Implication of AMP-Activated Protein Kinase and Akt-Regulated Survivin in Lung Cancer Chemopreventive Activities of Deguelin

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Abstract

Survivin plays important roles in maintaining cell proliferation and survival and promoting tumorigenesis. The present study was conducted to determine the stage of lung carcinogenesis at which survivin expression is induced and to investigate how survivin affects the chemopreventive action of deguelin. In in vitro studies, we observed higher levels of survivin expression in a subset of premalignant and malignant human bronchial epithelial (HBE) and non–small-cell lung cancer (NSCLC) cell lines than in normal HBE cells, and in in vivo studies, a higher level of survivin expression in specimen of human lung dysplasia than in normal lung specimens. Treatment with deguelin inhibited de novo synthesis of survivin protein and induced apoptosis, resulting in suppression of transformation phenotypes, in the premalignant and malignant HBE and NSCLC cell lines. Deguelin inhibited survivin expression in tuberous sclerosis complex 2 (TSC2) wild-type mouse embryonic fibroblasts (MEF) but not in TSC2-knockout MEFs in which mammalian target of rapamycin (mTOR) is constitutively active. Deguelin induced activation of AMP-activated protein kinase (AMPK) and inactivation of Akt. Overexpression of constitutively active Akt abolished deguelin-induced modulation of AMPK activity and survivin expression. Conversely, inactivation of AMPK by compound C or AMPKα1/2 small interfering RNA restored Akt and mTOR activities and survivin expression in deguelin-treated HBE cells. These results suggest that survivin expression is induced as an early event in lung carcinogenesis, and deguelin acts as a chemopreventive agent by inducing a reciprocal regulation between AMPK and Akt, resulting in the inhibition of mTOR-mediated survivin.

Introduction

In the United States, lung cancer leads all other cancers in rates of incidence and mortality rate (1). Despite recent advances in radiotherapy and chemotherapy modalities, the 5-year survival rates have not improved substantially (1). Chemoprevention has been proposed as a logical and obvious strategy for targeting key molecules involved in lung carcinogenesis. Dysregulated apoptosis is thought to contribute to the development of various human cancers, including lung cancer (2). Changes in the expression and/or activities of molecules that play a major role in cell survival and apoptosis have been implicated in the development and progression of cancer. One such molecule is survivin, a member of the inhibitor of apoptosis protein (IAP) family that inhibits the execution of apoptosis (3). Survivin also has an important role in controlling cell mitosis (4). Suppression of survivin expression has been shown to induce cell cycle arrest and apoptosis and to sensitize cancer cells to a spectrum of anticancer agents (3). Although survivin is rarely expressed in normal human tissues, it is frequently overexpressed in a variety of cancerous human tissues, suggesting that it has an important role in carcinogenesis (3, 5, 6).

Recent evidence suggests that the mammalian target of rapamycin (mTOR) induces apoptosis by inhibiting survivin expression (7, 8). Erk1/2 (9) or Akt (10) induces inactive phosphorylation of tuberous sclerosis complex 2 (TSC2), a negative mTOR regulator, resulting in activation of mTOR; on the other hand, AMP-activated protein kinase (AMPK; ref. 11) induces active phosphorylation of TSC2, resulting in inhibition of mTOR. Under conditions of ATP depletion, however, AMPK is allosterically activated by the binding of AMP to AMPK, which facilitates LKB1-mediated phosphorylation of AMPK on Thr172 (12, 13). Activated AMPK then induces the expression of p21, p27, and p53 proteins and suppresses the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (14–16). Conversely, when activated, Akt regulates the intracellular ATP level and acts as a negative regulator of AMPK. Furthermore, Akt has been shown to be an upstream negative regulator of AMPK and TSC2 and, in that capacity, induces the full inhibition of TSC2 and activation of mTOR (17). However, activated AMPK has also been shown to inhibit Akt activity through inactive phosphorylation of insulin receptor substrate 1 (15, 18, 19).

We previously showed that deguelin, a natural product, acts as a lung cancer chemopreventive agent by inhibiting PI3K/Akt–mediated signaling pathways (20–22). Deguelin has been shown to induce a rapid depletion of ATP levels and activation of AMPK (23, 24). On the basis of these findings, we hypothesized that survivin is involved in deguelin-mediated control of lung carcinogenesis. We designed the current study to investigate the induction of survivin expression during lung carcinogenesis and its role in malignant transformation of human bronchial epithelial (HBE) cells. We found increased levels of survivin expression in specimens of low-grade and high-grade dysplasia from patients with non–small-cell lung cancer (NSCLC) than in normal, hyperplasia, and squamous metaplasia specimens, indicating induction of survivin expression at an early stage during the multistep process of lung cancer progression. We also found evidence that survivin has a critical role in the survival and tumorigenic potential of premalignant and malignant HBE and NSCLC cells. In addition,
deguelin inhibited transformation of HBE cells by regulating mTOR-mediated survivin expression through its ability to regulate AMPK and Akt activities, thereby showing its cancer chemopreventive activities.

Materials and Methods

Cells and reagents. The following cell lines were used: the BEAS2B HBE cell line and its variants including premalignant 1799 and 1198 cells and tumorigenic 1170-T cells (provided by Dr. A. Klein-Szanto, Fox Chase Cancer Center, Philadelphia, PA), which have previously been described (25); BEAS2B cells, which were also treated with 4-(methylisoxazolino)-1-(3-pyridyl)-1-butanol (NNK, 10 μmol/L) for 75 or 90 days (BEAS2BNNK or BEAS2B1576, respectively); the NSCLC cell lines (American Type Culture Collection); and TSC2-knockout (TSC2-/-) and wild-type (TSC2+/+) mouse embryonic fibroblasts (MEF) immobilized by p53 knockout (gifts of Dr. D.J. Kwiatkowski, Brigham and Women’s Hospital, Boston, MA; ref. 26).

The following vectors were used: an adenoviral vector expressing wild-type survivin (gift of Dr. D.C. Altieri, University of Massachusetts Medical School, Worcester, MA); an adenoviral vector expressing a kinase-dead, dominant-negative Akt with a hemagglutinin (HA) tag (Ad-HA-Akt-DN; ref. 27); an adenoviral vector expressing constitutively active AMPK with a c-myc tag (Ad-myc-AMPK-CA; gift of Dr. J. Ha and Dr. I. Kang, Kang Hye University, College of Medicine, South Korea); an adenoviral vector expressing constitutively active Akt (Ad-HA-MyrAkt; ref. 21); and control adenoviral vectors expressing the luciferase gene (Ad-Luc) or no transgene insert (Ad-EV). These vectors were amplified as previously described (21).

The following reagents were used: deguelin (Gaia Chemical Corporation); ethanol (Midland Grain Products of Illinois); NNK (Midwest Research Institute); 12949002 (Cell Signaling Technology); 6-[4-(2-piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a]-pyrimidine (AMPK inhibitor, compound C; EMD Biosciences, Inc.); and 5-phosphoribosyl-5-aminomimidazole-4-carboxamide (AICAR; Toronto Research Chemicals, Inc.). Deguelin was dissolved in DMSO for the cell treatments.

Immunohistochemistry. Tissue specimens from bronchial epithelia were collected from 90 patients with previously untreated NSCLC. A total of 368 specimens (up to six specimens per patient), all of which were in tissue microarrays, were collected. Histologic classification of epithelial lesions was done using the 2004 WHO classification system (28) for lung cancer preneoplastic lesions. For survivin immunohistochemistry evaluation, one tissue microarray core (2 mm in size) was examined by epithelial site; each microarray contained at least ~1,000 cells. Survivin immunostaining was done as previously described (29), and survivin was quantified in the cytoplasm and nucleus of epithelial cells by a lung cancer pathologist (I.I.W.) using a four-value intensity score (0, 1+, 2+, and 3+) and the percentage (0–100%) of the reactivity extent. A final score was obtained by multiplying the intensity and by the extension values (range, 0–300). Preimmune serum was used as a negative control for immunostaining and H1299 NSCLC cells with high survivin expression served as a positive control.

Western blot analysis. Total protein isolation and Western blotting were done as previously described (29). All primary antibodies were purchased from Cell Signaling Technology unless otherwise indicated. Goat polyclonal anti-actin antibody and all horseradish peroxidase conjugate secondary antibodies were obtained from Santa Cruz Biotechnology. Finally, the proteins were visualized by enhanced chemiluminescence (Amersham Biosciences) and quantified by using NIH Image software version 1.61.

Transient transfection with small interfering RNAs. For the transient transfection with small interfering RNAs (siRNA), cells were plated in six-well plates at a density of 3 × 10^4 per well. AMPKα1/2 siRNA (siAMPK1a/2) or control siRNA (Dharmacon) was transfected with Oligofectamine reagent (Invitrogen) according to the manufacturer’s instructions. Transfected cells were used for Western blot analysis.

Cell treatment and cell growth determination. To determine the effect of deguelin on cell viability, cells were plated at a density of 5 × 10^4 per well in 96-well plates. The next day, cells were treated with deguelin (0–100 nmol/L) for 1 day in the growth medium. To determine the effects of survivin on deguelin-induced cell death, cells were uninfected or infected with the indicated adenosviruses and then incubated in the presence or absence of deguelin in the growth medium for 1 day. These cells were used for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, Western blotting, or flow cytometry analysis using a fluorescence-activated cell sorter as previously described (29).

Clonogenic assays. Cells were treated or untreated with deguelin in keratinocyte serum-free medium (KSFM) for 1 day in the absence of epidermal growth factor (EGF). To determine the effect of deguelin on anchorage-dependent colony formation, cells were replated at a density of 5 × 10^3 per well in six-well plates, cultured in growth medium for 7 days, stained with 0.1% Coomassie blue (Bio-Rad Laboratories, Inc.) in 30% methanol and 10% acetic acid, and the colonies were then counted. To determine the effect of deguelin on anchorage-independent colony formation, cells were suspended in 0.3% agar in KSFM at a density of 1 × 10^5/mL, replated in six-well plates precoated with 0.6% agar, and cultured for 20 days. Finally, colonies >0.2 mm in diameter were counted.

Cell cycle and apoptosis assays. Cells uninfected or infected with Ad-survivin or Ad-EV for 1 day were treated with deguelin in the absence of EGF for 1 day. Both floating and attached cells were collected, washed with 1× PBS, fixed in 1% paraformaldehyde and 70% ethanol, and stained with propidium iodide. The percentages of cells in specific phases of the cell cycle (G0, S, and G2-M) were determined as previously described by flow cytometry (21). The proportion of apoptotic cells was measured using an APO-BrdU kit (Phoenix Flow Systems) as previously described (21). The results of two independent experiments are reported.

Northern blot analysis. Approximately 5 × 10^5 premalignant 1198 cells in 100-mm dishes were treated with 100 nmol/L deguelin from 1 to 24 h. Total RNA was extracted with TRIzol reagent (Invitrogen) and Northern blotting was done using [α-32P]dCTP-labeled survivin cDNA as a probe, as described previously (21). We then examined the loading and integrity of each RNA sample by determining the intensity of 18S and 28S in ethidium bromide–stained gels.

35S metabolic labeling. Metabolic labeling of survivin was done as previously described (30). Briefly, premalignant 1198 cells were grown to 90% confluence in a six-well plate, incubated in a methionine- and cysteine-free medium for 2 h, and then cultured in a medium containing [35S]methionine/[35S]cysteine with or without 100 nmol/L deguelin for the indicated times. Metabolically labeled survivin was immunoprecipitated with an anti-survivin antibody, and the immune complex was recovered by binding with protein G-Sepharose (Upstate Biotechnology, Inc.). Immunoprecipitates were analyzed on 15% SDS-PAGE and then dried and autoradiographed. In addition, cell extracts were subjected to Western blotting with an antibody to ensure that equal amounts of protein were used.

Measurement of ATP level and ADP/ATP ratio. Levels of ATP and ADP/ATP ratio in 1198 cell extracts were determined using the ATP bioluminescence somatic cell assay kit (Sigma-Aldrich) and the ApoGlow assay kit (Cambrex).

Statistical analyses. Data from cell viability, anchorage-dependent colony formation, and anchorage-independent colony formation assays were analyzed by simple t tests or two-sided log-rank tests. Differences in cytosolic and nuclear survivin scores between any two groups of patients were analyzed with Spearman’s rank correlation coefficient. P < 0.05 was considered statistically significant.

Results

Survivin expression increases during lung carcinogenesis. To investigate the role of IAPs in lung carcinogenesis, we sought to determine the expression levels of survivin and X-linked IAP (XIAP), both of which have been shown to have an overall increased mRNA expression in human NSCLC (31), normal HBE cells, immortalized HBE (BEAS2B) cells, and derivatives of BEAS2B, including premalignant (1799 and 1198 cells) and malignant (1170-T) HBE cells, which together constitute an in vitro progressive lung carcinogenesis model (21, 22, 25, 32). In some instances, normal HBE cells showed undetectable levels of survivin.
expression (Fig. 1A). BEAS2B and its derivative HBE cell lines cultured in growth medium (KSFM + EGF for BEAS2B and 1799 cells; KSFM + 3% fetal bovine serum for 1198 and 1170-I cells) expressed survivin, although at lower levels in BEAS2B and 1799 cells than in 1198 and 1170-I cells (Fig. 1A). 1198 and 1170-I cell cultures in KSFM in the presence of EGF for 2 days expressed higher levels of survivin than 1799 and BEAS2B cells cultured in the same medium (data not shown). Most of the NSCLC cell lines that were examined (H1299, H460, A549, H596, H661, H322, H226B, and H226Br) showed greater levels of survivin expression than did 1170-I cells (Fig. 1B).

We then immunohistochemically analyzed survivin expression in bronchial epithelium specimens obtained from NSCLC patients who underwent bronchoscopic biopsy analysis. Specifically, we analyzed tissue specimens from bronchial epithelia with normal histology, basal cell hyperplasia, squamous metaplasia, low-grade squamous dysplasia, high-grade squamous dysplasia, and atypical adenomatous hyperplasia that were collected from patients with NSCLC before treatments such as chemotherapy or radiotherapy. The patients from whom these histologic specimens were obtained were well balanced in terms of age, ethnicity, and sex (Supplementary Table S1). Representative examples of survivin immunostaining in these histologic specimens are shown in Fig. 1C. Survivin was expressed in both the cytoplasm and nucleus of many human biopsy specimens, consistent with previous findings (33). Because cytosolic survivin plays an essential role in protecting cells from apoptotic stimuli and promoting tumorigenesis (34), we analyzed the cytosolic survivin expression in these tissue specimens.

Figure 1. Increased survivin expression during early lung carcinogenesis. A, normal HBE (NHBE), BEAS2B, 1799, 1198, and 1170-I cells were incubated in growth medium and then harvested. Total protein was extracted and subjected to immunoblot analysis of survivin and XIAP. Actin expression served as a loading control. B, 1170-I, H1299, H460, A549, H596, H661, H322, H226B, and H226Br cells were harvested and processed for immunoblot analysis of survivin and actin expression. C, human survivin expression was examined immunohistochemically in normal (n = 74), bronchial hyperplasia (H; n = 131), squamous metaplasia (SQM; n = 22), low-grade dysplasia (LGD; n = 12), high-grade dysplasia (HGD; n = 53), and atypical adenomatous hyperplasia (AAH; n = 76) tissue specimens. D, human survivin expression levels were presented as cytosolic mean scores. Mixed-effect models and Spearman’s rank correlation coefficient were used to assess differences in survivin mean scores between any two types of lesions. *, statistically significant compared with normal tissue; #, statistically significant compared with hyperplasia; †, statistically significant compared with squamous metaplasia. P < 0.05.
Surprisingly, cytosolic survivin expression was detected in normal specimens (47.3%). It was also detected in the specimens of epithelial hyperplasia (57.3%), squamous metaplasia (63.6%), low-grade squamous dysplasia (83.3%), high-grade squamous dysplasia (79.2%), and atypical adenomatous hyperplasia (35.5%). The mean cytosolic survivin score was significantly higher for the high-grade squamous dysplasia specimens than for the specimens of normal tissue (*P* < 0.001), epithelial hyperplasia (*P* < 0.001), and squamous metaplasia (*P* = 0.01). The mean cytosolic survivin score for the low-grade squamous dysplasia specimens was significantly higher than those for the specimens of normal tissue (*P* = 0.014) and epithelial hyperplasia (*P* = 0.004; Fig. 1D). These data suggest that survivin expression was induced early during lung carcinogenesis.

**Deguelin suppresses the transformation of HBE cells by inhibiting survivin expression.** In previous studies, we showed that deguelin effectively blocks tobacco-induced lung carcinogenesis *in vitro* and *in vivo* (21, 22). We then investigated the role of survivin in the lung cancer chemopreventive properties of deguelin using *in vitro* HBE cell system in which the tobacco component NNK induces transformation of the cells. We found that NNK induced survivin gene expression as early as 1 day after the treatment (data not shown). Indeed, previous studies have shown that treatment of NNK for more than 1 day stimulates cell transformation *in vitro* (35). To assess the effects of deguelin on HBE cells during the tobacco carcinogen–induced malignant transformation, BEAS2B cells were subjected to chronic exposure to NNK for 75 or 90 days before the deguelin treatment. As shown in Fig. 2A, the protein expression of survivin was obviously inhibited by deguelin in BEAS2B cells treated with NNK (10 μmol/L) for 90 days (BEAS2BN90d) in a dose-dependent manner; however, XIAP expression was not affected by deguelin (Fig. 2A). Treatment with deguelin also reduced survivin expression in 1198 and 1170-I cells that had been established through the *in vivo* exposure of BEAS2B cells to cigarette smoke condensate (25). We next examined the effects of deguelin on the transformation phenotypes of NNK-exposed HBE cells (e.g., decreased growth factor requirement and anchorage-dependent and anchorage-independent colony-forming abilities). In the absence of EGF, BEAS2BN75d and BEAS2BN90d cells showed less viability than did BEAS2B cells in response to deguelin treatment (BEAS2BN75d cells, 68%; BEAS2BN90d cells, 61%; and BEAS2B cells, 74%; Fig. 2B). Similarly, the BEAS2BN75d and BEAS2BN90d cells exhibited much less anchorage-dependent (Fig. 2C) and anchorage-independent (Fig. 2D) colony-forming abilities compared with BEAS2B cells after deguelin treatment.

We assessed the effects of deguelin on the cell cycle and apoptosis in NNK-exposed HBE cells. BEAS2BN75d and BEAS2BN90d cells treated with deguelin (10 μmol/L) in the absence of EGF showed no remarkable change in cell cycle progression compared with untreated cells (data not shown). In contrast, terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining showed a marked increase in the apoptotic cell population of BEAS2BN75d and BEAS2BN90d cells (35). To assess the effects of deguelin on the HBE cells during the tobacco carcinogen–induced malignant transformation, BEAS2B cells were subjected to chronic exposure to NNK for 75 or 90 days before the deguelin treatment. As shown in Fig. 2A, the protein expression of survivin was obviously inhibited by deguelin in BEAS2B cells treated with NNK (10 μmol/L) for 90 days (BEAS2BN90d) in a dose-dependent manner; however, XIAP expression was not affected by deguelin (Fig. 2A). Treatment with deguelin also reduced survivin expression in 1198 and 1170-I cells, which had been established through the *in vivo* exposure of BEAS2B cells to cigarette smoke condensate (25). We next examined the effects of deguelin on the transformation phenotypes of NNK-exposed HBE cells (e.g., decreased growth factor requirement and anchorage-dependent and anchorage-independent colony-forming abilities). In the absence of EGF, BEAS2BN75d and BEAS2BN90d cells showed less viability than did BEAS2B cells in response to deguelin treatment (BEAS2BN75d cells, 68%; BEAS2BN90d cells, 61%; and BEAS2B cells, 74%; Fig. 2B). Similarly, the BEAS2BN75d and BEAS2BN90d cells exhibited much less anchorage-dependent (Fig. 2C) and anchorage-independent (Fig. 2D) colony-forming abilities compared with BEAS2B cells after deguelin treatment.

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the protein level of survivin in association with a cleavage of poly(ADP-ribose) polymerase or caspase-3 in 1198, 1170-I, and H226Br cells in a time- or dose-dependent manner (Fig. 3B).

To determine whether deguelin-induced apoptosis is mediated through the inhibition of survivin expression, we infected 1198 and H226Br cells with adenovirus expressing survivin (Ad-survivin) and tested their susceptibility to deguelin. Western blot analysis (Fig. 3C, top) showed that Ad-survivin induced a dose-dependent increase in the expression of survivin in 1198 cells. The 1198 cells infected with 100 plaque-forming units (pfu)/cell of Ad-survivin showed increased cell viability after the deguelin treatment compared with those infected with the same doses of Ad-Luc or Ad-EV as controls (Fig. 3C, bottom). We also carried out a fluorescence-activated cell sorting analysis in the cells. About 52.2% and 43.0% of uninfected or Ad-EV-infected 1198 cells showed induction of apoptosis after the deguelin treatment compared with those infected with the same doses of Ad-Luc or Ad-EV as controls (Fig. 3C, bottom). We also carried out a fluorescence-activated cell sorting analysis in the cells. About 52.2% and 43.0% of uninfected or Ad-EV-infected 1198 cells showed induction of apoptosis after the deguelin treatment compared with those infected with the same doses of Ad-Luc or Ad-EV as controls (Fig. 3C, bottom).

regulated apoptosis in 1198 and H226Br cells by inhibiting survivin expression. These findings suggest that the lung cancer chemopreventive properties of deguelin stem, at least in part, from its regulation of survivin expression.

Inhibition of survivin protein synthesis by deguelin through the mTOR pathway. We investigated the mechanism responsible for deguelin-induced decrease in survivin expression. Northern blot analysis showed that survivin mRNA levels were mildly changed in 1198 cells after deguelin treatment (Fig. 4A, top). In contrast, metabolic labeling of deguelin-treated 1198 cells with [35S]Met-Cys revealed that the rate of survivin protein synthesis was markedly decreased compared with that in untreated cells (Fig. 4A, middle). A densitometric analysis of this experiment is shown in Fig. 4A, bottom.

Because the TSC1/2 complex plays a key role in controlling protein translation through the regulation of mTOR-mediated phosphorylation of eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) and 70-kDa ribosomal protein S6 kinase (p70S6K; ref. 10), we assessed the effects of deguelin on survivin protein expression in TSC2−/− and TSC2+/+ MEFs (Fig. 4B).
We found that the basal level of survivin, phosphorylated (activated) mTOR (pmTOR), p70S6K, and S6 protein expression was higher in TSC2−/− MEFs than in TSC2+/− MEFs. XIAP expression was clearly weaker in TSC2−/− MEFs than in TSC2+/+ MEFs, probably owing to the low activation of NF-κB in TSC2−/− MEFs (36). Expressions of survivin, pmTOR, pp70S6K, and pS6 proteins were concomitantly decreased by deguelin in TSC2+/+ MEFs in a dose-dependent manner. However, knockout of TSC2 completely blocked the effect of deguelin on survivin expression and phosphorylation of mTOR, p70S6K, and S6. These results indicate that the mTOR pathway is a major target for deguelin-mediated survivin expression.

Deguelin inhibits mTOR activation and survivin expression by activating AMPK and inactivating Akt. Akt and AMPK negatively and positively regulate TSC2, respectively (10, 11). Akt activation has been shown to inhibit AMPK activation by increasing ATP production and thus decreasing the ratio of AMP/ATP (17). In previous studies, AMPK was also found to suppress Akt activity (14, 15, 19). To understand the mechanism by which deguelin mediates survivin expression, we studied the function of Akt and AMPK in survivin expression in 1198 HBE cells.

Figure 4. Deguelin inhibits survivin protein synthesis through the mTOR pathway. A, Northern blotting and metabolic labeling were done on the 1198 cells treated with deguelin. 18S RNA and expression of actin were used as loading controls (top). The 35S-labeled survivin values were calculated after densitometric analysis of the immunoprecipitated bands (middle), and the fold increase in each band was compared with the intensity of the band in the first lane (bottom). B, TSC2−/+ and TSC2+/+ cells were treated with deguelin for 24 h. Total protein extracts were subjected to immunoblot analysis on pmTOR (Ser2448), pAkt (Ser473), pp70S6K (Thr389, Thr421/Ser424), and pS6 (Ser235/236). The unphosphorylated forms of these proteins plus survivin, XIAP, TSC2, and actin were also analyzed. C, 1198 cells were treated with the PI3K inhibitor LY294002 (10 μmol/L) for the indicated time (left) or infected with Ad-EV or Ad-HA-Akt-DN (200 pfu/cell; right). Total protein extracts were subjected to immunoblot analysis on pAMPKα (Thr172), pAkt (Ser473), Akt, AMPKα, HA, survivin, and actin. D, 1198 cells were transfected with Ad-EV or Ad-myc-AMPK-CA (50 pfu/cell) or treated with the indicated concentrations of AICAR, and then harvested for the immunoblot analysis. Total cell lysates were subjected to immunoblot analysis on pAMPKα (Thr172), pAkt (Ser473), pp70S6K (Thr389, Thr421/Ser424), or p4E-BP1 (Thr70). The unphosphorylated forms of these proteins plus c-myc, survivin, and actin expression were also analyzed.
AICAR (Fig. 4, D, right). The levels of unphosphorylated Akt, glycogen synthase kinase-3α/β (GSK-3α/β), p70S6K, 4E-BP1, and actin remained unchanged. These findings suggest the reciprocal regulation of Akt and AMPK in modulating mTOR activity and survivin expression.

We next studied the role of Akt and AMPK in the deguelin-mediated regulation of mTOR and survivin. Consistent with the depletion of ATP by deguelin seen in previous studies (23, 24), we observed that the amount of ATP was decreased and the ratio of ADP/ATP was increased by deguelin in a time-dependent manner in premalignant 1198 cells (Fig. 5A). In addition, deguelin induced decreases in the levels of pAkt, pmTOR, p4E-BP1, and survivin in BEAS2BN90d and 1198 cells in association with increases in the levels of pAMPKα and its substrate pACC, with marginal changes in the levels of unphosphorylated Akt, AMPK, mTOR, and 4E-BP1 (Fig. 5B). However, overexpression of the constitutively active Akt as the result of infection with Ad-HA-MyrAkt showed a decrease in the pAMPK level in association with restored survivin expression in the presence of deguelin (Fig. 5C). The efficiency of Ad-HA-MyrAkt infection was confirmed by Western blotting on HA and pGSK-3β, a substrate of Akt. These findings suggest that deguelin induces activation of AMPK and suppresses survivin expression by inactivation of Akt.

We then examined whether AMPK could act as an upstream negative regulator of Akt during deguelin-mediated inactivation of mTOR and suppression of survivin expression. We found that the pretreatment of 1198 cells with the AMPK inhibitor compound C (20 μmol/L, 1 h) before treatment with deguelin (100 nmol/L, 24 h) abolished both the basal and deguelin-induced increases in pAMPKα expression (Fig. 5D, left). Further, deguelin-mediated regulation of pAkt, pGSK-3β, p70S6K, p4E-BP1, and survivin was prohibited by pretreatment with compound C. Consistent with these results, deguelin-induced decreases in the levels of pAkt, pGSK-3β, p70S6K, and p4E-BP1, as well as survivin protein expression, were dramatically restored in the 1198 cells transfected with siRNA AMPKα1/2 (siAMPKα1/2) for 48 h and then cells were treated with deguelin for a further 24 h (right). Cell lysates were subjected to immunoblot analysis. The unphosphorylated forms of these proteins plus survivin and actin expression were also analyzed. Scr, control siRNA.

We conclude that deguelin induced depletion of ATP in association with activation of AMPK and inactivation of Akt, resulting in the effective modulation of the TSC2 and mTOR enzymatic activities and survivin protein synthesis.

Figure 5. Both Akt and AMPK are involved in the inhibition of mTOR-mediated survivin protein synthesis by deguelin. A, 1198 cells were treated with 100 nmol/L deguelin for the indicated times and then harvested for the determination of the ATP level and ADP/ATP ratio. The change in the total ATP level produced by deguelin was adjusted to the levels of ATP at 0 h. Columns, mean from triplicate experiments; bars, 95% confidence intervals. *, P < 0.05; **, P < 0.01; ***, P < 0.001. B, BEAS2BN90d and 1198 cells were treated with deguelin in the absence of growth factors and then subjected to immunoblot analysis on indicated proteins. The unphosphorylated forms of these proteins, as well as survivin and actin expression, were also analyzed. C, 1198 cells were infected with Ad-EV or Ad-HA-MyrAkt (200 pfu/cell) and then treated with deguelin. Total protein extracts were subjected to immunoblot analysis on indicated proteins. D, premalignant 1198 cells were pretreated with 20 μmol/L compound C and then treated with 100 nmol/L deguelin for a further 24 h (left). Premalignant 1198 cells were transfected with siRNA AMPKα1/2 (siAMPKα1/2) for 48 h and then cells were treated with deguelin for a further 24 h (right). Cell lysates were subjected to immunoblot analysis. The unphosphorylated forms of these proteins plus survivin and actin expression were also analyzed. Scr, control siRNA.
Discussion

Recent findings have shown that expression of survivin, one of the major regulators of cell division and apoptosis (3, 37, 38), is induced by a tobacco component that activates the PI3K/Akt pathway and subsequently stimulates E2F and NF-κB, an important pair of transcription factors involved in survivin expression (39). This activity implies a potential role of survivin in malignant transformation of HBE cells and lung epithelial neoplasia during tobacco-induced lung carcinogenesis. These data suggested the possibility that the agents that target survivin could be useful for controlling lung cancer, especially lung cancer induced by tobacco carcinogens. On the basis of this notion, this study was designed to investigate the roles of survivin in malignant transformation of HBE cells and to assess its involvement in chemopreventive actions of deguelin. Specifically, we showed that (a) survivin expression is induced at an early stage of human lung carcinogenesis, (b) deguelin induces apoptosis in premalignant and malignant HBE and NSCLC cell lines by suppressing survivin expression, (c) deguelin exhibits orchestrated actions on AMPK and Akt activities and thus effectively suppresses the mTOR-mediated de novo synthesis of survivin protein, and (d) the reciprocal actions of AMPK and Akt further contribute to the deguelin-mediated inhibition of survivin expression.

A growing body of evidence implicates survivin in the inhibition of apoptosis. We found the obvious increased expression of survivin in premalignant HBE cells and in vivo in bronchial dysplasia samples. Survivin protects cells from apoptosis induced by different apoptotic stimuli, including a broad spectrum of anticancer agents (3). Conversely, suppression of survivin expression induces cell cycle arrest and apoptosis and sensitizes cells to chemotherapy (38). In addition, previous studies showed that down-regulation of survivin expression by antisense oligonucleotides induced apoptosis in A549 lung adenocarcinoma cells (40) and that a DNA vaccine targeting survivin eradicated or suppressed pulmonary metastases of NSCLC in both prophylactic and therapeutic settings in C57BL/6j mice (41). These previous findings and the results from our current study implicated survivin in the progression of lung cancer, providing a potential role of survivin as a target for lung cancer chemoprevention.

In our efforts to identify chemopreventive agents targeting survivin, we found that deguelin, a natural product, inhibits the expression of survivin in premalignant and malignant HBE cells as well as NSCLC cells. Deguelin has been shown to block tobacco carcinogen–induced lung carcinogenesis by inhibiting Akt activation in cell cultures and in animal models (21, 22). We recently found that deguelin binds to the ATP-binding pocket of heat shock protein 90 (Hsp90) and disrupts Hsp90 function, leading to the ubiquitin-mediated degradation of Hsp90 client proteins including survivin, Akt, and hypoxia-inducible factor 1α, a transcription factor for survivin gene transcription (42–44). In a recent study, deguelin markedly inhibited survivin mRNA expression in breast cancer cells (45). On the basis of these findings, we assumed that deguelin might regulate survivin expression at the transcriptional or posttranslational level; however, it seems that survivin mRNA was only marginally changed by deguelin in HBE cells. This difference could be cell type specific in response to deguelin treatment. Furthermore, the proteasome inhibitor MG132 did not restore survivin expression in the presence of deguelin (data not shown). These findings indicate the presence of other mechanism used by deguelin to decrease survivin expression.

Given the role of the PI3K/Akt pathway in protein synthesis, it was plausible that deguelin could regulate de novo synthesis of the survivin protein. Indeed, deguelin dramatically inhibited the translation of the survivin protein in premalignant 1198 cells. Furthermore, a deguelin-induced decrease in survivin expression was detected in TSC2+/+ MEFs, but not in TSC2−/− MEFs, in which the mTOR pathway is constitutively active. Therefore, it seems that the mTOR pathway is the major target of deguelin in the regulation

![Figure 6](https://www.aacrjournals.org)  
**Figure 6.** Schematic model of the inhibition of mTOR-mediated survivin protein synthesis by deguelin. In this model, deguelin activates AMPK through ATP depletion and suppresses Akt in an AMPK-dependent and/or AMPK-independent manner; reversely, the inhibited Akt activates AMPK. This results in the full activation of the tumor suppressor TSC2, which in turn inhibits mTOR and its target protein-p70S6 kinase and activates another mTOR target protein, 4E-BP1. The latter events suppress survivin translation, which induces apoptosis, the ultimate chemopreventive effect of deguelin on lung carcinogenesis.
of survivin expression and subsequent induction of apoptosis in premalignant HBE cells. Considering the ability of deguelin to induce ATP depletion, we further hypothesized that the deguelin-mediated regulation of mTOR activity and the resultant decrease in survivin protein expression are caused by the dual modulation of AMPK and Akt activities. Our hypothesis was supported by the following findings in premalignant HBE cells: (a) deguelin induced decreases in pAkt (Ser472) and increases in pAMPK in association with decreases in the ATP level, an increase in the ratio of ADP/ATP, and inactivation of mTOR. (b) inhibition of PI3K/Akt activated AMPK and suppressed survivin expression, and the activation of AMPK by the overexpression of constitutively active AMPK or AICAR treatment suppressed mTOR-mediated survivin expression; (c) overexpression of constitutively active Akt inactivated AMPK and restored survivin expression in the presence of deguelin; and (d) blockade of AMPK with siRNA or its inhibitor compound C restored survivin expression in the presence of deguelin.

Taken together, we provide evidence that survivin expression is induced in normal bronchial epithelium at an early stage of lung carcinogenesis and has an important role in malignant transformation of HBE cells. We also show that the dual role of deguelin in modulating the activities of AMPK and Akt effectively activated TSC2, leading to the suppression of mTOR-mediated survivin protein translation and thus blockade of malignant transformation of HBE cells (Fig. 6). These findings also suggest that agents that induce activation of AMPK and/or inactivation of Akt could provide effective strategies for controlling cancer, including lung cancer. In fact, AMPK activators, including AICAR (15), selenium (46), thiazolidinediones (rosiglitazone and troglitazone; ref. 47), and metformin (48), have shown an anticarcinogenesis potential. The roles of AMPK, Akt, and survivin in cancer cell metabolism, proliferation, and survival indicate that deguelin can induce a variety of anticancer activities through multiple mechanisms. Thus, deguelin is likely a promising chemopreventive/therapeutic agent for human cancers, including lung cancer. Clinical trials testing both the chemopreventive and therapeutic actions of deguelin are warranted.

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