A Chemical Screen Identifies Anisomycin as an Anoikis Sensitizer That Functions by Decreasing FLIP Protein Synthesis

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Abstract

Malignant epithelial cells with metastatic potential resist apoptosis that normally occurs upon loss of anchorage from the extracellular matrix, a process termed "anoikis." Resistance to anoikis enables malignant cells to survive in an anchorage-independent manner, which leads to the formation of distant metastases. To understand the regulation of anoikis, we designed, automated, and conducted a high-throughput chemical screen for anoikis sensitizers. PPC-1 anoikis–resistant prostate cancer cells were seeded in hydrogel-coated ultralow binding plates for suspension conditions and standard tissue culture plates to promote adhesion. After seeding, cells were treated with aliquots from a library of previously characterized small molecules, and viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt, assay. From this chemical screen, we identified anisomycin that induced apoptosis in suspension conditions, but was not toxic to these cells grown under adherent conditions. Anisomycin sensitized cells to anoikis by decreasing levels of the caspase-8 inhibitor FLIP and subsequently activating the death receptor pathway of caspase activation. Although anisomycin activated c-Jun-NH_2-kinase and p38, these kinases were not functionally important for the effect of anisomycin on anoikis and FLIP. Rather, anisomycin decreased FLIP and sensitized cells to anoikis by inhibiting its protein synthesis. Finally, we showed that anisomycin decreased distal tumor formation in a mouse model of prostate cancer metastases. Thus, a novel chemical screen identified anisomycin as an anoikis sensitizer that acts by decreasing FLIP protein synthesis. Our results suggest that FLIP is a suppressor of anoikis and inhibiting FLIP protein synthesis may be a useful antimetastatic strategy. [Cancer Res 2007;67(17):8307–15]

Introduction

In advanced tumors, malignant cells with metastatic potential can gain independence from the extracellular matrix (ECM) and can therefore survive in an anchorage-independent manner (1–6). This anchorage-independent survival enables metastatic cells to survive after detachment from their resident tumor and migrate to the lymphatic and circulatory systems. Once within the circulation, cells with high metastatic potential can evade immune surveillance, invade distal organs, and initiate *de novo* tumor growth. Although metastasis is a multistep process, anchorage-independent survival represents a critical step in the early development of metastatic disease (2, 7). Therefore, the identification of pathways that regulate anchorage-independent survival is important to understanding the process of metastases and may provide novel targets for antimetastatic strategies.

Nonmalignant cells undergo apoptosis upon detachment from the ECM, a self-initiated process termed "anoikis" (8, 9). Studies in endothelial cells, nonmalignant epithelial cells, and, more recently, malignant epithelial cells, suggest that anoikis can occur through self-initiated activation of the death receptor signaling pathway (6, 9–11). Apoptosis that proceeds through the death receptor pathway is generally mediated by the upstream caspase, caspase-8, that induces apoptosis by activating downstream effector caspases such as caspase-3.

One mechanism of resistance to death receptor pathway activation is overexpression of the endogenous dominant negative homologue of caspase-8, FLIP [c-Fas–associated death domain-like interleukin-1 (IL-1)–converting enzyme-like inhibitory protein]; (12–15). In contrast to nonmalignant cells, cancerous epithelial cells with metastatic potential have increased levels of FLIP and fail to down-regulate this protein after detachment from the ECM (6, 16).

To better understand the regulation of anoikis, we developed a novel high-throughput screen to identify small molecules that can sensitize resistant malignant cells to this process. By screening a library of well-characterized compounds, we identified anisomycin as a potent and specific anoikis sensitizer. Anisomycin initiated anoikis in a caspase-8–dependent manner, due to its inhibition of FLIP protein synthesis and independent of its ability to activate c-Jun-NH_2-kinase (JNK) and p38. Consistent with its activity as an anoikis sensitizer, anisomycin also reduced the metastatic potential of human prostate cancer cells seeded in the mouse circulation. Together, these studies define anisomycin as a potent and specific anoikis sensitizer and suggest that suppression of FLIP synthesis may be a useful strategy to reduce metastases.

Materials and Methods

Reagents. The LOPAC chemical library (1,280 compounds) was purchased from Sigma-Aldrich and the Prestwick chemical library (1,120 compounds) was purchased from Prestwick Chemical. Anisomycin, homoharringtonine, cycloheximide, and emetine were purchased from Sigma-Aldrich. z-VAD-fmk, SP6000125, and SB203580 were purchased from Calbiochem.

Cell culture. E1A transformed wild-type, and caspase-8−/− mouse embryonic fibroblasts (MEF; gifts from S. Benchimol, York University, Toronto, Canada) were maintained in DMEM medium. PPC-1, PC-3, DU-145, LNCaP, and OVCAR-3 cells were maintained in RPMI 1640. MB-MDA468 cells were maintained in DMEM medium. All culture media was supplemented with 10% fetal bovine serum (Hyclone) and antibiotics.

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dsRED-PPC-1 cells were engineered by transfecting PPC-1 cells with pdsRed2-C1 (Clontech Laboratories). Cells stably expressing dsRed2 protein were selected with 1 mg/mL G418 for 5 days, and G418-resistant colonies were pooled and enriched for dsRed2 expression using a MoFlo fluorescent activated cell sorter (DAKO). All cells were grown on tissue culture–treated polystyrene for adherent conditions or on hydrogel-coated ultralow binding plates for suspension conditions (Corning). Cells in suspension were gently pipetted at least once during the suspension culture to prevent the formation of stable cell spheroids. All cells were cultured at 37°C in a humid atmosphere with 5% CO_2.

High-throughput anoikis assay. Liquid handling was done by a Biomek FX Laboratory Automation Workstation (Beckman Coulter). PPC-1 cells were seeded into 96-well polystyrene and 96-well ultralow binding hydrogel-coated tissue culture plates to represent anchorage-dependent (adherent) and anchorage-independent (suspension) conditions, respectively. Compounds were transferred from the chemical libraries to cells in adherent and suspension conditions in parallel to a final compound concentration of 5 μmol/L and a final DMSO concentration (compound solvent) of 0.2%. Cells were incubated in standard tissue culture conditions for 20 h. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) reduction assay (Promega) on a SpectraMax384 spectrophotometer (Molecular Devices) according to the manufacturer's protocols. Cell viability was calculated relative to vehicle-treated (0.2% DMSO) control wells on each plate. Hits were defined empirically as compounds that preferentially reduced viability under suspension conditions but not adherent conditions and defined mathematically as (relative viability under suspension conditions) / (relative viability under adherent conditions) ≥3 SDs below the mean of the population of compounds tested.

Cell viability, apoptosis, colony formation, and caspase activation assays. Cell viability was assessed using the MTS reduction assay according to the manufacturer's protocols and as previous described (17). Apoptosis was measured by flow cytometry to detect cell surface Annexin V expression and propidium iodide uptake (Biovision) as previously described (18). Clonogenic growth after suspension culture was measured using colony formation assays as previously described (6). Briefly, equal volumes of suspension-cultured cells were seeded into six-well plates and grown in adherent conditions for 1 week. Colonies were fixed, stained with methylene blue, and counted. Caspase activation in intact cells was measured by flow cytometry using FITC-labeled, cell-permeable peptides that bind preferentially and reversibly to caspase-3, caspase-8, or caspase-9 (Cell Technologies), according to the manufacturer's directions and as previously described (18).

Small interfering RNA transfections. Cells (3.0×10^6) were seeded in 100-mm dishes and transfected the next day using LipofectAMINE 2000 (Invitrogen) and double-stranded small interfering RNAs (siRNA) targeting either FLIP (siFLIP) or luciferase (siCtrl; Smartpool, Dharmacon). Cells were reseeded 6 h posttransfection into 24-well plates at 7.5×10^4 per well, in either adherent or suspension conditions for 16 to 20 h, and then assayed for viability by Annexin V and propidium iodide staining.

Reverse-transcriptase real-time PCR. First-strand cDNA was synthesized from 1 μg of DNase-treated total cellular RNA using random primers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocols. Real-time PCR assays were done in triplicate with 100 ng of RNA equivalent cDNA, SYBR Green PCR Master mix (Applied Biosystems), and 400 nmol/L of gene-specific primers. Reactions were processed and analyzed on an ABI 7700 Sequence Detection System (Applied Biosystems). Forward/reverse PCR primer pairs for human cDNAs were as follows: Flip L forward 5′-CCTAGGAATCTGCCTGATAATCGA-3′, reverse 5′-TGGGATATACCATGCATACTGAGATG-3′; Flip S forward 5′-GCAGCAATCCAAAAGAGTCTCA-3′, reverse 5′-TTTTCCAAGAATTTTCAGATCAGGA-3′; and 18S forward 5′-AGGAATTGACGGAAGGGCAC-3′, reverse 5′-GGACATCTAAGGGCATCACA-3′. Relative mRNA expression was determined using the $\Delta\Delta C_T$ method as described (17).

Immunoblot analysis. Cells were lysed with radioimmunoprecipitation assay buffer [10 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, and 5 mmol/L EDTA] containing Complete protease inhibitors (Roche) and phosphatase inhibitors (0.4 mmol/L sodium orthovanadate, 10 mmol/L sodium fluoride, and 10 mmol/L tetrasodium PPi), all from Sigma. Immunoblot assays were done as described previously (18). Briefly, protein lysates were quantified (Protein Assay Dye Reagent or Dc Protein Assay, Bio-Rad), resolved by electrophoresis through 10% SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Membranes were incubated with mouse monoclonal anti-human FLIP (clone NF6, 1:500 v/v dilution, Alexis), mouse monoclonal anti-human caspase-8 (clone 3-1-9, 1:1,000 v/v dilution, BD Biosciences), mouse monoclonal anti-human JNK-1 (1:200, v/v dilution, Santa Cruz Biotechnologies), mouse monoclonal anti-human phosphorylated JNK (1:1,000 v/v dilution, Cell Signalling), mouse monoclonal anti-human p38 (1:1,000 v/v dilution, Cell Signalling), mouse monoclonal anti-human phosphorylated p38 (1:1,000 v/v dilution, Cell Signalling), mouse monoclonal anti-human phosphorylated c-Jun (1:1,000 v/v dilution, Cell Signalling), mouse monoclonal anti-human actin (clone AC-15, 1:30,000 v/v dilution, Sigma-Aldrich), or monoclonal anti-human tubulin (1:30,000 v/v dilution, Sigma). Primary antibodies were detected with horseradish peroxidase (HRP)–conjugated secondary antibodies [goat anti-mouse IgG (Bio-Rad), protein A HRP (GE), and goat anti-mouse IgG (GE)] and enhanced chemiluminescence (West Pico Reagent, Pierce).

Protein synthesis assay. PPC-1 cells (1.0×10^5) were seeded in 24-well plates. The next day, cells were treated with increasing concentrations of anisomycin. One hour after treatment, 10 μCi [^{3}H]leucine (Perkin-Elmer) was added to each well and the cells were incubated for an additional hour. After incubation, cells were harvested, washed once with PBS, and protein synthesis was terminated by exposing cells to 15% ice-cold trichloroacetic acid for 20 min. Cells were washed with water to remove trichloroacetic acid and unbound [^{3}H]leucine and then lysed with 0.1 mol/L NaOH-0.1% SDS. [^{3}H]leucine incorporation was measured with a scintillation counter.

***In vivo* studies.** The effects of anisomycin on distant tumor formation *in vivo* were evaluated essentially as previously described (6). Briefly, dsRED-PPC-1 cells that stably express dsRed2 fluorescent protein were treated in culture with anisomycin (5 μmol/L) or buffer control for 16 h. After treatment, 3.5×10^6 viable cells (as measured by trypan blue exclusion assay) were either injected via the tail vein or s.c. into the hind limbs into sublethally irradiated (3.5 Gy) male severe combined immunodeficient (SCID) mice between ages 5 and 7 weeks. The number of cells injected was at least 3-fold above the minimum threshold required for distal tumor formation (data not shown). Mice injected with tumor cells s.c. were maintained for 3 weeks and then sacrificed via carbon dioxide inhalation. Tumors were excised and weighed. Mice injected with tumor cells i.v. were maintained for 5 weeks after injection or until moribund, at which time the animals were sacrificed via carbon dioxide inhalation and were dissected. Red fluorescent tumors were detected via whole-body imaging and whole-organ imaging using a Leica MZ FLIII fluorescent stereomicroscope with a 100 W mercury lamp, a 560/40 excitation filter, and a 610 long-pass emission filter. Images were acquired using a Leica DC350 digital camera at ×0.8 magnification and analyzed using Image Pro Plus 6.0 (MediaCybernetics). A single common threshold was applied to identify and measure fluorescence in each organ (19). The number of fluorescent spots and the corresponding pixel area were recorded for each lung lobe. All quantification was done on unmanipulated images.

Mice were obtained from an in-house breeding program and housed in laminar-flow cage racks under standardized environmental conditions with *ad libitum* access to food and water. All experiments were done according to the regulations of the Canadian Council on Animal Care.

Statistics. For the *in vivo* studies, nonparametric methods were used to test for differences in the number of metastases. For comparisons of two groups, the Mann-Whitney rank-sum test was used. In studies of distant tumor formation in mice, survival times were compared with a log-rank statistic, where the day of sacrifice was considered a censored event.

Results

Identification of small molecules that sensitize resistant cells to anoikis. Resistance to anoikis permits malignant cells to

survive after loss of attachment to their ECM and facilitates metastases. Small molecules that restore sensitivity to anoikis could serve as useful molecular probes to better understand the regulation of this pathway. To identify such molecules, we developed, automated, and conducted a high-throughput chemical screen for anoikis sensitizers. In the optimized and automated assay, PPC-1 cells were seeded in parallel in 96-well hydrogel-coated plates to prevent adhesion and 96-well standard tissue culture plates to promote adhesion. Cells under suspension or adherent conditions were treated with aliquots from the Prestwick (n = 1,120) and LoPAC (n = 1,200) library of off-patent drugs and chemicals (final concentration 5 μmol/L and <0.2% DMSO). Twenty hours after incubation, cell viability was measured by MTS assay. The Z factor of the combined suspension and adherent assay was 0.8, where the Z factor is defined as 1 − [(3SD sample + 3SD of negative control) / (mean of sample − mean of negative control)]. The Z factor is a measure of the variability of the data and a Z factor of 1 is ideal and a Z factor of 0.5 denotes a very robust screening assay (20). The results of the screen are shown in Fig. 1*A*. The top hit from this screen was anisomycin that was 8.1 SDs below the mean of the population of compounds tested (Fig. 1*B*). Anisomycin is an inhibitor the 28S rRNA that is known to inhibit protein synthesis and activate the kinases JNK and p38 (21–25).

Anisomycin sensitizes resistant cells to anoikis. To validate anisomycin as an anoikis sensitizer, PPC-1 cells were treated with increasing concentrations of anisomycin under suspension and adherent conditions, after which viability was measured by the MTS assay. Anisomycin sensitized PPC-1 cells to anoikis with an LD_{50} of 0.54 ± 0.05 μmol/L (Fig. 1*C*). In contrast, its LD_{50} under adherent conditions was >50 μmol/L. Sensitization to anoikis was confirmed using a colony formation assay (Fig. 1*D*). To assess the spectrum of activity of anisomycin as an anoikis sensitizer, we tested the effects of this compound on five additional malignant cell lines (Fig. 1*D*). Anisomycin sensitized PC-3 and DU-145 prostate cancer and OVCAR-3 ovarian cancer cell lines to anoikis. Conversely, anisomycin was less active in LNCaP prostate cancer and MB-MDA-468 breast cancer cells.

Anisomycin sensitizes to anoikis by activating the death receptor pathway of caspase activation. We next explored the mechanism by which anisomycin sensitized cells to anoikis. To determine whether anisomycin sensitized cells to anoikis through a caspase-dependent mechanism, PPC-1 cells were treated under suspension conditions with increasing concentrations of anisomycin in the presence or absence of the pan-caspase inhibitor z-VAD-fmk (100 μmol/L). z-VAD-fmk inhibited the ability of anisomycin to promote anoikis, demonstrating a caspase-dependent mechanism of anoikis (Fig. 2*A*).

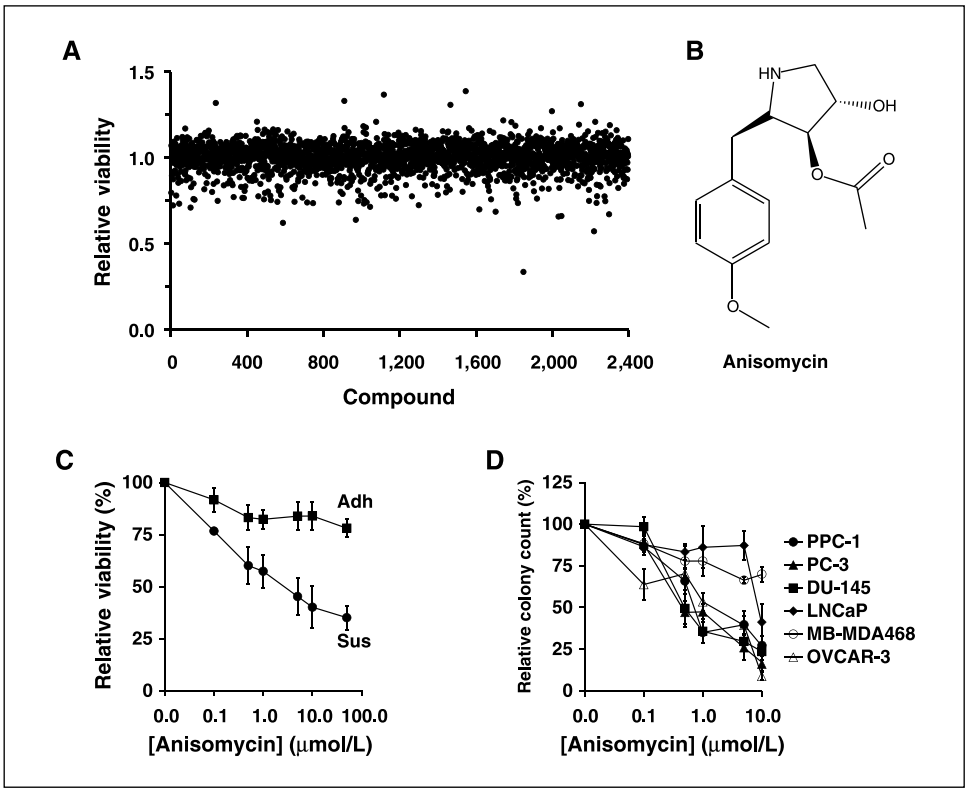

Figure 1. High-throughput screening identifies anisomycin as an anoikis sensitizer. *A,* PPC-1 cells were seeded in 96-well polystyrene or hydrogel-coated plates to represent anchorage-dependent (adherent) and anchorage-independent (suspension) conditions, respectively. Compounds from chemical libraries were added to adherent and suspension cells in parallel for a final concentration of 5 μmol/L. Twenty hours later, cell viability was assessed using an MTS assay. Data were expressed as the relative viability of suspension-cultured cells divided by the relative viability of adherent-cultured cells for each compound tested. Hits were defined empirically as compounds that preferentially reduced viability under suspension conditions but not adherent conditions and defined mathematically as (relative viability under suspension conditions) / (relative viability under adherent conditions) ≥3 SDs below the mean of the population of compounds tested. *B,* chemical structure of anisomycin. *C,* PPC-1 cells (1.5×10^4) were seeded overnight in 96-well plates in adherent (Adh) or suspension (Sus) conditions. Cells were then cultured with increasing concentrations of anisomycin for 20 h. After incubation, cell viability was measured by the MTS assay. *Points,* mean percent viable cells relative to untreated controls; *bars,* SE. *D,* PC-3, DU-145, and LNCaP prostate; MB-MDA-468 breast; and OVCAR-3 ovarian cancer cells were cultured under suspension conditions with increasing concentrations of anisomycin. Twenty-hours after incubation, equal volumes of suspension-cultured cells were replated in a colony formation assay. *Points,* mean percentage of colonies compared with untreated controls; *bars,* SE.

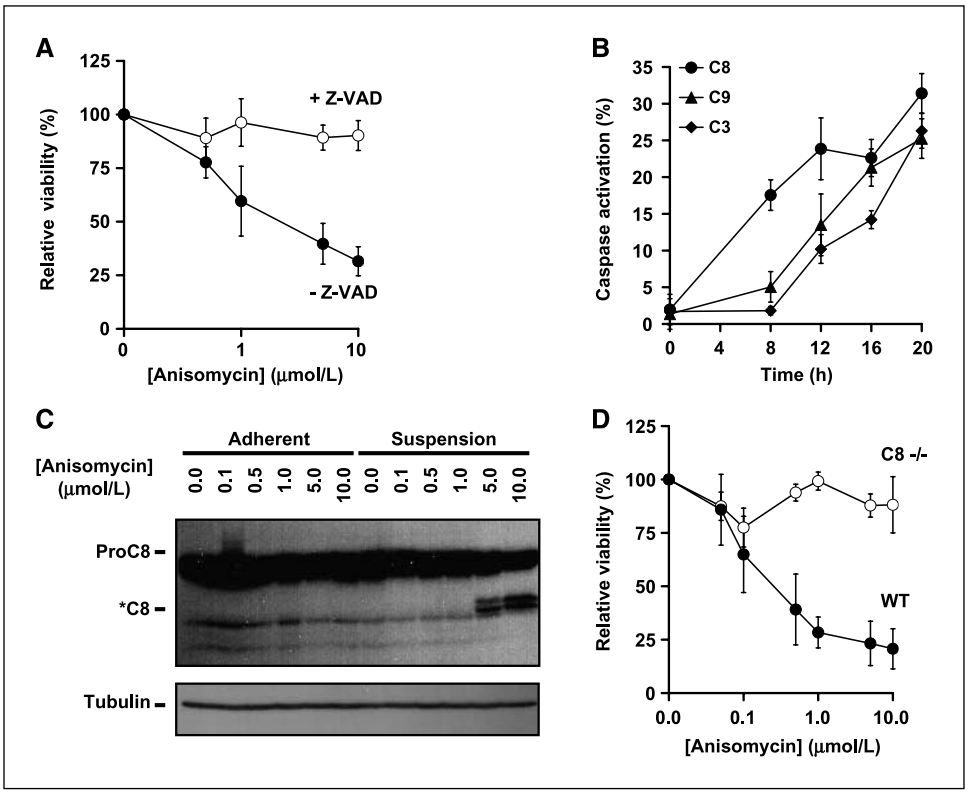

Figure 2. Anisomycin initiates anoikis via a caspase-8–dependent mechanism. *A,* PPC-1 cells (7.5×10^4) were seeded in 24-well plates under suspension conditions and treated with increasing concentrations of anisomycin in the presence (●) or absence (○) the pan-caspase inhibitor z-VAD-fmk (100 μmol/L). Twenty hours after incubation, anoikis was determined by flow cytometry to detect cell surface Annexin V expression and propidium iodide uptake. *Points,* mean percent of viable cells compared with untreated controls; *bars,* SE. *B,* PPC-1 cells (7.5×10^4) were seeded in 24-well plates under suspension and treated with anisomycin (5 μmol/L). At increasing times after treatment, caspase activation was detected with FITC-labeled peptides that bind preferentially and irreversibly to caspase-3/7 (C3), caspase-8 (C8), or caspase-9 (C9). Caspase activation was monitored by flow cytometry to quantify the percentage of fluorescently labeled cells. *Points,* mean percentage of cells with active caspases relative to untreated cells; *bars,* SE. *C,* PPC-1 cells (3.0×10^6) were seeded in 100-mm dishes in either adherent or suspension conditions overnight. Cells were then treated with increasing concentrations of anisomycin. Twenty hours after incubation, total cellular protein was isolated and analyzed by SDS-PAGE immunoblotting using anti–caspase-8 and anti-tubulin antibodies. *ProC8,* uncleaved (inactive) procaspase-8; **C8,* cleaved (active) caspase-8. *D,* E1A-transformed wild-type (*WT*) and caspase-8−/− (C8−/−) MEFs (1.0×10^4) were cultured in suspension conditions with increasing concentrations of anisomycin. Ten hours after incubation, cell viability was measured using the MTS assay. *Points,* mean percentage viable cells compared with untreated controls; *bars,* SE.

To determine the sequence of caspase activation in anisomycin-mediated anoikis, PPC-1 cells were treated with anisomycin (5 μmol/L) or buffer under suspension conditions. At increasing times after treatment, caspase activation was detected using cell-permeable, FITC-labeled peptides that bind preferentially and irreversibly to active caspases. Active caspase-8 was detected before activation of caspase-3/7 and caspase-9 (Fig. 2*B*). These data suggest that anisomycin can sensitize cells to anoikis by activating the death receptor pathway of caspase activation. Consistent with this observation, immunoblot analysis revealed that anisomycin activated caspase-8 in cells cultured under suspension but not adherent conditions (Fig. 2*C*).

To further evaluate the dependence on caspase-8 for anisomycin-mediated sensitization to anoikis, E1A-transformed wild-type and caspase-8–deficient MEFs were treated with increasing concentrations of anisomycin under suspension conditions and cell viability was determined by the MTS assay. Caspase-8−/− MEFs were resistant to anisomycin-mediated anoikis compared with wild-type cells. (Fig. 2*D*). Thus, anisomycin sensitizes resistant cells to anoikis by activating the death receptor pathway of caspase activation.

Anisomycin sensitizes cells to anoikis by decreasing levels of FLIP protein. FLIP is an endogenous inhibitor of caspase-8 (26). We have previously shown that increased levels of FLIP contribute to anoikis resistance in malignant cells (6). Therefore, we measured the effects of anisomycin on FLIP protein expression. PPC-1 cells were treated with increasing concentration of anisomycin under adherent and suspension conditions. After incubation, FLIP protein and mRNA levels were measured by immunoblotting and reverse transcription-PCR (RT-PCR), respectively. Under both adherent and suspension conditions, anisomycin decreased levels of FLIP protein but did not change FLIP mRNA (Fig. 3*A* and *B*, and data not shown). To determine whether a reduction in FLIP protein was functionally important for anoikis sensitization, we tested whether FLIP knockdown using siRNA recapitulated the effects of anisomycin (Fig. 3*C*). We found that FLIP knockdown, anisomycin, or a combination of these treatments did not influence viability under adherent conditions, whereas each treatment alone sensitized cells to anoikis. Furthermore, combining the two treatments did not further sensitize cells to anoikis. Thus, we concluded that anisomycin sensitizes resistant cells to anoikis by decreasing levels of FLIP protein.

Activation of JNK and p38 by anisomycin is not necessary or sufficient to sensitize cells to anoikis. Through its ability to inhibit the 28S rRNA, anisomycin can activate JNK and p38, and these effects can be dissociated from its ability to inhibit protein synthesis (21–25). Through the E3 ligase ITCH, JNK promotes the degradation of FLIP (27–29). Therefore, we tested the effects of

anisomycin on JNK activation and its functional significance in decreasing FLIP and sensitizing cells to anoikis. PPC-1 cells were treated with anisomycin (5 μmol/L) and levels of JNK, phosphorylated JNK, p38, phosphorylated p38, FLIP, and caspase-8 were measured by immunoblotting at increasing times of incubation (Fig. 4*A*). Rapid increases in phosphorylated JNK and phosphorylated p38 were detected at times that preceded the decrease in FLIP and activation of caspase-8.

We then determined whether anisomycin-mediated activation of JNK and p38 were functionally important for the effects of anisomycin on FLIP and anoikis. PPC-1 cells under suspension conditions were treated with increasing concentrations of anisomycin along with the chemical JNK inhibitor SP600125. After incubation, cell viability was measured by Annexin V staining and levels of FLIP were measured by immunoblotting (Fig. 4*B*). Despite pretreatment with SP600125, anisomycin continued to sensitize cells to anoikis and decrease FLIP. However, pretreatment with SP600125 inhibited anisomycin-mediated phosphorylation of the JNK target c-Jun (Fig. 4*C*). Similar to the effects of JNK inhibition, pretreatment with the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 did not block the effects of anisomycin on anoikis and FLIP (data not shown). Thus, these results show that anisomycin activates JNK and p38 MAPK, but activation of these kinases is not necessary for the effects of anisomycin on anoikis or FLIP.

Anisomycin inhibits FLIP synthesis. FLIP protein has a short half-life, so decreased FLIP protein and sensitization to anoikis could be an early sign of protein synthesis inhibition. As anisomycin induces cell stress through a ribotoxic mechanism (24), we tested whether anisomycin sensitized cells to anoikis by inhibiting protein synthesis. PPC-1 cells were treated with the protein synthesis inhibitor cycloheximide, anisomycin, or both cycloheximide and anisomycin, and changes in levels of FLIP protein were measured by immunoblotting over time. Inhibition of protein synthesis by cycloheximide caused a rapid decrease in FLIP protein within 1.5 h of treatment. The rate of reduction of FLIP was the same after anisomycin treatment and no further decrease in FLIP levels were observed when anisomycin was added to cycloheximide (Fig. 5*A*). We also measured protein synthesis after treatment of cells with increasing concentrations of anisomycin. Anisomycin reduced protein synthesis at concentrations associated with reductions in FLIP and sensitization to anoikis (Fig. 5*B*). Finally, we tested the effects of other known inhibitors of protein synthesis on anoikis sensitization. PPC-1 cells were

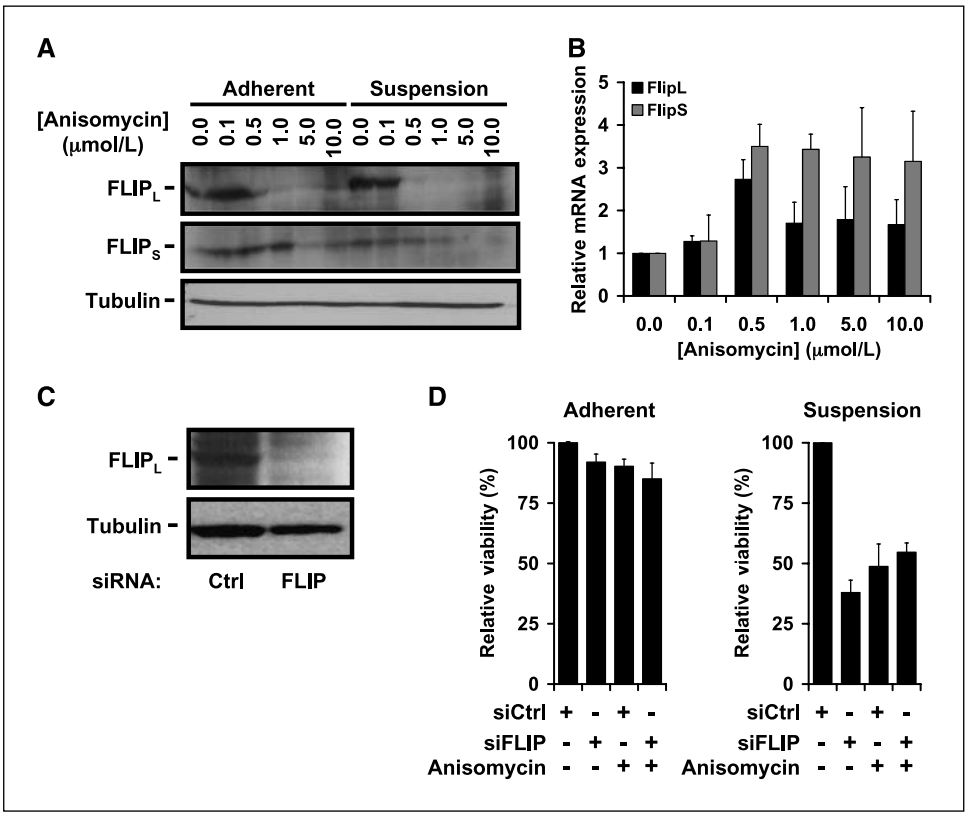

Figure 3. Anisomycin induces anoikis by decreasing FLIP protein through a posttranslational mechanism. *A,* PPC-1 cells (3.0×10^6) were seeded overnight in adherent or suspension conditions and then treated with increasing concentrations of anisomycin. Twenty hours after incubation, total cellular protein was isolated and analyzed by SDS-PAGE immunoblotting using anti-FLIP and anti-tubulin antibodies. *B,* PPC-1 cells were cultured in the presence of increasing concentrations of anisomycin. Twenty hours after incubation, total cellular RNA was isolated. FLIP long (*FLIP-L*) and FLIP short (*FLIP-S*) mRNA expressions were measured relative to 18S RNA by real-time RT-PCR. *Columns,* mean percentage of FLIP-L/18S or FLIP-S/18S expression relative to untreated controls ($\Delta\Delta C_T$ normalization); *bars,* SE. *C,* PPC-1 cells (3.0×10^6) were seeded in 100-mm dishes under adherent conditions overnight and then transfected with anti-luciferase siRNA (*siCtrl*, 50 nmol/L) or anti-FLIP siRNA (*siFLIP*, 50 nmol/L). Forty-eight hours after transfection, total cellular protein was isolated and analyzed by SDS-PAGE immunoblotting using anti-FLIP and anti-tubulin antibodies. *D,* PPC-1 cells were transfected with siRNAs as in (*C*). Eight hours after transfection, cells were subcultured into 24-well plates (7.5×10^4 per well) under adherent or suspension conditions and incubated overnight. Cells were then treated with anisomycin (5 μmol/L) for 20 h. After incubation, apoptosis/anoikis was determined by flow cytometry to detect cell surface Annexin V expression and propidium iodide uptake. *Columns,* mean percentage of viable cells compared with untreated controls; *bars,* SE.

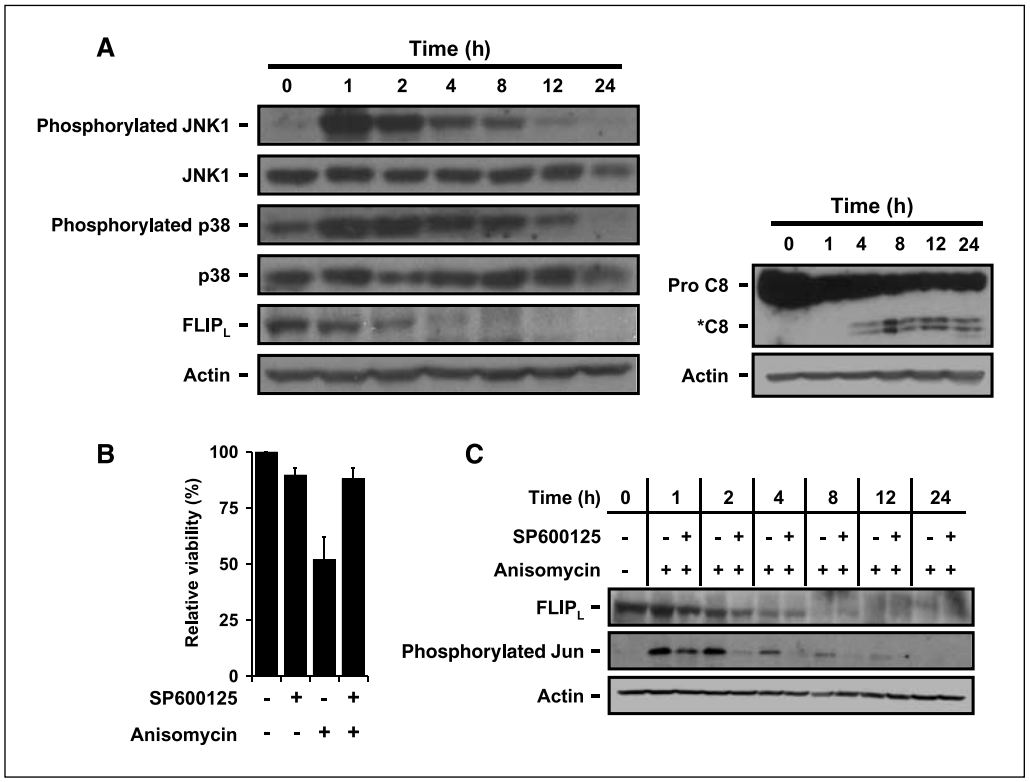

Figure 4. Anisomycin activates JNK and p38, but this activation is not functionally important for the effects of anisomycin on anoikis and FLIP. *A,* PPC-1 cells (3.0×10^6) were seeded in 100-mm dishes for 8 h under suspension conditions and then treated with anisomycin (5 μmol/L). Total cellular protein was isolated at increasing times after treatment and analyzed by SDS-PAGE immunoblotting using antibodies against JNK, phosphorylated JNK, FLIP, p38, phosphorylated p38, caspase-8, and actin. *B,* PPC-1 cells (7.5×10^4) were cultured in 24-well plates in suspension conditions for 8 h. Cells were then pretreated with a JNK inhibitor (*SP610025*, 25 μmol/L) for 1 h followed by treatment with anisomycin (5 μmol/L). Twenty hours after incubation, anoikis was determined by flow cytometry to detect cell surface Annexin V expression and propidium iodide uptake. *Columns,* mean percentage of viable cells compared with untreated controls; *bars,* SE. *C,* PPC-1 cells were cultured as in (*B*). Twenty hours after incubation, total cellular protein was isolated and analyzed by SDS-PAGE immunoblotting using anti-FLIP, anti–c-Jun, and anti-actin antibodies.

treated under adherent or suspension conditions with increasing concentrations of the known protein synthesis inhibitors emetine (30), homoharringtonine (31), and cycloheximide (30), and cell viability was measured by the MTS assay. All three protein synthesis inhibitors induced cell death under suspension but not adherent conditions similar to anisomycin (Fig. 5*C*). Interestingly, emetine and cycloheximide were both weak hits in the initial screen for anoikis sensitizers. Taken together, these results suggest that inhibiting protein synthesis sensitizes resistant cells to anoikis by decreasing FLIP synthesis.

Anisomycin decreases the *in vivo* survival of circulating prostate cancer cells. The presence of circulating cancer cells after adjuvant chemotherapy is prognostic for an increased risk of relapse and the development of metastatic disease (32–34). Anoikis blocks metastasis by preventing normally adherent cells from surviving after detachment from their primary site, and FLIP contributes to anoikis resistance. Therefore, we tested whether reducing FLIP levels with anisomycin could decrease distal tumor formation *in vivo*. PPC-1 cells labeled with a red fluorescent protein (dsRed2) were treated with anisomycin (5 μmol/L) or buffer control in culture under adherent conditions. After treatment, cells were injected i.v. into sublethally irradiated SCID mice. Five weeks after i.v. injection or when moribund, mice were sacrificed and tumor formation in the organs was imaged with fluorescent microscopy. Invasion of prostate cancer cells was detected in the lung, bone, and liver, which are clinically relevant sites of metastases in prostate cancer. Treatment with anisomycin decreased tumor formation in these organs compared with controls (Fig. 6*A*). It is important to note that both treated and control cells were >85% viable at the time of injection and remained viable for at least 72 h under adherent conditions.

Metastasis of dsRed–PPC-1 cells to the lung was readily quantifiable using image-based analysis (19, 35). Compared with control, mice injected with anisomycin-treated cells had a decreased median total tumor count and median total tumor area within the lung (Fig. 6*B*). Median survival of mice injected with anisomycin-treated cells was significantly longer than mice injected with control treated cells (35 versus 25 days, respectively, $P = 0.0001$ by log-rank statistic, $n = 27$). Similar reductions in tumor growth were observed in the bones and liver.

In contrast to the decrease in tumor formation after i.v. injection, no significant difference in tumor weight was detected after s.c. injection of dsRed–PPC-1 cells treated with anisomycin or buffer alone (128 ± 57 versus 193 ± 85 mg; $P = 0.25$, by t test; Fig. 6*C*). These results further validate that the PPC-1 cells were viable at the time of injection.

Taken together, these results indicate that inhibiting FLIP synthesis with anisomycin decreases the survival of circulating tumor cells and thereby decreases tumor formation in distant organs. Thus, the inhibition of FLIP and protein synthesis inhibitors may be an effective antimetastatic strategy and a useful adjunct to chemotherapy.

Discussion

Anoikis serves as a barrier to metastasis. Resistance to anoikis permits cancer cells to survive in the systemic circulation and facilitates their metastasis to distant organs. In fact, patients with circulating tumor cells in the peripheral blood after conventional chemotherapy have a worse prognosis compared with patients without these circulating cells (32, 34). Therefore, therapeutic strategies that specifically target anoikis resistance pathways have the potential to decrease metastasis and thereby improve patient survival.

To better understand the mechanisms that regulate the process of anoikis, we developed, automated, and conducted a chemical screen that identified anisomycin as a small molecule that sensitizes resistant tumor cells to anoikis. Anisomycin binds and inhibits 28S rRNA, leading to activation JNK and p38 MAPK (21–25). By binding the 28S subunit, anisomycin also inhibits the peptidyl-transferase activity of ribosomes and thereby blocks protein synthesis (36, 37). The effects on protein synthesis can be dissociated from its activation of kinases such as JNK (25).

We then used anisomycin as a chemical probe to understand control points within the anoikis pathway and showed that anisomycin sensitizes resistant cells to anoikis by decreasing FLIP protein synthesis and activating the death receptor pathway of caspase activation. FLIP is a short half-life protein as it is rapidly ubiquitinated and degraded by the proteasomal system (38). Thus, decreased levels of FLIP would be an early sign of inhibition of protein synthesis as degradation proceeds without continued synthesis. In our study, we observed that anisomycin activated both JNK and p38; however, activation was not necessary for decreases in FLIP protein or sensitization to anoikis. This finding is consistent with earlier studies in nonmalignant cells showing that JNK activation is not requisite for activation of anoikis upon detachment from the ECM (39).

As a protein synthesis inhibitor, anisomycin would be expected to decrease the synthesis of many proteins and is not a specific inhibitor of FLIP expression. Although the studies with FLIP siRNA support a critical role for FLIP in anoikis resistance, we cannot exclude the possibility that protein synthesis inhibitors sensitize cells to anoikis by decreasing additional proteins. Rather, these results suggest that continued protein synthesis within suspended tumor cells permits them to escape anoikis and highlights protein synthesis inhibition as a potential strategy to inhibit metastases.

Whereas anisomycin has not been used clinically, the protein synthesis inhibitor homoharringtonine is under development as a new therapeutic agent. Although this drug has activity as a single agent in hematologic malignancies (40, 41), it did not reduce the size of established solid tumors in clinical studies (42, 43). Our report might provide an explanation for the lack of benefit of homoharringtonine in solid tumors. We showed that inhibiting protein synthesis did not reduce the viability of malignant cells adherent to their ECM, but induced apoptosis after the cells detached. Thus, our study suggests that protein synthesis inhibitors could be of clinical benefit for the treatment of solid tumors when used to induce apoptosis of circulating tumor cells.

Studies in animals and patients with malignancies have shown that the presence and phenotype of malignant cells in the circulation can predict metastasis and survival (33, 34). To study the effect of inhibition of FLIP synthesis on the survival and distant

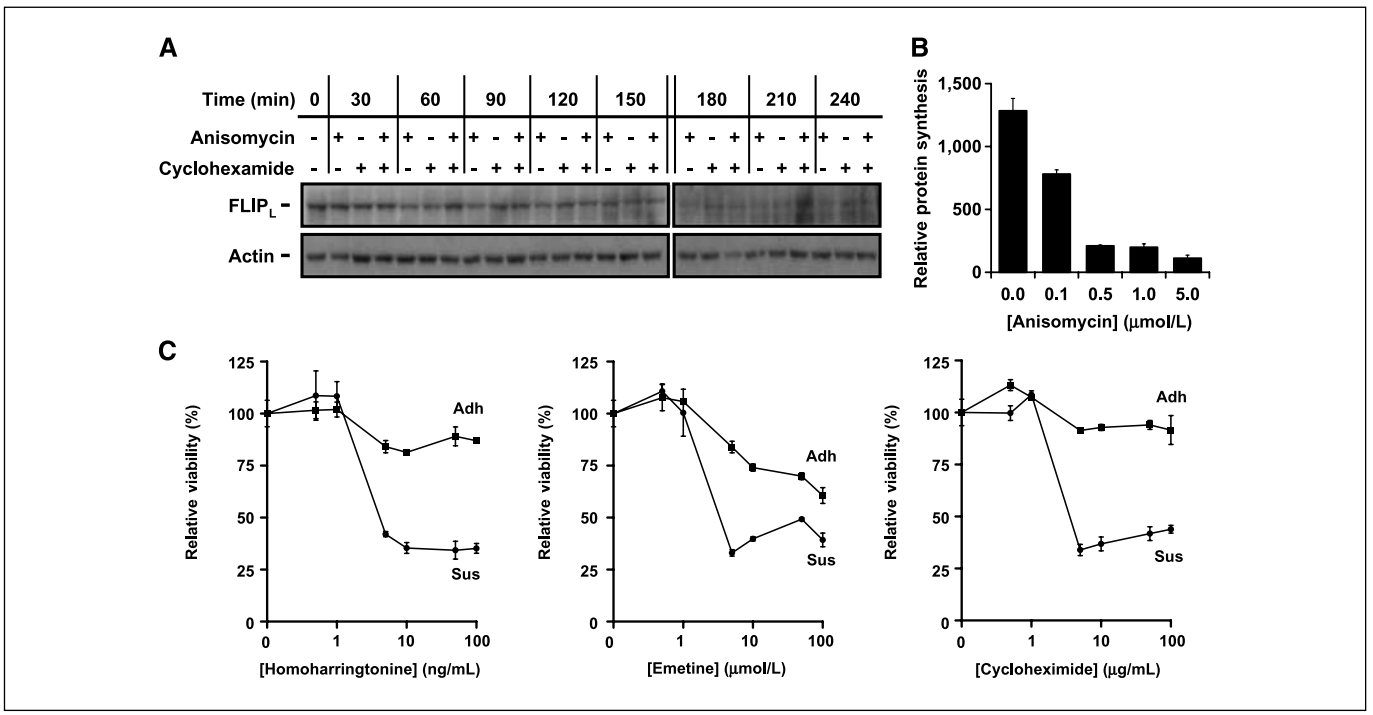

Figure 5. Anisomycin sensitizes cells to anoikis by decreasing FLIP synthesis. *A,* PPC-1 cells (3.0×10^6) were seeded overnight in adherent conditions and then treated with anisomycin (5 μmol/L), cycloheximide (5 μg/mL), or a combination of both. At increasing times after incubation, total cellular protein was isolated and analyzed by SDS-PAGE immunoblotting using anti-FLIP and anti-actin antibodies. *B,* PPC-1 cells (1.0×10^5) were seeded in 24-well plates. The next day, cells were treated with increasing concentrations of anisomycin. One hour after treatment, [^{3}H]leucine (10 μCi) was added to each well and the cells were incubated for an additional hour. After incubation, cells were harvested, washed, lysed, and [^{3}H]leucine incorporation was measured with a scintillation counter. *Columns,* mean radioactive counts normalized to the number of cells per well; *bars,* SD. *C,* PPC-1 cells (1.5×10^4) were seeded in 96-well plates in adherent (*Adh*) or suspension (*Sus*) conditions and incubated overnight. Cells were then cultured with increasing concentrations of homoharringtonine, emetine, or cycloheximide for 20 h. After incubation, cell viability was measured by the MTS assay. *Points,* mean percentage viable cells relative to untreated controls; *bars,* SE.

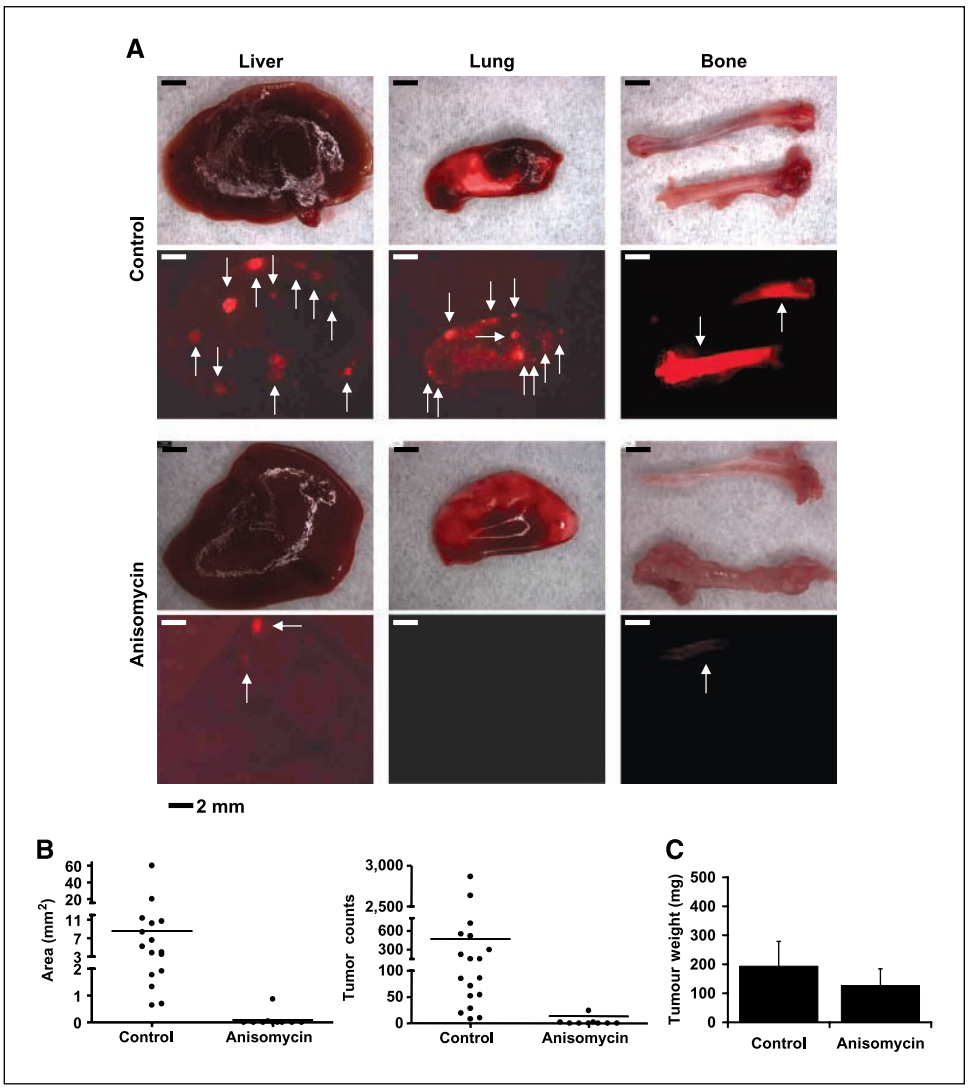

Figure 6. Anisomycin diminishes the *in vivo* survival and growth of circulating prostate cancer cells. *A,* fluorescent dsRed–PPC-1 cells were treated under adherent conditions with anisomycin (5 μmol/L) or buffer control. Sixteen hours after treatment, cells were harvested and 3.5×10^6 viable cells were injected into the tail veins of sublethally irradiated SCID mice. Five weeks after injection, or when mice became moribund, mice were sacrificed and their organs were imaged using a fluorescent microscope ($n = 27$). Representative images of tumor formation in the lung, bone, and liver. *B,* the number and area of distant tumors from mice described above were quantified using image analysis software. Tumors were quantified from all five lobes of the lungs. *Points,* measurements from each mouse; *bars,* median of the population. *C,* fluorescent dsRed–PPC-1 cells were treated under adherent conditions with anisomycin (5 μmol/L) or buffer control. Sixteen hours after treatment, cells were harvested and 3.5×10^6 viable cells were injected s.c. into sublethally irradiated SCID mice. Three weeks after injection, mice were sacrificed, and the tumors were excised and weighed. *Columns,* mean tumor weight; *bars,* SD.

tumor formation of malignant cells *in vivo*, we used our previously described mouse model that measures the ability of circulating human prostate cancer cells to form distant tumors (6). We showed that inhibition of FLIP with anisomycin reduced the number and area of distant tumors. In this model, FLIP protein levels were decreased in adherent dsRed–PPC-1 cells in culture before mice were injected, but the cells were viable at the time of injection and remained viable when cultured under adherent conditions for extended periods of time. Furthermore, we note that no difference was detected in tumor growth after s.c. injection of adherent cells treated with anisomycin or control.

It would have been interesting to determine the mechanism by which anisomycin prevents distant tumor formation *in vivo*. Unfortunately, anisomycin suppresses FLIP protein expression for a maximum of 24 h (data not shown), so anisomycin was no longer active by the time the metastatic tumors were detected. Therefore, it was not possible to compare levels of FLIP in the metastatic tumors from mice injected with treated and untreated cells to determine whether there had been target knock down. We attempted to collect dsRed-labeled PPC-1 cells 20 h after injection into the tail veins of SCID mice as described, but even at this time point we could not detect enough dsRed-labeled cells in the circulation to permit analysis of viability by flow cytometry. Nonetheless, we feel that our xenograft model provides a proof-of-concept that inhibition of protein synthesis within circulating tumor cells may decrease their ability to form distant tumors.

In summary, we used a chemical biology approach to identify mechanisms that influence anoikis. Our study shows that inhibition of protein synthesis can sensitize cells to anoikis by decreasing FLIP protein levels. Thus, protein synthesis inhibition may be a useful strategy to decrease metastases and could have clinical benefit when combined with chemotherapy. In the adjuvant setting, the chemotherapy would induce apoptosis of any micrometastases that have already seeded distant sites, whereas protein synthesis inhibitors would induce apoptosis in chemoresistant circulating tumor cells.

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A Chemical Screen Identifies Anisomycin as an Anoikis Sensitizer That Functions by Decreasing FLIP Protein Synthesis

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