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Targeting NF-κB signaling pathway suppresses tumor growth, angiogenesis, and metastasis of human esophageal cancer

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Abstract

Esophageal cancer is the eighth most common malignancy, and one of the leading causes of cancer-related deaths worldwide. The overall 5-year survival rate of patients with esophageal cancer remains low at 10% to 40% due to late diagnosis, metastasis, and resistance of the tumor to radiotherapy and chemotherapy. NF-κB is involved in the regulation of cell growth, survival, and motility, but little is known about the role of this signaling pathway in the tumorigenesis of human esophageal squamous cell carcinoma (ESCC), the most common form of esophageal cancer. This study aims to explore the functions of NF-κB in human ESCC progression and to determine whether targeting the NF-κB signaling pathway might be of therapeutic value against ESCC. Our results from human ESCC cell lines and ESCC tissue indicated that NF-κB is constitutively active in ESCC. Exposure of ESCC cells to two NF-κB inhibitors, Bay11-7082 and sulfasalazine, not only reduced cancer cell proliferation, but also induced apoptosis and enhanced sensitivity to chemotherapeutic drugs, 5-fluorouracil, and cisplatin. In addition, Bay11-7082 and sulfasalazine suppressed the migration and invasive potential of ESCC cells. More importantly, the results from tumor xenograft and experimental metastasis models showed that Bay11-7082 had significant antitumor effects on ESCC xenografts in nude mice by promoting apoptosis, and inhibiting proliferation and angiogenesis, as well as reduced the metastasis of ESCC cells to the lungs without significant toxic effects. In summary, our data suggest that NF-κB inhibitors may be potentially useful as therapeutic agents for patients with esophageal cancer. [Mol Cancer Ther 2009;8(9):2635–44]

Introduction

Esophageal squamous cell carcinoma (ESCC) is the most common form of esophageal cancer throughout the Asia-Pacific region, including mainland China and Hong Kong. In spite of the use of modern surgical techniques combined with various adjuvant treatment modalities, such as radiotherapy and chemotherapy, the overall 5-year survival rate of ESCC patients remains low at 10% to 40% due to advanced disease, metastasis, and the resistance of the tumor to radiotherapy and chemotherapy (1–3). Recent advances in understanding the biology of human cancer have led to novel targeted therapeutic approaches for the treatment of human cancer (4). However, esophageal cancer unfortunately remains one of the least studied cancers worldwide, and up to now the molecular mechanisms and signaling pathways involved in the initiation, promotion, and progression of esophageal cancer are not fully understood. Thus, there is an urgent need for a more thorough understanding of esophageal cancer biology to identify crucial molecular pathways associated with this disease and to develop new therapeutic options for esophageal cancer to improve patient survival.

The NF-κB transcription factor family is composed of p50, p52, RelA/p65, c-rel, and Rel B. The homodimers and heterodimers are sequestered in the cytoplasm as an inactive form by the inhibitor of kappa B (IκB). Upon stimulation, the IκB kinase complex (IKK) phosphorylates the κB inhibitor, which then releases NF-κB and allows its phosphorylation, nuclear translocation, binding, and subsequent activation of target genes involved in the regulation of cell proliferation, survival, angiogenesis, and metastasis (6). Constitutively active NF-κB is common in human cancer cell lines as well as tumor tissues derived from patients, but is rare in normal cells (7). In some types of human cancer, there is strong evidence of NF-κB being involved in cancer progression (8), thus making NF-κB and its downstream signals promising targets for therapeutic intervention. However, the role of the NF-κB signaling pathway in the tumorigenesis of human esophageal cancer, in particular ESCC, is not fully understood. We previously established that NF-κB plays a role in protecting ESCC cells from tumor necrosis factor-α–induced apoptosis (9), but its role in esophageal cancer growth and progression (i.e., invasion and metastasis) is unclear. Although some articles reported that inhibition of NF-κB can increase the drug sensitivity of

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esophageal cancer cells *in vitro* (10–12), there is very little clinical or *in vivo* experimental data on the effects of NF-κB inhibition on tumor growth and metastasis. Thus, the feasibility of targeting NF-κB as a potential treatment for esophageal cancer remains underexplored.

In this study, our goal was to study the significance of NF-κB in esophageal cancer progression, and to determine whether inhibition of the NF-κB signaling pathway has anticancer effects *in vitro* and *in vivo*. Our results from esophageal cancer cell lines and human esophageal cancer tissue microarray indicated that NF-κB is constitutively active in ESCC. The results from *in vitro* experiments and *in vivo* animal models showed that blockade of the NF-κB signaling pathway inhibited esophageal cancer proliferation, enhanced the resistance to chemotherapeutic drugs and, more importantly, suppressed esophageal cancer tumor growth and metastasis.

Materials and Methods

Cell Culture and Drugs

ESCC cell lines KYSE30, KYSE150, KYSE270, and KYSE510 (obtained from DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; ref. 13), and HKESC-1 and HKESC-2 (14) were maintained in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Gaithersburg, MD) at 37°C in 5% CO2. Normal esophageal epithelial cell lines immortalized by expression of human papilloma virus E6/E7 and/or human telomerase reverse transcriptase (hTERT), namely, NE1-E6E7 (15), NE2-hTERT, NE083-hTERT (16), and NE3-hTERT-E6E7, were used as reference cell lines for comparison of gene expressions and were maintained in defined keratinocyte serum-free medium (Invitrogen). Bay11-7082, sulfasalazine, 5-fluorouracil (5-FU), and cisplatin were purchased from Calbiochem (Darmstadt, Germany) and diluted in culture medium to obtain the desired concentration.

MTT Assay

Cell viability was measured with the use of MTT proliferation assay. Briefly, about 1,000 cells were seeded in 96-well plates and cultured for 24 h. Then, Bay11-7082, sulfasalazine, 5-FU, cisplatin, or their combinations were added. Cell viability was examined at the indicated time points after treatment. A 20-μL aliquot of MTT labeling reagent (5 mg/mL in PBS; Sigma) was added, and the cells were incubated for 5 h at 37°C. Then, 200 μL of DMSO was added, and the plate was further incubated for 5 min at 37°C to dissolve the formazan crystals. The absorbance was measured at a wavelength of 570 nm on a Labsystems Multiskan microplate reader (Merck Eurolab, Dietikon, Switzerland). The results represented the absorbance ratio between the treated and untreated cells at indicated time points. Each data point represented the mean and SD of three assays.

Colony Formation Assay

About 1,000 cells were seeded per well in 6-well plates 24 h before the addition of drugs. After 14 d, the cells were fixed in 70% ethanol and stained with 10% (volume for volume) Giemsa. Colonies consisting of >50 cells were counted. The colony-forming ability after drug treatment was calculated as the ratio between the number of colonies in the treated wells and that in the untreated controls, multiplied by 100. The results represented the average of three independent experiments.

Soft Agar Assay

Cells were seeded in 6-well plates (5×10^5 cells per well), and different concentrations of Bay11-7082 or sulfasalazine were added 24 h later. The cells were treated for 24 h, trypsinized and suspended in RPMI 1640 containing 0.33% agar and 10% fetal bovine serum, and then layered on RPMI 1640 containing 0.6% agar and 10% fetal bovine serum in a 6-well plate.

Colonies were photographed 20 d later, and colony-forming ability was calculated as the ratio between the number of colonies in the treated wells and that in the untreated controls, multiplied by 100. The results represented the average of three independent experiments.

Western Blot Analysis

Preparation of whole cell lysates and immunoblotting were described previously (17). Tumor samples were homogenized in radioimmunoprecipitation assay buffer. The following primary antibodies were used: phospho-IκB ($Ser^{32/36}$), phospho-p65 (Ser^{536}), Bcl2, Bcl-xL, Bax, caspase-3, cleaved caspase-3, and poly(ADP-ribose) polymerase purchased from Cell Signaling Technology (Beverly, MA); cyclin D1 and E-cadherin from BD Biosciences (Bedford, MA); NF-κB/p65, CDK2, β-catenin, and actin from Santa Cruz Biotechnology (Santa Cruz, CA); vascular endothelial growth factor (R&D Systems, Minneapolis, MN); and cytokeratin 8 (Epitomics, Burlingame, CA).

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling

End labeling of exposed 3′OH ends of DNA fragments was undertaken with the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) *In situ* Cell Death Detection kit Fluorescein (Roche Diagnostics, Mannheim, Germany) as described previously (9). Briefly, the cells were fixed with 4% paraformaldehyde for 1 h and permeabilized with 0.2% Triton X-100 in PBS for 10 min before incubating in TUNEL reaction mixture. The cells were subsequently stained with 4′, 6-diamidino-2-phenylindole (DAPI) and visualized under fluorescence microscopy with ×40 objective. Eight representative areas were randomly selected. At least five hundred DAPI-positive cells were scored, and the percentage of apoptotic cells (apoptotic index) was determined by dividing the number of TUNEL-positive cells by the total number of cells (DAPI-positive cells) in the corresponding area.

Wound-Healing Assay

A wound was induced on the confluent monolayer cells by scraping a gap with the tip of a micropipette, and the speed of wound closure was monitored every 12 h. Photographs were taken with the use of phase contrast microscopy immediately after wound incision and at later time points.

In vitro Cell Migration and Invasion Assays

In vitro cell migration assays were done as described previously with the use of uncoated Transwell chambers

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(8 μmol/L pore size; Corning, New York, NY; ref. 18). Cells in serum-free medium containing the drug(s) were seeded to the upper chamber. Complete medium was added to the bottom wells of the chambers. After 24 h, the cells that had not migrated were removed from the upper surface of the filters with the use of cotton swabs. The cells that had migrated to the lower surface were fixed with methanol and stained with 10% (volume for volume) Giemsa. Images of three different fields were captured from each membrane, and the number of migrated cells was counted. The mean of triplicate assays for each experimental condition was calculated. Similar protocol was carried out to determine invasive potential with the use of BD BioCoat Matrigel Invasion Chambers (BD Biosciences).

Mouse Models of Tumor Growth and Metastasis

Female BALB/c nude mice ages 6 to 8 wk were purchased from the Animal Laboratory Unit of The University of Hong Kong. The mice were maintained under standard conditions and cared for according to the institutional guidelines for animal care. All the animal experiments were approved by the Committee on the Use of Live Animals in Teaching and Research of The University of Hong Kong. KYSE30 cells (1×10^6) were suspended in PBS, mixed with an equal volume of Matrigel (Basement Membrane Matrix, BD Biosciences), and were injected s.c. into the flank of mice. The mice were randomized into treatment and control groups when the tumor reached 5-mm diameter. The treatment group received i.p. injection thrice per week of 5 mg/kg Bay11-7082 dissolved in DMSO/PBS buffer (vehicle, 1:1; volume for volume), whereas the control group received the vehicle only. The two groups were treated for 16 d. Tumor size was measured with calipers every 3 d, and tumor volumes were calculated with the equation $V = (\text{length} \times \text{width}^2)/2$. The body weight of mice was monitored weekly during the experiments to evaluate overall health, and all animals were euthanized at the end of the study. Tumors, together with pieces of liver, lung, and kidney, were removed, stored at −80°C for Western blot, or fixed in 4% paraformaldehyde and paraffin-embedded for subsequent histologic and immunohistochemistry analyses. The effects of Bay11-7082 on metastasis of esophageal cancer cells were determined by injecting KYSE150 cells (1×10^6 in PBS) through the lateral tail vein of nude mice. After 24 h, the animals were treated with Bay11-7082 (4 mg/kg, i.p., twice per week) or vehicle for 8 wk.

Immunohistochemistry

A human esophageal cancer tissue microarray (Biomax, Rockville, MD) containing 60 cases of matched ESCC and adjacent normal tissue was used. Paraffin-embedded sections of human esophageal cancer tissue microarray and tumor samples from mice were deparaffinized in toluene, rehydrated in a graded series of ethanol solutions, and incubated with 0.3% hydrogen peroxide for 30 min. Antigen retrieval was done by heating the slides for 15 min in citrate buffer (pH 6.0) in a microwave oven. After blocking with normal serum corresponding to the origin of the secondary antibodies for 30 min, the slides were incubated overnight at 4°C with primary antibodies against Ki-67 (Dako, Mississauga, ON), CD31 (Santa Cruz Biotechnology), phospho-p65 (Ser^{276}; Cell Signaling Technology), or cytokeratin 8 (Epitomics, Burlingame, CA). After washing with PBS, the slides were incubated with biotinylated secondary antibodies for 30 min at room temperature, followed by peroxidase-conjugated avidin-biotin complex and 3, 3′-diaminobenzidine (Dako) as chromogen. Finally, the sections were counterstained with hematoxylin.

Evaluation of Immunostaining

For phospho-p65, the staining intensity in the esophageal cancer specimens was compared with the corresponding adjacent tissue specimens, and scored as stronger (1), same (0), or weaker (−1). The stained sections were reviewed independently by two investigators. For tumors for which there was disagreement, another review was done by both investigators to obtain a consensus. For tumor xenograft specimens, the Ki-67 proliferation index was determined by dividing the number of positively stained nuclei in a minimum of six randomly selected fields from representative tumor sections by the total number of cells counted. Microvessel density was determined by CD31 immunostaining, which is specific for endothelial cells of blood vessels, and was estimated by the mean number of vessels stained in six random fields from representative tumor sections.

Statistical Analysis

The results were analyzed with the use of SPSS (Aspire Software International, Leesburg, VA). The data from each experiment (expressed as the mean ± SD) were compared by ANOVA. *P*-values < 0.05 were deemed significant.

Results

NF-κB Signaling Pathway Is Constitutively Activated in Esophageal Cancer Cell Lines and Tumor Tissues

To determine whether the NF-κB pathway is activated in clinical tissues from patients with esophageal cancer, the nuclear expression level of phospho-p65 (i.e., the active form of NF-κB subunit p65) in human ESCC tissue microarray was detected immunohistochemically. Among 57 analyzable cases of ESCC and matched adjacent tissues, phospho-p65 nuclear staining was detected in 29 ESCC cases (50.9%), of which 25 (43.9%) had stronger phospho-p65 nuclear staining compared with corresponding normal tissues; only 6 (10.5%) cases of normal tissues showed positive phospho-p65 nuclear staining (Fig. 1A). We further confirmed the activation state of NF-κB in cell lines. A panel of six human esophageal cancer cell lines was analyzed for expression of phospho-IκB, phospho-p65, and p65, and five of them showed increased phospho-IκB and phospho-p65 expression compared with the normal esophageal epithelial cell lines (Fig. 1B). These results suggest that the NF-κB signaling pathway is constitutively activated in human esophageal cancer. Two inhibitors were used in this study to block the NF-κB signaling pathway. First, two human esophageal cancer cell lines, KYSE30 and KYSE150, were treated with 5 or 20 μmol/L Bay11, or vehicle (DMSO) for different durations (0, 6, 12, or 24 h), and the cell lysates were prepared for Western blot analysis to assess the effects

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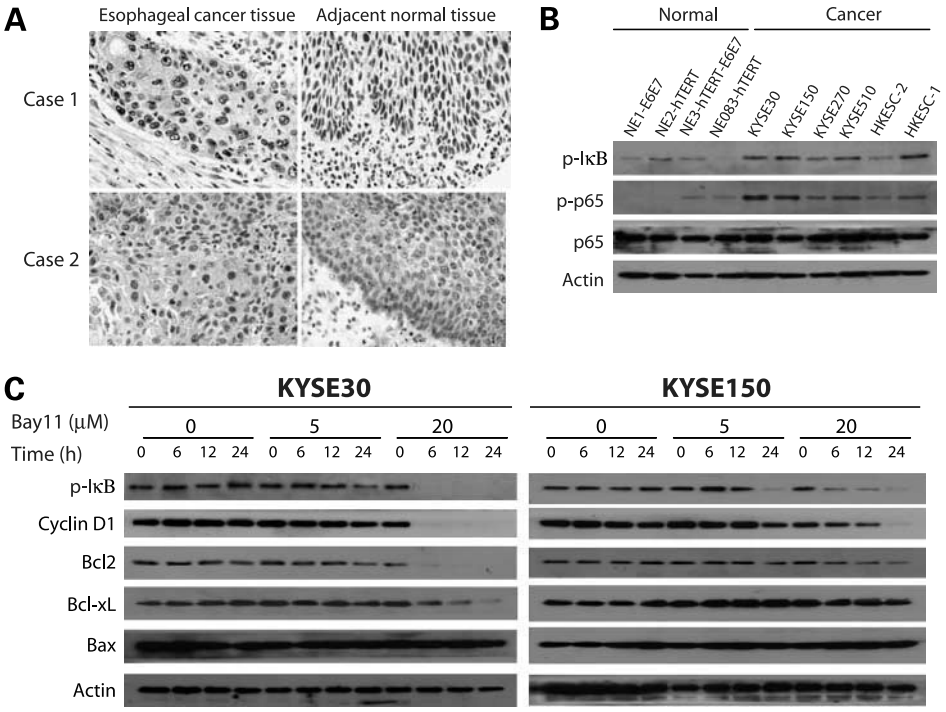

Figure 1. NF-κB activation in human esophageal cancer and blockade of NF-κB pathway by Bay11 in human esophageal cancer cells. **A,** immunohistochemical staining for phospho-p65 (p-p65) in a human ESCC tissue array. Two pairs of cancer and matching adjacent normal tissue showing overexpression of p-p65 in the nuclei of cancer cells were shown. **B,** Western blot analysis of protein expression of phospho-IκB (p-IκB) in immortalized normal esophageal epithelial cell lines and esophageal cancer cell lines. Actin was included as internal loading control. The expression level of p-IκB was higher in the cancer cell lines compared with the normal cell lines. **C,** Western blot analysis of p-IκB, cyclin D1, Bcl2, Bcl-xL, and Bax expressions in the cell lysates of KYSE30 and KYSE150 treated with different concentrations of Bay11 or vehicle (DMSO) for different durations.

on NF-κB and the downstream targets. As shown in Fig. 1C, Bay11 treatment at 20 μmol/L reduced the expression levels of the phospho-IκB, together with down-regulation of downstream proliferation-associated or survival-associated targets, including cyclin D1, Bcl2, and Bcl-xL, although the Bax expression level remained unchanged. However, Bay11 treatment at a lower concentration of 5 μmol/L had only a marginal effect on the protein expressions. We repeated these experiments with another NF-κB inhibitor, sulfasalazine, at doses of up to 1 mmol/L in the two cell lines and obtained similar results (Supplementary Fig. S1). These data indicated that Bay11 and sulfasalazine had time-dependent and dose-dependent inhibition effects on the NF-κB signaling pathway in human esophageal cancer cells.

Bay11 and Sulfasalazine Inhibit Proliferation and Induce Apoptosis of Esophageal Cancer Cells

To investigate the involvement of the NF-κB pathway in esophageal cancer proliferation and apoptosis, we exposed KYSE30 and KYSE150 cells to different concentrations of Bay11 and sulfasalazine. The results from the MTT (Fig. 2A; Supplementary Fig. S2A), colony formation (Fig. 2B; Supplementary Fig. S2B), soft agar (Fig. 2C; Supplementary Fig. S2C), and TUNEL (Fig. 2D; Supplementary Fig. S2D) assays showed that inhibition of the NF-κB pathway by the two inhibitors significantly suppressed cell proliferation and induced apoptosis in human esophageal cancer cells *in vitro*. Even at a relatively low dose of 5 μmol/L and 0.5 mmol/L, respectively, Bay11 and sulfasalazine had significant effects on cell proliferation (MTT) within 72 hours of treatment (Fig. 2A; Supplementary Fig. S2A).

Bay11 and Sulfasalazine Enhance the Sensitivity of Esophageal Cancer Cells to Chemotherapeutic Drugs

Resistance to chemotherapy is one of the reasons for the low survival rate of esophageal cancer. We next studied the effects of NF-κB inhibitors on the sensitivity of esophageal cancer cells to the chemotherapeutic drugs 5-FU and cisplatin. Three parallel experiments, including MTT assay, colony formation assay, and TUNEL, were done after exposure of KYSE30 and KYSE150 cells to different treatments, including Bay11, 5-FU, cisplatin, Bay11 in combination with 5-FU, and Bay11 combined with cisplatin. The results showed that, whereas low-dose 5-FU (2.5 μmol/L) or cisplatin (10 μmol/L) alone had no effects on cancer cell proliferation and apoptosis, combination with Bay11 (5 μmol/L) had significant synergistic effects on inhibiting cell growth (Fig. 3A) and colony formation (Fig. 3B), as well as inducing apoptosis (Fig. 3C). The experiments were repeated using sulfasalazine (0.5 mmol/L) in place of Bay11, and consistent results were obtained (Supplementary Fig. S3), indicating that the NF-κB inhibitors Bay11 and sulfasalazine potentiated the anticancer effects of 5-FU and cisplatin on ESCC cells.

Effects of NF-κB Inhibition on Esophageal Cancer Cell Migration and Invasion

Because signaling through NF-κB is thought to play an essential role in the mobility of cancer cells, including esophageal cancer cells, we evaluated the effects of inhibition of NF-κB on the migration and invasion potential of human esophageal cancer *in vitro*.

We found that Bay11 and sulfasalazine at low concentrations of 5 μmol/L and 0.5 mmol/L, respectively, which had no significant effects on cell proliferation within a 24-hour time frame (Fig. 2A; Supplementary Fig. S2A), significantly

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decreased the migration and invasive potential of both KYSE30 and KYSE150 cell lines, as indicated by the results of the wound-healing assay (Fig. 4A; Supplementary Fig. S4A), and *in vitro* cell migration and invasion assays (Fig. 4B and C; Supplementary Fig. S4B and C) 24 hours after treatment (Fig. 4C; Supplementary Fig. 4C). A time-dependent induction in the expression levels of E-cadherin and β-catenin was detected after treatment with these inhibitors (Fig. 4D; Supplementary Fig. S4D), indicating that the effects of NF-κB inhibition on migration and invasion in esophageal cancer cells may involve the reversal of epithelial-mesenchymal transition.

Bay11 and Sulfasalazine Inhibit Human Esophageal Cancer Xenograft Growth in Nude Mice

To evaluate the biological significance of NF-κB inhibition in esophageal cancer, we tested the effects of Bay11 on established

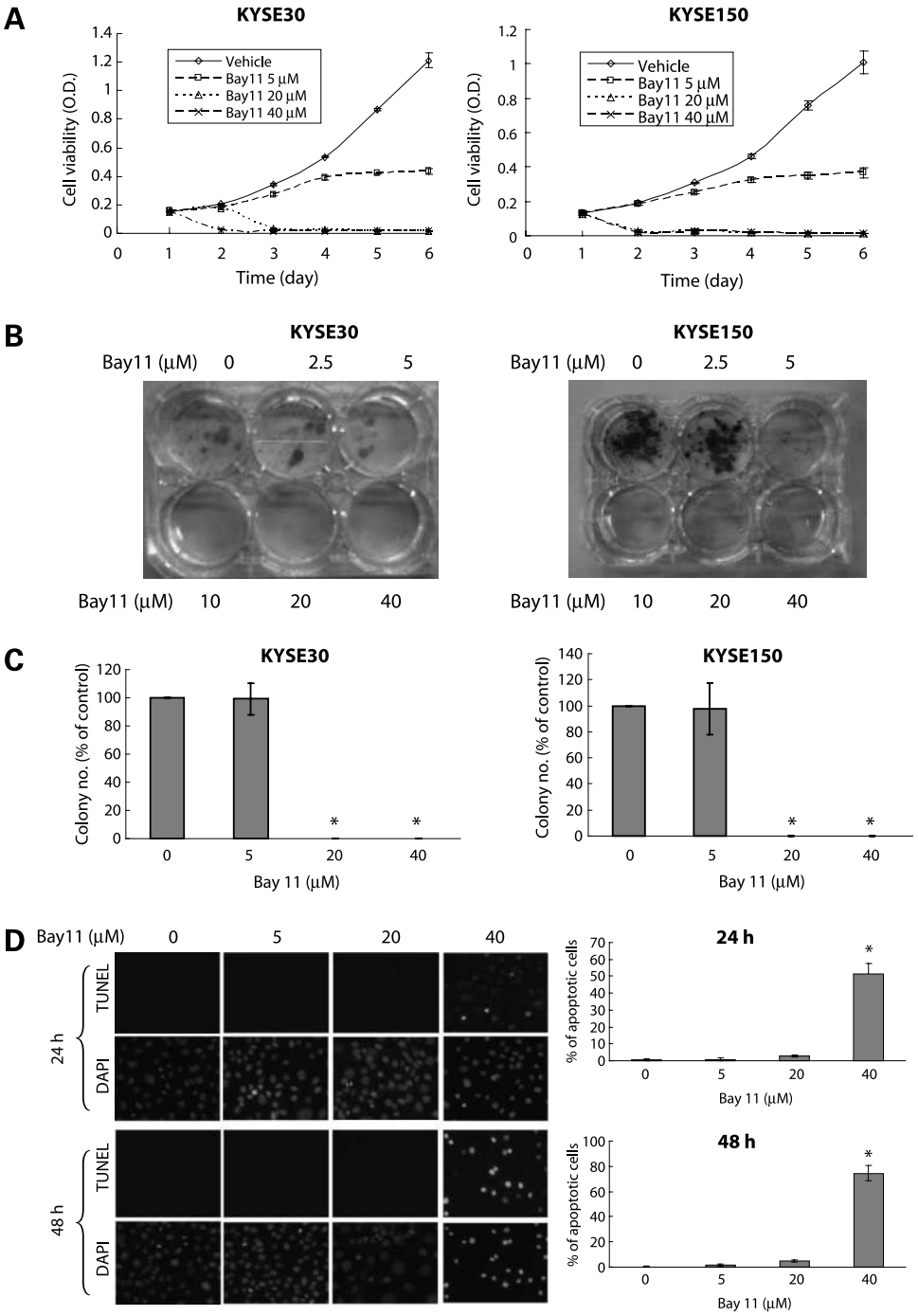

Figure 2. Effects of Bay11 on esophageal cancer cell growth and death. **A,** MTT assay was used to determine the effects of Bay11 on the viability of the esophageal cancer cell lines KYSE30 and KYSE150. Note that exposure of the cells to 20 and 40 μmol/L Bay11 completely abrogated cell viability within 2 d, whereas 5 μmol/L of Bay11 significantly reduced cell viability after 72-h treatment ($P < 0.001$). **B** and **C,** colony formation assay and soft agar assay, respectively, showed that high concentrations of Bay11 (20 and 40 μmol/L) significantly decreased both anchorage-dependent and anchorage-independent colony formation ability of KYSE30 and KYSE150 cells, whereas treatment of the cells with a low concentration of Bay11 (5 μmol/L) for only 24 h did not affect anchorage-independent growth in soft agar. **D,** representative TUNEL-stained images (*left*) and apoptotic indices of KYSE30 cells treated with 5, 20, or 40 μmol/L Bay11, or DMSO control for 24 or 48 h. Treatment with 40 μmol/L Bay11 significantly increased the apoptotic index at both time points. Bars, SD; *, $P < 0.001$.

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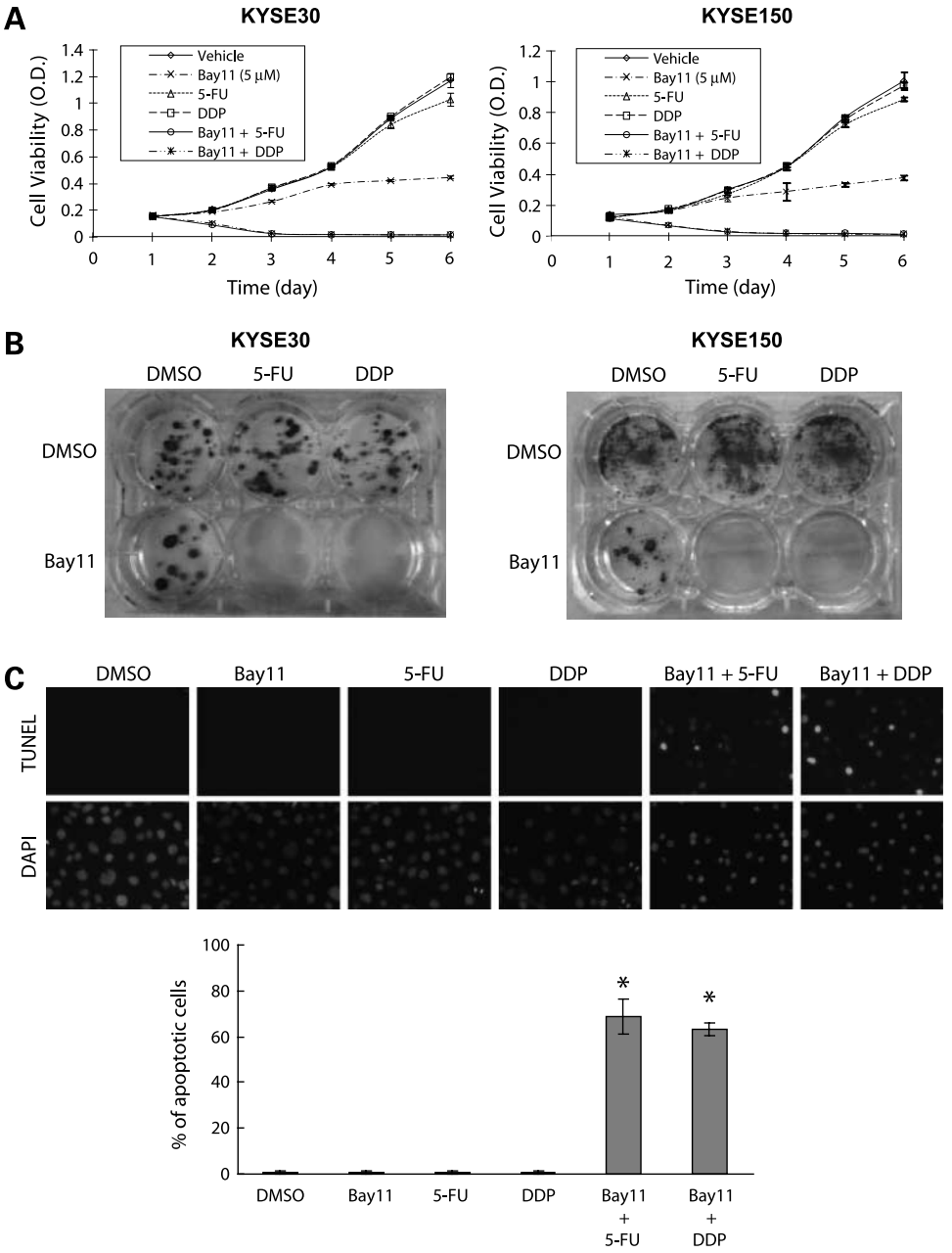

Figure 3. Effects of Bay11, alone or in combination with chemotherapeutic drugs, on esophageal cancer cells. **A,** cell viability was measured by MTT assay. Note that single drug treatment with 5-FU (2.5 μmol/L), cisplatin (DPP; 10 μmol/L), or Bay11 (5 μmol/L) for 24 h had no inhibitory effect on the proliferation of KYSE30 and KYSE150 cells, but combination of FU or cisplatin with Bay11 significantly suppressed the growth of human esophageal cancer cells ($P < 0.001$). **B,** colony formation assay showed that exposure of KYSE30 and KYSE150 cells to a low concentration of Bay11 decreased resistance to chemotherapeutic drugs 5-FU and cisplatin. **C,** synergistic effect of Bay11 with chemotherapeutic drugs on apoptosis in KYSE30 cells measured by TUNEL 24 h post treatment. Note that Bay11 significantly enhanced the drug sensitivity of the cells. Bars, SD; *, $P < 0.001$.

tumors after s.c. inoculation of the esophageal cancer cells in the flank of nude mice. As shown in Fig. 5A, treatment with Bay11 (5 mg/kg) for 16 days significantly inhibited tumor growth by ~71% (from 657.2 ± 229.6 mm^3 to 191.3 ± 103.3 mm^3; $P < 0.001$) without detectable toxic effects on body weight, or the lungs, liver, or kidneys (Fig. 5B).

Tumor xenografts harvested from the two groups of mice were processed for immunohistochemistry. Comparison of the Ki-67 proliferation and apoptotic indices indicated that the suppressive effect of Bay11 on the growth of esophageal tumor xenografts was due to decreased cell proliferation rate, as indicated by the significantly lower ($P < 0.005$) percentage of Ki-67–positive cells (19.4% ± 3.0%) compared with the vehicle-treated group (29.3% ± 5.6%), as well as increased apoptosis (mean apoptotic index increased from 4.3% ± 1.0% in the vehicle-treated group to 28.6% ± 3.1% in the Bay11-treated group; $P < 0.001$; Fig. 5C). In addition, a ~70% reduction in tumor microvessel density in the Bay11-treated group compared with the vehicle-treated group ($P < 0.001$) indicated that Bay11 treatment also inhibited tumor angiogenesis (Fig. 5C).

To confirm that the growth-suppressive effects of Bay11 were correlated with inhibition of the NF-κB signaling pathway, Western blot was carried out to compare the protein expression profiles of tumor xenografts from the

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Bay11-treated and control groups. As shown in Fig. 5D, there was decreased expression of phospho-IκB, and the downstream targets Bcl2, Bcl-xL, and vascular endothelial growth factor. The down-regulation of vascular endothelial growth factor expression might be the mechanism by which Bay11 inhibited angiogenesis in the tumor xenograft. In addition, the increased expression of cleaved caspase-3 in the Bay11-treated group corroborated the results of TUNEL staining (Fig. 5C), indicating increased apoptosis.

Bay11 and Sulfasalazine Suppress Metastasis of Esophageal Cancer Cells *In vivo*

Tumor metastasis is one of the important reasons why cancer of the esophagus often has a poor survival rate. Because the NF-κB signaling pathway is involved in the regulation of tumor metastasis, and our results above showed that blockade of NF-κB with the use of inhibitors decreased the migration and invasive potential of human esophageal cancer cells *in vitro*, we next evaluated the effects of Bay11 on metastasis of esophageal cancer cells *in vivo*. Nude mice were inoculated i.v. with esophageal cancer cells, and given Bay11 treatment at a lower dosage and frequency (4 mg/kg, twice per week) than that used in tumor growth assay. Compared with vehicle treatment, the drug significantly reduced the metastatic spread to the lungs (Fig. 6A), as evidenced by the smaller number of metastatic tumor nodules in the lungs and the lower expression of human-specific cytokeratin 8 protein in the lung extracts of Bay11-treated animals compared with the vehicle-treated control (Fig. 6B and C).

Discussion

The NF-κB signaling pathway has a crucial role in cancer development and progression, including proliferation, survival, angiogenesis, and metastasis (6). The significance of NF-κB and its therapeutic value as a target for cancer therapy have been investigated in several types of human cancer (4, 19). Only a handful of studies to date have explored the involvement of NF-κB activation in esophageal cancer, and these are mostly on esophageal adenocarcinoma (20–25) in which the NF-κB status correlates positively with metastasis, resistance to chemotherapy, and patient survival (23–25). Far less is known about the role of NF-κB in ESCC. To our knowledge, the present study is the first to provide *in vitro* and *in vivo* data to show constitutive activation of the NF-κB pathway in ESCC tumor tissues.

Our results also showed that NF-κB is involved in promoting the key steps of ESCC tumorigenesis and tumor progression, including increased cell proliferation, resistance to apoptosis, angiogenesis, invasiveness, and metastasis, thus making it an attractive therapeutic target. We showed that Bay11-7082 and sulfasalazine, which inhibit IκB phosphorylation and NF-κB activation (26, 27), successfully induced apoptosis of ESCC cells by down-regulating the antiapoptotic proteins Bcl-2 and Bcl-xL. The two inhibitors also exerted anticancer effects by inhibiting cell proliferation, migration, and invasion. In addition, the results from our animal experiments showed that Bay11 not only inhibited tumor growth *in vivo* but also reduced tumor blood supply (indicated by decreased microvessel density) through down-regulation of vascular endothelial growth factor. Intratumoral microvessel density and vascular endothelial growth factor expression have been identified as significant prognostic predictors of the overall survival of ESCC patients (28). Further research into the use of these anti–NF-κB agents in the management of ESCC patients is therefore justified.

Despite advances in treatment modalities, the mortality rate of esophageal cancer remains exceptionally high due to late diagnosis, early metastasis, and recurrence. There is still no effective treatment for advanced and metastatic esophageal cancer. Our novel finding on the *in vivo* effects of NF-κB blockade on inhibiting metastasis of ESCC cells to

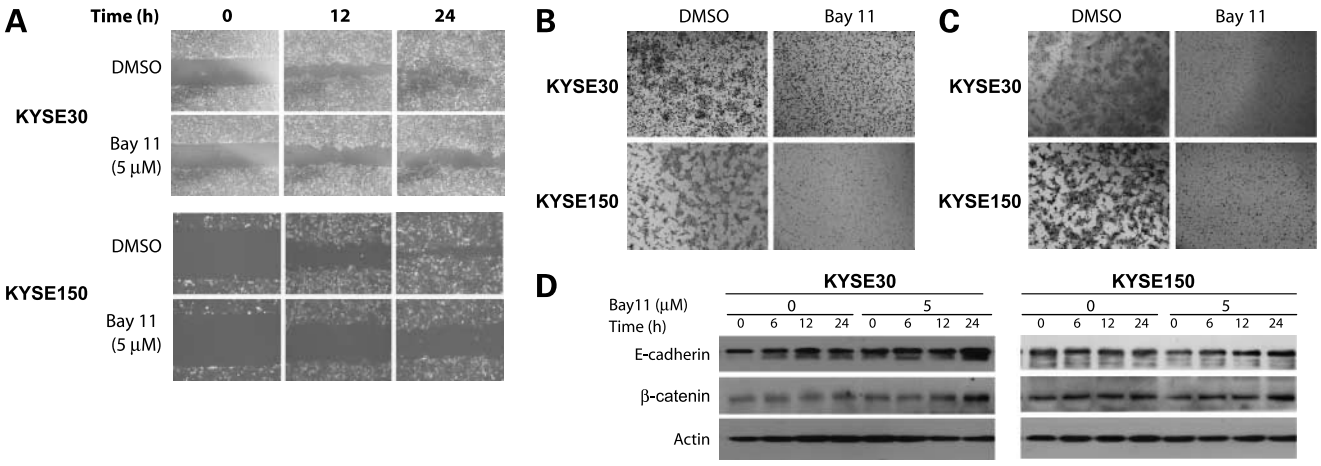

Figure 4. Effects of Bay11 on esophageal cancer cell migration and invasion. Treatment of KYSE30 and KYSE150 cells with Bay11 (5 μmol/L) reduced the migration and invasion potential as determined by wound-healing assay (**A**), cell migration assay (**B**), and cell invasion assay (**C**). **D,** Western blot analysis of protein expressions of E-cadherin and β-catenin in KYSE30 and KYSE150 cells treated with Bay11 (5 μmol/L) for the indicated time points. Actin was included as internal loading control. The expression levels of E-cadherin and β-catenin were increased after treatment with Bay11 for 24 h compared with the cells treated with vehicle control (DMSO).

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lungs, therefore, has special implications for esophageal cancer therapy.

A recent immunohistochemical study suggests that NF-κB activity is correlated with metastatic progression in esophageal adenocarcinoma (23). Although this correlation has not been reported in ESCC specimens, the relatively low dose of Bay11 required to effectively prevent lung metastasis in our experimental metastasis model and the apparent lack of toxicity for the animals certainly lend support to the feasibility of NF-κB–targeted therapy. In addition, our results showing increased expression of the epithelial markers E-cadherin and β-catenin in Bay11-treated and sulfasalazine-treated ESCC cells provide the first evidence that the suppressive effects of Bay11-7082 and sulfasalazine on the migration and invasion of ESCC cells may be attributed to a reversal of the epithelial-mesenchymal transition, which is a faithful *in vitro* correlate of metastasis.

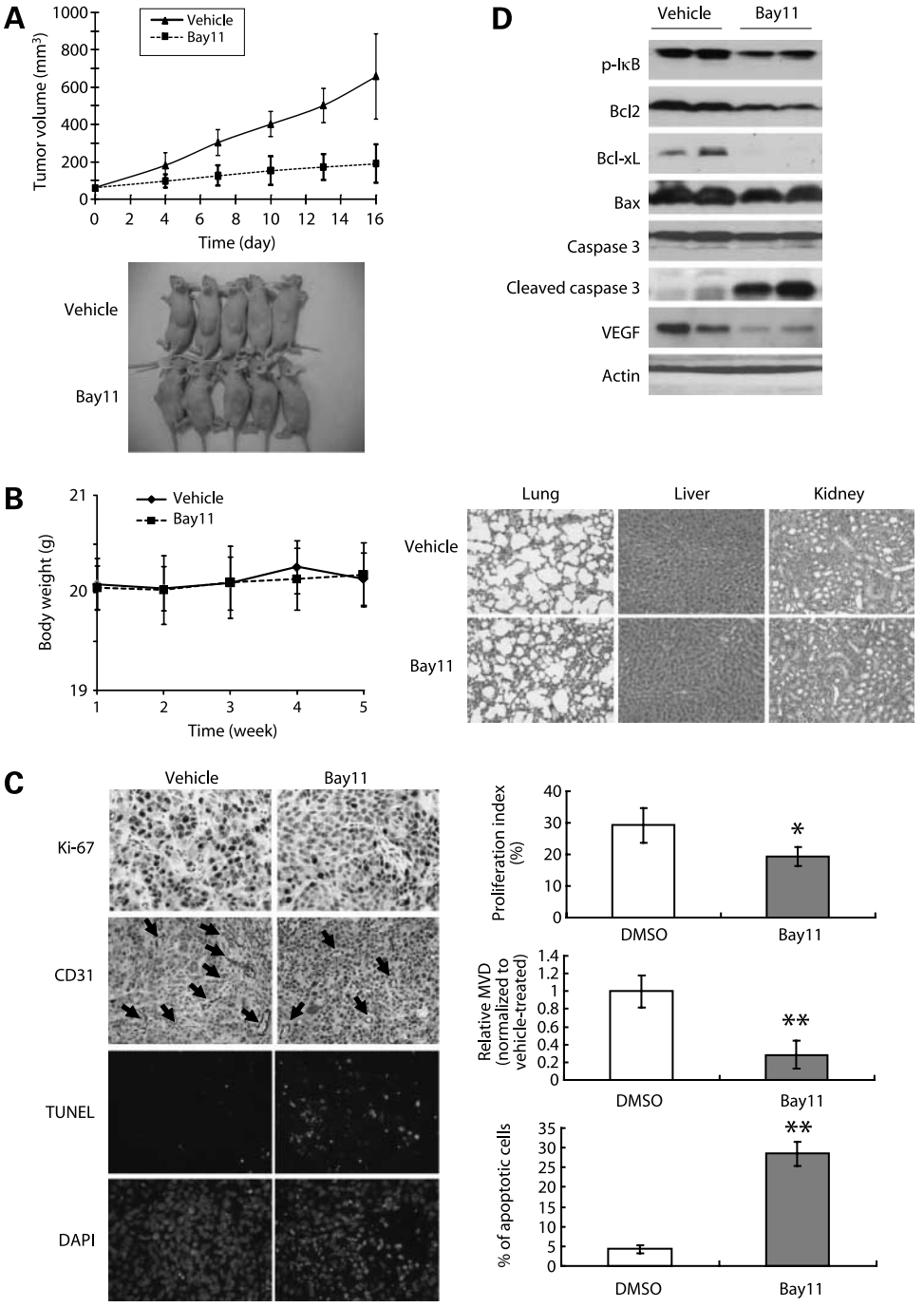

Figure 5. Effects of Bay11 on suppressing growth of human esophageal cancer xenograft in nude mice. **A,** growth curves and representative photograph of s.c. tumors in nude mice treated with Bay11 or DMSO. The mice bearing KYSE30 xenografts received 5 mg/kg Bay11 (n = 9) or DMSO (n = 9) through i.p. injection thrice per week. **B,** comparison of body weight of Bay11-treated mice, and histologic examination of lung, liver, and kidney specimens (H&E stained) with that of DMSO-treated control animals indicated no toxic effects **C,** Ki-67, CD31 immunostaining, and TUNEL staining of tumor xenografts from the mice treated with Bay11 or DMSO (*left*); tumor proliferation index, tumor microvessel density (*MVD*), and apoptosis index (*right*). Bars, SD; *, $P < 0.005$; **, $P < 0.001$ compared with DMSO-treated mice. **D,** Western blot analysis showed decreased expression of p-IκB, Bcl2, Bcl-xL, and vascular endothelial growth factor (VEGF), as well as increased cleaved caspase-3 in the Bay11-treated tumor xenografts, compared with the DMSO-treated tumor xenografts.

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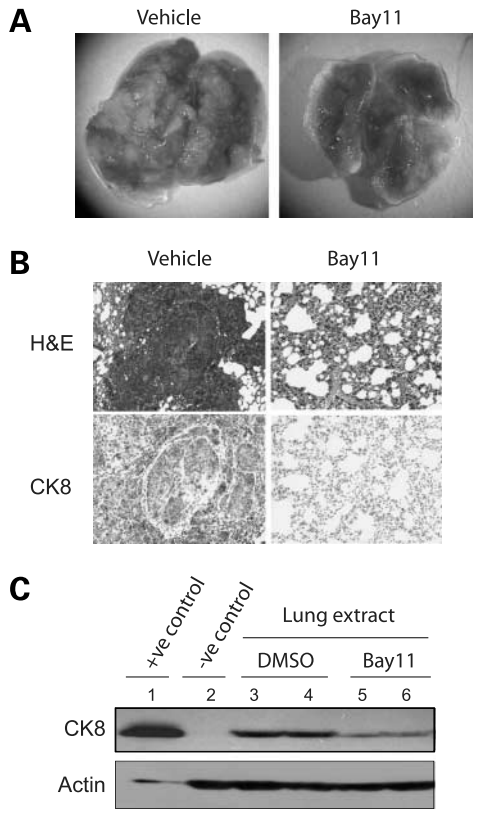

Figure 6. Experimental metastasis assay showing that Bay11 significantly inhibited metastasis of KYSE150 esophageal cancer cells *in vivo*. **A,** nude mice receiving i.v. injection of KYSE150 cells through the tail vein ($n = 8$ per group), and then treated with Bay11-7082 24 h later (4 mg/kg, i.p., twice per week) or DMSO for an 8-wk period. Representative images of lungs harvested 8 wk post injection are shown. Note that Bay11 significantly reduced the number and size of the metastatic nodules in the lungs. **B,** representative H&E-stained lung sections from the two groups of mice showing metastastic cancer cells in the lungs. A human-specific anticytokeratin 8 monoclonal antibody was used to distinguish them from the surrounding mouse pulmonary tissue. **C,** Western blot analysis of expression of human cytokeratin 8 in the lungs of mice treated with Bay11 or DMSO. Human esophageal cancer cells KYSE150 and lung tissue from normal untreated mice were included as positive and negative controls, respectively.

Recently, much evidence has shown that constitutive activation of NF-κB induces expression of antiapoptotic proteins and mediates drug resistance in cancer cells (8). In addition to examining the efficacy of Bay11 and sulfasalazine as single agents in cancer treatment, we also tested the combinatory effects of these NF-κB inhibitors with chemotherapeutic drugs on esophageal cancer cells. The cytotoxic drugs 5-FU and cisplatin are commonly used in adjuvant therapy for esophageal cancer (29). Like other cancer types, esophageal cancer may develop resistance during the therapy, leading to treatment failure. Furthermore, the success of adjuvant chemotherapy is often hindered by the compound effects of reduced performance status, postoperative complications, and chemotoxicity. It had been reported that inhibition of NF-κB by adenoviral delivery of a superrepressor of IκB-α made esophageal adenocarcinoma cells more sensitive to 5-FU (11) and that Bay11 sensitizes esophageal cancer cells to paclitaxel treatment (10). Here, we have further shown the effectiveness of both Bay11 and sulfasalazine in potentiating the effects of low-dose 5-FU and cisplatin on ESCC cells, thus supporting their application in postoperative adjuvant therapy.

In conclusion, our results from *in vitro* and *in vivo* studies suggest that two NF-κB inhibitors, namely, Bay11 and sulfasalazine, may be potentially useful in the development of new treatment strategies for patients with esophageal cancer. As increasing evidence indicates that NF-κB regulates tumorigenesis and cancer progression, many strategies and compounds have been developed to target this pathway, including I kappa B kinase inhibitors, proteasome inhibitors, acetylation inhibitors, gene transfer of inhibitory proteins, antisense RNA, small interfering RNA, anti-inflammatory agents, and chemopreventive agents (7). Neither Bay11 nor sulfasalazine has significant renal, hepatic, or pulmonary tissue toxicity in animal models (4, 19, 30). As soluble compounds, they also have an edge in terms of drug delivery. More studies are required to determine the potential tumor specificity and the effects of anti–NF-κB agents on other aspects of cancer progression regulated by the NF-κB pathway, such as angiogenesis and metastasis. More importantly, further preclinical and clinical studies are needed to define efficacious doses and appropriate treatment regimes for NF-κB inhibitors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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