inducing agent staurosporine. We also assessed PARP cleavage, which occurs downstream of caspase activation, as an additional specific marker of apoptosis by western blotting. We were able to detect cleaved PARP after 10 μM
SSA treatment but not after 5 μM treatment (Figure 1D). A dose-dependent increase in PARP cleavage was also observed after SS treatment. As an additional marker of cell death, we measured Annexin-V and PI labeling after SSA treatment by flow cytometry. Interestingly, we observed a significant and dose-dependent increase in the extent of Annexin-V surface staining and PI labeling in all cell lines after 24 hours of SSA treatment at concentrations that suppressed growth and lower than those required for caspase activation (Figure 1E). SSA was also able to significantly and dose-dependently inhibit DNA synthesis in lung tumor cells as measured by EdU incorporation (Figure 1F). Furthermore, SSA treatment led to a dose-dependent increase in the percentage of cells in the G₀/G₁ phase of the cell cycle at concentrations that suppressed growth (Figure 1G). Significant inhibition of DNA synthesis and G₀/G₁ cell cycle arrest were also observed after single-dose SS treatment at 100 μM. These results indicate that the growth inhibitory activity of SSA is associated with inhibition of proliferation but not apoptosis after treatment at its IC₅₀ value, suggesting the involvement of alternative mechanisms of cell death.

**SSA induces autophagy in lung adenocarcinoma cells**

SSA-treated cells displayed a striking accumulation of vesicle-like structures within the cytoplasm that was readily apparent by phase contrast microscopy (Figure 2A, left panel). As shown by hematoxylin and eosin staining (Figure 2A, middle panel), SSA treatment resulted in extensive intracellular vacuolization characterized by multiple small vesicles (arrowheads), and large vacuoles (arrow) typically observed in cells undergoing autophagy. To determine if SSA induced an autophagic response in lung cancer cells, we used the Lyso-ID dye, which labels acidic compartments within
the cytoplasm including autophagosomes. SSA treatment (5 μM, 24 hours) of A549 cells increased both the number and size of acidic vesicles within the cytoplasm compared to vehicle treatment (Figure 2A, right panel). To confirm that the acidic vesicles represented autophagosomes, A549 cells were transfected with a plasmid that expresses GFP-tagged microtubule-associated protein 1 light-chain 3 (LC3), a cytoplasmic protein, which, at the onset of autophagy, is cleaved, lipidated and incorporated into the membranes of autophagosomes (29). GFP-LC3 can be used to visualize and quantify autophagosomes, which appear as punctate dots within the cytoplasm. After SSA treatment (5 μM, 24 hours), the number of LC3-positive vesicles was significantly increased compared to vehicle control in both A549 and H1299 cells, confirming that SSA-treated cells were undergoing autophagy (Figures 2B and 2D). SSA treatment also induced dose- and time-dependent cleavage of cytosolic LC3-I to autophagosome-associated LC3-II (Figure 2C). The concentrations required to induce autophagy were comparable to the concentrations required for the inhibition of growth and proliferation, suggesting that autophagic vacuole accumulation may contribute to the tumor cell growth inhibitory activity of SSA.

To determine whether autophagy is contributing to SSA-induced cytotoxicity, the morphological changes induced by SSA treatment in A549 cells were examined using electron microscopy. Cells treated with SSA (7.5 μM, 24 hours) displayed extensive intracellular vacuolization with multiple vesicles, distorted nuclei and a reduced number of mitochondria (Figure 2E). Consistent with autophagy, multiple small autophagosomes distinguished by their characteristic double-membranes could be identified (arrowheads), as well as large single-membraned vacuoles (arrows).
containing electron-dense deposits, indicating mature autophagosomes. Higher magnification images clearly demonstrate that these autophagic vacuoles contained degrading organelles (Figure 2F). Similar ultrastructural features were also observed in H1299 cells treated with SSA (data not shown).

Figure 2G shows additional TEM images that capture the progression of autophagic degradation in A549 cells from early to advanced stages (clockwise). These images demonstrate a dynamic process from the induction stage with multiple smaller vesicles (top left), to the engulfment of organelles (arrowheads), docking and fusion of mature autophagosomes (arrows) to large autophagolysosomes, and finally the complete degradation of cytoplasmic material (bottom left). Remarkably, even at the most advanced stages, this intense vacuolization was not paralleled by classical signs of apoptosis such as nuclear fragmentation, cell shrinkage, or chromatin condensation. These results indicate that SSA is able to induce a distinct type of cell death involving autophagy with an absence of morphological features of apoptosis. Furthermore, the Annexin V labeling results are consistent with previous reports indicating that phosphatidylserine exposure on the cell surface may be a common feature of apoptotic and autophagy-associated cell death (30, 31).

**SSA increases autophagic flux**

The accumulation of autophagic vacuoles (AVs) in response to SSA treatment could result from either an increase in the synthesis of autophagosomes or from a blockage of lysosomal fusion and degradation. Elevated autophagic flux, whereby there is both active synthesis and degradation of autophagosomes, has to be demonstrated in
cells undergoing autophagy-mediated cell death (32). To distinguish these effects, A549 cells were incubated with SSA and bafilomycin A1, a specific inhibitor of vacuolar type H^+-ATPase that blocks the last step of autophagic degradation (33). As shown in Figure 3A, LC3-II levels were higher in cells treated with the combination of drugs compared with cells treated with SSA only, indicating that SSA induces the synthesis of autophagosomes instead of blocking degradation. The 20 nM dose of bafilomycin A1 was included to demonstrate that complete blockage of lysosomal fusion was achieved at the 10 nM concentration used for the combination treatment, and higher concentrations were unable to induce further AV accumulation. Disappearance of the ubiquitin-binding factor p62 (SQSTM1), which targets polyubiquitinated proteins to autophagosomes for degradation, can also be used as a marker of increased autophagic turnover (34). Western blotting demonstrated a time-dependent decrease of p62 after SSA treatment indicating that autophagic flux is increased (Figure 3B).

The tfLC3 imaging assay was used as an additional marker of increased autophagic flux (35, 36). Over-expression of the tfLC3 plasmid results in tandem expression of both mRFP-LC3 and GFP-LC3. When autophagosomes fuse with the lysosomes, GFP-LC3 fluorescence is quenched due to the acidic environment. However, a compromise in lysosomal function stabilizes GFP-LC3 fluorescence leading to GFP-LC3 and mRFP-LC3 co-localization. Accordingly, earlier autophagosomes would show co-localization of GFP and mRFP, but have diminished GFP as they mature and become acidified. Images from cells treated with vehicle show one cell that has lost most of its GFP and a second cell that displays GFP-LC3 and mRFP-LC3
colocalization. While GFP-LC3 and mRFP-LC3-positive vesicles are apparent after SSA treatment (5 µM, 24 hours), many others that have lost GFP (arrows) and can be viewed as fusing with the lysosomes are observed (Figure 3C). These findings indicate that autophagosomes in SSA-treated cells are able to fuse with the lysosomes and that autophagic flux or turnover is increased.

**Autophagy contributes to SSA-induced growth inhibition**

The ability of SSA to induce autophagy in lung tumor cells lead us to next investigate the precise role of autophagy in its anticancer activity. Cancer cells, including lung cancer cells can undergo autophagy in response to various anticancer therapies. This autophagic response can serve as a survival mechanism for the cell, or conversely, in some cases, as a non-apoptotic mechanism of programmed cell death (37). To determine whether the autophagy seen in SSA- treated lung cancer cells is involved in cell death, we used siRNA to knock down Atg7, an essential protein for the induction of autophagy (38). Atg7 siRNA was able to attenuate SSA-induced autophagy in A549 cells compared to scramble siRNA-transfected controls, as measured by western blotting (Figure 4A). Furthermore, knockdown of Atg7 resulted in a significant increase in cell viability after SSA treatment for 24 hours (Figure 4B). To assess the role of apoptotic cell death in SSA-treated lung cancer cells, we used pan-caspase inhibitor Z-VAD-FMK (40 µM) to block caspase activity in A549 and H1299 cells. Blocking caspase activation did not result in an increase in viability in either cell line after SSA treatment (Figure 4C). In contrast, addition of Z-VAD-FMK was able to reduce cytotoxicity induced by SS and known apoptosis-inducing anticancer drug etoposide in both cell lines. These results suggest that the growth
inhibitory activity of SSA is predominantly mediated through autophagy and that cell death can occur independent of apoptosis.

**SSA modulates autophagy and loss of viability in part by inhibiting Akt/mTOR/p70S6k signaling**

The Akt/mTOR pathway, a known regulator of autophagy and apoptosis, has been reported to be a target for sulindac sulfide (39, 40), as well as the selective COX-2 inhibitor celecoxib (41, 42). In addition, several chemotherapeutic agents have been shown to induce autophagy by inhibiting Akt and mTOR kinases (43). Therefore, we evaluated the effects of SSA treatment on the phosphorylation of these kinases. As shown in Figure 5A, SSA inhibited the phosphorylation of Akt at the Ser 473 residue in a dose- and time-dependent manner without affecting total Akt levels in both A549 and H1299 cells. The inhibition of Akt phosphorylation became apparent starting at 4 hours and reached maximal levels between 8 and 16 hours of SSA treatment. In the same samples, we also probed for the effects of SSA on mTOR kinase activity by measuring ribosomal protein p70S6 kinase (p70S6k) phosphorylation, a well-known mTOR substrate that is preferentially phosphorylated by mTOR at the Thr 389 residue (44). SSA was able to potently suppress p70S6 kinase phosphorylation at the Thr 389 in both cell lines (Figure 5A). The inhibitory effect was apparent after only 2 hours of treatment in A549 and H1299 cells, indicating a relatively rapid suppression of mTOR kinase activity. Similar effects on Akt/mTOR signaling were also observed in HOP62 cells (data not shown). To determine if the inhibition of Akt/mTOR signaling by SSA was involved in autophagy induction and growth inhibition, lung cancer cells were transfected with a plasmid expressing the
myristoylated and constitutively-active form of the Akt1 protein (Myr-Akt) (45). Overexpression of activated Akt was able to prevent the decrease in phosphorylated Akt levels, and partially block SSA-induced autophagy in A549 cells (Figure 5B). We obtained similar results with H1299 cells (data not shown). Furthermore, the addition of Myr-Akt1 significantly inhibited SSA-induced cytotoxicity in both cell lines (Figure 5C). These results suggest that the induction of autophagy and cell death by SSA is mediated through the Akt pathway. However, it needs to be noted that the blockage of autophagy and the rescue of viability are incomplete and additional mechanisms may be involved.

SSA is a more potent and effective inducer of autophagy than SS

We also evaluated SS for its ability to induce autophagy and inhibit Akt/mTOR signaling. Figure 6A shows that while SSA induced autophagy at concentrations equivalent to its IC₅₀ value for growth inhibition (5 µM), SS was unable to induce autophagy at its IC₅₀ value of 50 µM in A549 cells as determined by LC3-I to LC3-II conversion. However, SS caused LC3-II accumulation after 100 µM treatment. These results indicate that induction of autophagy is a common feature among SS and SSA but that higher concentrations are needed in the case of SS, which exceed its IC₅₀ value for growth inhibition.

As shown in Figure 6B, 100 µM SS treatment resulted in an inhibition of Akt phosphorylation comparable to 2.5 µM SSA treatment. Phosphorylation of mTOR kinase, as well as its effector p70S6k were also inhibited by SS treatment. Another well-known effector protein of mTOR kinase is the translation repressor protein
4EBP-1. SSA and SS were able to reduce the inactivating hyperphosphorylation of 4E-BP1 indicating that both compounds inhibit global protein translation, a feature of autophagy. A more detailed examination of other regulators of this pathway showed that phosphorylation of AMPK, which suppresses mTOR activity by activating the TSC 1/2 complex (46) was induced upon SSA-treatment. This induction in AMPK activity was closely mimicked by 100 µM SS-treatment.

In addition, SSA inhibited the phosphorylation of MDM2 oncoprotein at the Akt-specific Ser 166 residue, which increases its interaction with p300 allowing MDM2-mediated ubiquitination and degradation of p53 (47). However, the effect of SS on MDM2 phosphorylation was minimal at the 100 µM concentration. Finally, levels of the anti-apoptotic and autophagy-inhibiting protein, survivin, were measured following SSA and SS treatment. We and others have reported that SS can suppress survivin levels leading to apoptosis and in some cases autophagic cell death (27, 48). We found that SSA and SS potently suppress survivin levels in A549 lung tumor cells. These results indicate that SSA is a potent inhibitor of Akt and mTOR effectors and suggest that these activities contribute to its antineoplastic activity.

Discussion
Preclinical, clinical and epidemiological studies have demonstrated promising antineoplastic properties for a number of NSAIDs. Unfortunately, toxicity resulting from COX-1 and/or COX-2 inhibition limits their long-term use for cancer indications. However, several lines of evidence suggest that COX-independent mechanisms may contribute to or be fully responsible for their antineoplastic activity. Perhaps the most
compelling evidence for this possibility comes from studies showing that the dose of a given NSAID to suppress tumor cell growth in vitro or in vivo far exceeds those required to inhibit COX-1 or COX-2 (49, 50). Moreover, NSAID analogs or metabolites that lack COX-inhibitory activity retain or have improved tumor cell growth inhibitory activity (17, 26, 51). Our results with SSA suggest that it is feasible to chemically modify sulindac for developing safer and more efficacious drugs for lung cancer chemoprevention or therapy.

Despite lacking COX inhibitory activity, SSA was able to inhibit lung tumor cell growth much more potently than sulindac sulfide. The increase in potency was appreciable with a shift from IC₅₀ values of 44-52 µM for SS to IC₅₀ values of 2-5 µM for SSA. Although further studies are necessary to determine if SSA can inhibit lung tumor growth in suitable preclinical models and is sufficiently safe for human use, previous studies using a human colon xenograft mouse model demonstrated in vivo antitumor efficacy at dosages that appeared to be well tolerated (26).

Similar to previous results with SS, the tumor cell growth inhibitory activity of SSA was associated with the inhibition of DNA synthesis and induction of cell cycle arrest. The ability of SS to induce apoptosis has been demonstrated in multiple cell types and is generally associated with the antineoplastic properties of sulindac and other NSAIDs (51-53). However, in contrast to SS, appreciably lower concentrations of SSA equivalent to its IC₅₀ value for growth inhibition caused a distinct type of cell death in lung adenocarcinoma cells involving the surface exposure of phosphatidylserine, as evident by AnnexinV labeling, but in the absence of significant
caspase cleavage or the classical morphological features of apoptosis such as nuclear fragmentation. Instead, SSA-induced cell death was characterized by extensive intracellular vacuolization and bulk degradation of cytoplasmic material with an apparently intact nucleus even at late stages. The intracellular vacuoles induced by SSA-treatment were confirmed to be autophagic vacuoles due to their acidic nature, translocation of autophagosome-associated LC3-II protein into their membranes, and the presence of digested organelle material within their lumen as shown by transmission electron microscopy. Furthermore, we showed that the increase in autophagic markers was due to an increase in autophagic flux and not associated with the inhibition of lysosomal function. We explored the role of apoptosis and autophagic cell death by using specific caspase inhibitors and knockdown of autophagy through siRNA studies. Our results demonstrate that autophagy, but not apoptosis participates in SSA-induced loss of cell viability at or around its IC$_{50}$ value for growth inhibition. Although SS also induced autophagy, higher concentrations were required compared with concentrations required to suppress tumor cell growth.

On the basis of growing evidence suggesting that Akt/mTOR signaling can inhibit drug-induced autophagy, we explored the role of this pathway on SSA-induced autophagy and cell death. SSA was able to potently inhibit the Akt/mTOR/p70S6k pathway at or around its IC$_{50}$ value for growth inhibition. We were able to demonstrate, through the use of a constitutively-active Akt overexpression vector, that Akt signaling mediates SSA-induced autophagy and related cytotoxicity. We observed the inhibition of Akt/mTOR signaling after SS treatment as well, albeit at higher concentrations. These results suggest that SSA and SS may share a similar
mechanism of action that impinges upon Akt/mTOR signaling. It is therefore plausible that the improved potency of SSA to inhibit Akt signaling may underlie its improved potency to induce autophagy and cell cycle arrest, and hence inhibit growth by dual mechanisms involving increased tumor cell death and the suppression of proliferation. In contrast, the predominant mechanism of cell death after SS treatment appears to be apoptosis.

The role of autophagy in tumor suppression is complex and likely involves several, sometimes paradoxical functions. Established tumors may utilize autophagy as an adaptive response against metabolic stress such as starvation, hypoxia, oxidative damage or chemotherapy (54). In contrast, numerous researchers have demonstrated using in vitro and in vivo models that in response to certain chemotherapeutic agents, autophagy can also mediate cell death as a genuine effector mechanism (55, 56). This has been dubbed autophagic cell death or programmed cell death type II. Alternatively, autophagy has been shown to act as an initial response mechanism for other agents, subsequently triggering apoptotic events (57). As autophagy and apoptosis share some common effectors (e.g. Akt, Bcl-2, mTOR), it has been proposed that the nature and intensity of the initial stimulus may determine which form of cell death will arise. In certain contexts of apoptosis resistance, for example, autophagy has been shown to mediate cell death in response to agents that would otherwise lead to apoptosis in naïve cells (58). This suggests that autophagy can function as a distinct cell death modality, which can be exploited for novel anticancer drug strategies to circumvent resistance.
The ability of SSA to induce autophagy has important implications for its development as a therapeutic agent for lung cancer, alone or in combination with standard chemotherapy. Our findings demonstrate that SSA can eliminate NSCLC cells through the induction of autophagy. We propose that this is a desirable property especially in the case of lung cancer chemotherapy because drugs commonly used in the clinic such as paclitaxel, gemcitabine and EGFR inhibitors often lead to the development of apoptosis-resistant tumors (59). Recent studies have shown that apoptosis resistance can develop as a result of Akt or Bcl-2 upregulation, and that these tumors can be chemosensitized by inducers of autophagy (60-62).

In summary, our findings support the potential of SSA as a novel agent for the prevention and/or treatment of lung cancer. We provide proof-of-concept evidence that the induction of autophagy through the Akt/mTOR pathway may represent a largely unexplored antineoplastic property that could be targeted to develop safer and more efficacious NSAID derivatives. Further studies are therefore warranted in order to determine the exact mechanism of action of SSA and to evaluate in vivo antitumor efficacy in suitable models of lung cancer. Identifying the specific molecular target(s) has implications not only for the development of SSA and analogs, but also can be used to identify additional small molecules in order to develop more selective and potent clinical candidates.
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Figure Legends:

Figure 1. Inhibition of growth, induction of apoptosis and inhibition of cell proliferation in lung adenocarcinoma cells by SSA. A, structures of SS and SSA. B, dose-dependent growth inhibitory activity of SS and SSA in A549, H1299 AND HOP-62 cells after 72 hours of treatment. C, apoptosis induction as measured by caspase-3 and -7 activation and D, PARP cleavage after 24 hours of treatment with SSA and SS (100 μM) (+:1 μM Staurosporine). E, AnnexinV/PI labeling after 24 hours of SSA treatment. F, dose-dependent inhibition of DNA synthesis as measured by EdU incorporation, and G, induction of G0/G1 cell cycle arrest in A549, H1299 and HOP62 cells after 24 hours of treatment with SSA or SS (100 μM).

Figure 2. SSA induces autophagy in lung adenocarcinoma cells. A, Phase-contrast images (left panel) and acidic vesicle-specific dye labeling (right panel) of A549 cells treated with vehicle or 5 μM SSA for 24h. Hematoxylin and eosin staining of floating cells after 10 μM SSA treatment for 24h (middle panel). Arrows indicate intracellular vesicles. B, eGFP-LC3 imaging of A549 and H1299 cells after vehicle or 5 μM SSA treatment for 24h. Autophagic vacuoles (AVs) are identified as punctuate dots within the cytoplasm. Scale bars: 10 μM. C, dose- and time-dependent induction of autophagy in A549 and H1299 cells as indicated by LC3-I to LC3-II conversion. Cells were treated either with the indicated concentrations of SSA for 24 hours or with 5 μM SSA for the indicated time periods and subjected to Western blotting. GAPDH is used as loading control. Results shown are representative of two independent experiments. D, Quantification of eGFP-LC3-positive puncta in A549 and H1299 cells after 24 hours of SSA treatment. E, Representative electron micrographs of A549 cells.
cells treated with vehicle or 7.5 μM SSA for 24 hours. Nuclei are labeled N. Arrows indicate autophagic vacuoles. Scale bars: 2 μm. F, Magnified image shows detailed AV structure and residual digested cellular material within their lumen. Scale bars: 500 nM. G, Representative electron micrographs of A549 cells at different stages of autophagy after 7.5 μM SSA treatment for 24 hours. Images show progressive autophagic degradation (clockwise) and an absence of morphological features of apoptosis. Scale bars: 2 μm.

**Figure 3.** SSA increases autophagic flux. A, LC3-II accumulation in A549 cells treated with 5 μM SSA with or without 10 nM Bafilomycin A1. Cells were pretreated with BafA1 for 1 h and then incubated with SSA for an additional 4 hours. LC3-II levels reached a ceiling effect at 10 nM and higher concentrations (20 nM) did not further increase LC3-II levels. B, time-dependent p62 downregulation after 5 μM SSA treatment in A549 cells. C, Effects of SSA on autophagic flux was measured using the ptfLC3 plasmid that simultaneously expresses mRFP- and GFP-tagged LC3 protein. A549 cells were treated with vehicle or 5 μM SSA for 24 hours. Early autophagosomes show GFP-mRFP co-localization whereas late, acidic autophagosomes (arrows) lose GFP and appear red. Scale bars: 10 μM.

**Figure 4.** Autophagy mediates SSA-induced cancer cell death. A, Modulation of SSA-induced autophagy by knockdown of Atg7. A549 cells were transfected with either Atg7 siRNA or a nonsilencing control siRNA for 24 hours and then treated with 5 μM SSA for 4 hours. Levels of Atg7 and LC3-II were measured by western blotting.
Knockdown of autophagy can attenuate SSA-induced cytotoxicity. A549 cells were transfected with either Atg7 or control siRNA for 24 hours and then treated with SSA for an additional 24 hours before measuring viability by CTG assay. Inhibition of apoptosis does not lead to an increase in viability after SSA treatment. A549 and H1299 cells were pretreated with 40 μM of pan-caspase inhibitor Z-VAD-FMK for 1 hour and then further incubated with different doses of SSA, SS (100 μM) and Etoposide (100 μM).

**Figure 5.** SSA modulates autophagy and cell death by inhibiting Akt/mTOR/p70S6k signaling. A, dose- and time-dependent downregulation of Akt and p70S6k phosphorylation after SSA treatment. Cells were treated either with the indicated concentration of SSA for 24 hours or with 5 μM SSA for indicated time periods and subjected to Western blotting. GAPDH is used as loading control. Images are representative of two separate experiments. B, Inhibition of SSA-induced autophagy by the overexpression of a constitutively active form of Akt (Myr-Akt). Cells were transfected with an Akt overexpression (Myr-Akt) or empty vector (pcDNA3) plasmid for 24 hours and then treated with 5 μM SSA for 4 hours. Levels of phosphorylated Akt, total Akt and LC3-II were measured by western blotting. C, Inhibition of SSA-induced cell death by Akt overexpression in A549 and H1299 cells. Cells were transfected with Myr-Akt or pcDNA3 plasmids for 24 hours and then treated with SSA for an additional 24 hours before measuring viability by CTG assay.

**Figure 6.** SSA inhibits the Akt/mTOR pathway and induces autophagy more potently than parent compound sulindac sulfide. A, LC3-II levels after 50 and 100 μM SS
treatment in A549 cells. Cells were treated for 24 hours. 5 μM SSA is included as a control and for comparison. Autophagy activation can be seen after dosing cells with 100 μM SS.  

B, Effects of SSA and SS on additional mediators of autophagy signaling. A549 cells were treated at the indicated concentrations of SSA, or 100 μM SS for 24 hours. SSA was able to dose-dependently inhibit Akt, mTOR, p70S6k, 4E-BP1 and MDM2 phosphorylation. SSA treatment induced AMPK phosphorylation while downregulating Survivin levels. SS was able to mimic SSA effects, albeit with low potency.
Figure 2.

A. 

[Images showing effects of SSA on cells, including images of cells under different conditions and immunofluorescence staining.]

B. 

[Images comparing A549 and H1299 cells under different conditions, showing cell morphology and staining patterns.]
Figure 3.

(A) SSA (μM) and Baf.A1 (nM) concentrations with respective LC3-I, LC3-II, and GAPDH bands. 
(B) A549 cell line with time (h) and Veh.(24h) conditions for p62 and GAPDH bands. 
(C) Confocal images showing mRFP-LC3 and eGFP-LC3 with a Merge view.
Figure 4.

A. 

<table>
<thead>
<tr>
<th>SSA (μM)</th>
<th>siCtrl</th>
<th>siAtg7</th>
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<tr>
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Atg7

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LC3-II

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<tr>
<td>1.1</td>
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</table>

B. 

% Growth (compared to control)

\[
\begin{align*}
\text{SSA (μM)} & \quad \text{siCtrl} & \quad \text{siAtg7} \\
0 & 100 & 100 \\
2.5 & 90 & 90 \\
5 & 80 & 80 \\
7.5 & 70 & 70 \\
10 & 60 & 60 \\
\end{align*}
\]

* p < 0.05
** p < 0.01

C. 

A549

% Growth (compared to control)

\[
\begin{align*}
\text{SSA (μM)} & \quad \text{A549} \\
0 & 100 \\
2.5 & 90 \\
5 & 80 \\
7.5 & 70 \\
10 & 60 \\
\end{align*}
\]

* p < 0.05
** p < 0.01

H1299

% Growth (compared to control)

\[
\begin{align*}
\text{SSA (μM)} & \quad \text{H1299} \\
0 & 100 \\
2.5 & 90 \\
5 & 80 \\
7.5 & 70 \\
10 & 60 \\
\end{align*}
\]

* p < 0.05
** p < 0.01
Figure 5.

A.

<table>
<thead>
<tr>
<th>SSA (μM)</th>
<th>A549</th>
<th>H1299</th>
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<tbody>
<tr>
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<td>![Image of blot for A549 at 0 μM SSA]</td>
<td>![Image of blot for H1299 at 0 μM SSA]</td>
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<tr>
<td>2.5</td>
<td>![Image of blot for A549 at 2.5 μM SSA]</td>
<td>![Image of blot for H1299 at 2.5 μM SSA]</td>
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<tr>
<td>5</td>
<td>![Image of blot for A549 at 5 μM SSA]</td>
<td>![Image of blot for H1299 at 5 μM SSA]</td>
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<td>![Image of blot for A549 at 7.5 μM SSA]</td>
<td>![Image of blot for H1299 at 7.5 μM SSA]</td>
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- p-Akt<sub>Ser473</sub>
- Akt1/2/3
- p-p70S6K<sup>Thr389</sup>
- p70S6k
- GAPDH

<table>
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<tr>
<th>Time (h)</th>
<th>Veh.(24h)</th>
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<td>![Image of blot for A549 at 2h]</td>
<td>![Image of blot for A549 at 4h]</td>
<td>![Image of blot for A549 at 8h]</td>
<td>![Image of blot for A549 at 16h]</td>
<td>![Image of blot for A549 at 24h]</td>
</tr>
<tr>
<td>H1299</td>
<td>![Image of blot for H1299 at 24h Veh]</td>
<td>![Image of blot for H1299 at 2h]</td>
<td>![Image of blot for H1299 at 4h]</td>
<td>![Image of blot for H1299 at 8h]</td>
<td>![Image of blot for H1299 at 16h]</td>
<td>![Image of blot for H1299 at 24h]</td>
</tr>
</tbody>
</table>

- p-Akt<sub>Ser473</sub>
- Akt1/2/3
- p-p70S6K<sup>Thr389</sup>
- p70S6k
- GAPDH

B.

<table>
<thead>
<tr>
<th>SSA (μM)</th>
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<th>Myr-Akt</th>
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<tr>
<td>5</td>
<td>![Image of blot for pcDNA3 at 5 μM SSA]</td>
<td>![Image of blot for Myr-Akt at 5 μM SSA]</td>
</tr>
</tbody>
</table>

- p-Akt (long exposure)
- p-Akt (short exposure)
- Akt1/2/3
- LC3-I
- LC3-II
- GAPDH

C.

<table>
<thead>
<tr>
<th>SSA (μM)</th>
<th>A549</th>
<th>H1299</th>
</tr>
</thead>
<tbody>
<tr>
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<td>![Graph for A549 at 0 μM SSA]</td>
<td>![Graph for H1299 at 0 μM SSA]</td>
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<tr>
<td>0</td>
<td>![Graph for A549 at 0 μM SSA]</td>
<td>![Graph for H1299 at 0 μM SSA]</td>
</tr>
</tbody>
</table>

- pcDNA3
- Myr-Akt

% Growth compared to control:

![Graph showing % Growth for A549 and H1299 at various SSA concentrations]

* p<0.05
** p<0.01
Molecular Cancer Therapeutics

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