

Increased Expression of Apoptosis Inhibitor Protein XIAP Contributes to Anoikis Resistance of Circulating Human Prostate Cancer Metastasis Precursor Cells

Olga Berezovskaya,¹ Aaron D. Schimmer,⁵ Anna B. Glinskii,¹ Clemencia Pinilla,² Robert M. Hoffman,^{3,4} John C. Reed,⁵ and Gennadi V. Glinsky¹

¹The Sidney Kimmel Cancer Center, ²The Torrey Pines Institute for Molecular Studies, ³AntiCancer, Inc., ⁴Department of Surgery, University of California, San Diego, California and ⁵The Burnham Institute, La Jolla, California

Abstract

Survival in lymph or blood is an essential prerequisite for metastasis of carcinoma cells to distant organs. Recently, we reported isolation and initial biological characterization of circulating metastatic cells in a fluorescent, orthotopic, metastatic nude-mouse model of human prostate cancer. Here we show that the metastatic human prostate carcinoma cells selected for survival in the circulation have increased resistance to anoikis, which is apoptosis induced by cell detachment. Using gene silencing and gene transfer techniques, we show that increased expression of the apoptosis-inhibitory protein XIAP contributes to anoikis resistance of the circulating metastatic human prostate carcinoma cells. We also provide initial preclinical data on the antimetastatic efficacy of recently discovered small-molecule antagonists of XIAP. (Cancer Res 2005; 65(6): 2378-86)

Introduction

Epithelial cancer cells have very low survival rates in the circulation (1–4). Even in the presence of high numbers of circulating cancer cells, there may be no clinical or pathohistologic evidence of metastatic colonization of target organs (5–12). The fate of cancer cells in the circulation includes a rapid phase of intravascular cancer cell death, which is completed in <5 minutes, and which accounts for 85% of the circulating cancer cells. For example, the number of tumor cells in the lungs declined very rapidly after i.v. injection such that after 3 days generally less than 1% remained (13–18). This decline is due to a rapid degeneration of cancer cells (14, 19).

The term “anoikis” is proposed to indicate apoptosis induced by disruption of cell attachment and cell-matrix interactions (20–32). Matrix-independent survival of metastatic cancer cells during passage through the blood and/or lymph compartment is an essential component of the metastatic cascade. Thus, anoikis resistance can contribute to metastasis, allowing epithelial cells to survive in a suspended state, thereby promoting their hematogenous or lymphatic dissemination (28, 32). Many cancer cell types selected for increased metastatic potential *in vivo* are resistant to apoptosis compared with the parental cells or less metastatic counterparts (23–26). Apoptosis-resistant tumor cells have also

recently been shown to survive and possibly grow intravascularly, when adherent to endothelial cells, in distal capillary beds (33).

Failure to activate caspases or excess suppression of the caspases causes resistance to apoptosis. The inhibitors of apoptosis proteins (IAP) represent the only known family of endogenous caspase inhibitors (34). There are eight known IAP-encoding genes in the human genome, several of which are overexpressed in cancers (35–37). Markedly increased expression of several IAPs was recently shown as a frequent and early event associated with human prostate cancer and in a transgenic mouse model of prostate cancer (38). However, little is known about the biological role of altered IAP expression in resistance to anoikis.

We have previously identified and isolated circulating tumor cells from mice bearing orthotopic human prostate cancer xenografts (1). Tumor cells isolated from the circulation of this model have enhanced metastatic capability. We show here that the circulating metastatic cells possess increased resistance to anoikis and also show increased expression of several IAPs. Using gene silencing and gene transfer techniques, we show that increased expression of the apoptosis inhibitory protein XIAP contributes to the anoikis resistance of circulating carcinoma cells. We also provide preclinical data on the efficacy of recently discovered small-molecule antagonists of the apoptosis suppressor XIAP (39).

Materials and Methods

Cell Lines and Culture. The human prostate carcinoma cell line PC-3, expressing green fluorescent protein (GFP), and sublines established from circulating tumor cells of mice with PC-3-derived tumors (1) including PC-3-32 and PC-3-33 were grown in DMEM supplemented with 10% fetal bovine serum (both from Irvine Scientific, Santa Ana, CA), penicillin (100 units/mL), streptomycin (100 µg/mL), and amphotericin B (0.25 µg/mL; Invitrogen, Grand Island, NY). Cells were routinely maintained in 75-cm² vented tissue culture flasks at 37°C in a humidified atmosphere of 5% CO₂. Cell viability was monitored by trypan blue dye and propidium iodide exclusion.

Cell Culture Conditions for Anoikis Assay. For culture under nonadherent conditions, cells (1.7×10^5) were plated in 1 mL of serum-free medium in 24-well ultralow-attachment polystyrene plates (Corning Inc., Corning, NY) and incubated at 37°C and 5% CO₂ overnight. The initial viability of cells before detachment culture was >95% in the trypan blue dye exclusion test. Each measurement was carried out in quadruplicate and repeated at least twice to ensure reproducibility.

Apoptosis Assay. Apoptotic cells were identified and quantified using the Annexin V-FITC kit (BD Biosciences PharMingen, <http://www.bdbiosciences.com>) per instructions of the manufacturer. The following controls were used: (a) unstained cells; (b) cells stained with Annexin V-FITC (no propidium iodide); and (c) cells stained with propidium iodide (no Annexin V-FITC). Each measurement was carried out in quadruplicate and each

Note: A.D. Schimmer is currently in the Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Ontario, Canada.

Requests for reprints: Gennadi V. Glinsky, Sidney Kimmel Cancer Center, 10835 Altman Row, San Diego, CA 92121. Phone: 858-450-5990; Fax: 858-623-2740; E-mail: gglinsky@skccc.org.

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experiment was repeated at least twice to ensure reproducibility. Adherent cultures served as controls for viability and apoptosis determinations.

Annexin V-FITC-positive cells were scored as early apoptotic cells; both Annexin V-FITC- and propidium iodide-positive cells were scored as late apoptotic cells; unstained Annexin V-FITC and propidium iodide-negative cells were scored as viable or surviving cells. In selected experiments, apoptotic cell death was documented using the terminal deoxynucleotidyl transferase-mediated nick-end-labeling assay as previously described (23, 24).

Western Blot Analysis. Adherent cultures at 70% confluence were harvested by 5-minute digestion with 0.25% trypsin/0.02% EDTA (Irvine Scientific), washed, and subjected to analysis. Cells were washed with cold PBS and lysed on ice in hypotonic buffer containing 20 mmol/L HEPES (pH 7.2), 10 mmol/L KCl, 1 mmol/L EDTA, 1.5 mmol/L MgCl₂, 1 mmol/L DTT, and 0.1 mmol/L phenylmethylsulfonyl fluoride. Lysates were then clarified by centrifugation at 14,000 × *g* for 20 minutes at 4°C. Total protein concentration was measured using the Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA). Lysates (20 µg/lane) were then boiled in sample loading buffer containing 42 mmol/L Tris-HCl, 10% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue, and 1.0% (v/v) 2-mercaptoethanol. Equal amounts of protein were subjected to SDS-PAGE (4–20% gradient gels from ISC BioExpress, Kaysville, UT) and transferred to Protran nitrocellulose membranes with pore size of 0.45 µm (Schleicher & Schüll, Dassel, Germany) using standard procedures. Membranes were blocked with 10% nonfat dry milk, 0.2% goat serum (Sigma, St. Louis, MO), and 0.1% Tween 20 in PBS for 1 hour at room temperature and then exposed to monoclonal mouse-antihuman XIAP (0.25 µg/mL, Transduction Laboratories, Lexington, KY) at a dilution of 1:250 at 4°C overnight. After several washings in PBS containing 10% (v/v) Tween 20 and 1% (w/v) nonfat dry milk, membranes were incubated with sheep antimouse horseradish peroxidase-conjugated immunoglobulin G (Transduction Laboratories) at 1:3,000 dilution for 1 hour at room temperature. Blots were developed by autoradiography using an enhanced chemiluminescence substrate system for detection of horseradish peroxidase, enhanced chemiluminescence TM Western Blotting Detection Reagent (Amersham Biosciences, United Kingdom, England). After several washings, membranes were reprobed for a loading control with mouse monoclonal antibodies against α -tubulin (Sigma) at a dilution of 1:1,000 (v/v). Images were scanned and densitometry was done using ImageJ software available in the public domain at <http://rsb.info.nih.gov/ij>. The expression values were normalized to the level in parental PC-3 cells.

XIAP Inhibitors. Polyphenylurea compounds 1396-12 and 1396-34 that display XIAP-inhibitory activity and a nonactive structural analogue 1396-28 have been previously described (39). After testing for cytotoxicity in adherent cultures and selection of the maximum nontoxic dose, each compound was used in anoikis experiments at a final concentration of 7 µmol/L. *In vivo* experiments were carried out using an orthotopic metastatic fluorescent model of human prostate cancer in nude mice (1). Male nude mice bearing PC-3-GFP-derived human xenografts implanted in their prostates (three animals per treatment group) were continuously treated for 2 weeks with i.p. daily injections of compounds (12.5 mg/kg) and monitored for tumor growth. Four weeks after tumor cell inoculation into the prostate, the animals were sacrificed and analyzed by GFP imaging as previously described (1). Quantitative measurements of tumor growth were done by measuring the pixel area of the imaged primary tumor area and metastasis which we have previously shown to correlate with the direct measurements of tumor volume (40).

Transient Transfection. PC-3 cells were seeded in six-well plates at a density of 2×10^5 cells/well the day before transfection to achieve 70% confluence. Cells were transfected with control pcDNA3, pcDNA3 myc *hXIAP* plasmid (41), or pEGFP. Transfection was done using SuperFect reagent (Qiagen, Inc., Valencia, CA) following the instructions of the manufacturer. Cells were allowed to recover after transfection for 48 hours before anoikis assays. Efficiency of transfection was confirmed by estimating the number of GFP-positive cells under fluorescence microscopy (~35–40%). Two independent transfection experiments were carried out to confirm the reproducibility of the findings.

RNA Interference. PC-3-32 circulating metastatic prostate carcinoma cells were seeded at a density of 10^5 cells/well in six-well tissue culture plates the day before transfection to achieve 50% to 60% confluence. Transfections were done with 70 nmol/L of siRNA duplex using RNAiFect (Qiagen) according to the instructions of the manufacturer. Subsequent transfections were carried out every 24 hours.

The antisense oligonucleotide was designed to the region 5'-AATAGTGCCACGCAGTCTACA-3' corresponding to residues 331 to 351 of human *XIAP* cDNA (U45880). Predesigned siRNA duplexes 5'-AACG-TACGCGGAATACTTCGA-3' for luciferase were used as a negative control. Both oligonucleotides were synthesized and purified by Qiagen-Xeragon (Germantown, MD). Three independent *XIAP* silencing experiments were carried out to confirm the reproducibility of the findings.

Flow Cytometry. Cells were washed in cold PBS and stained according to the instructions of the manufacturer using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA). Flow analysis was done with a FACS Calibur instrument (BD Biosciences). Cell Quest Software was used for data acquisition and analysis. All measurements were done under the same instrument setting, analyzing 10,000 cells per sample.

Quantitative Reverse Transcription-PCR. Total RNA was extracted using the RNeasy mini-kit (Qiagen) following the instructions of the manufacturer. Total RNA (4 µg) was used as a template for cDNA synthesis with SuperScript II (Invitrogen, Carlsbad, CA). Quantitative PCR primer sequences were selected for each cDNA with the aid of Primer Express software (Applied Biosystems, Foster City, CA). PCR amplification was done with the following gene-specific primers: *GAPDH*, 5'-CCCTCAACGAC-CACCTTGTCA-3' and 5'-TTCCTCTGTGCTCTTGCTGG-3'; *XIAP*, 5'-GGCA-GATTATGAAGCACGGATC-3' and 5'-GGCTTCCAATCAGTTAGCCCTC-3'; *cIAP-1*, 5'-GCAGACACATGCAGCTCGAAT-3' and 5'-ACACCTCAAGCCAC-CATCACA-3'; *cIAP-2*, 5'-ACACATGCAGCCCGCTTTA-3' and 5'-CTCCA-GATTCCTCAACACTGA-3'; *survivin*, 5'-CACTGCCCCACTGAGAACGA-3' and 5'-AAGGAAAGCGCAACCGGAC-3'; *EZH2*, 5'-CGCTTTTCTGTAGGC-GATGTTT-3' and 5'-TGCTGGTAACACTGTGGTCCA-3'. Quantitative PCR reactions and measurements were done with the SYBR-Green and ROX as a passive reference using the ABI 7900HT Sequence Detection System (Applied Biosystems). Conditions for the PCR were as follows: one cycle of 15 minutes at 95°C; 45 cycles of 20 seconds at 94°C, 20 seconds at 60°C, and 20 seconds at 72°C; dissociation stage of 15 seconds at 95°C, 15 seconds at 60°C, and 15 seconds at 95°C. The results were normalized to the relative amounts of *GAPDH*.

Statistical Analysis. Statistical analysis was done using paired two-tailed Student's *t* test. Significance was assumed for values of $P < 0.05$.

Results and Discussion

Increased Resistance to Anoikis of Circulating Metastatic Human Prostate Carcinoma Cells. We compared anchorage-independent survival *in vitro* of viable circulating metastatic human prostate carcinoma cells isolated from mice bearing orthotopic human PC xenografts and their nonselected parental prostate carcinoma cells. In these experiments, two independent isolates of circulating metastatic human prostate carcinoma cells and a parental cell line were subjected to cell detachment and monitored for cell viability (Fig. 1A) and apoptosis (Fig. 1B–D). In adherent cultures the basal level of apoptosis was below 5%, and there was no statistically significant difference between parental and circulating human prostate carcinoma cells. In contrast to adherent cultures, circulating human prostate carcinoma cells in suspension consistently had increased survival (Fig. 1A) and diminished apoptosis (Fig. 1B–D) compared with the parental cell line. Adherent cultures of parental and circulating metastatic cells cultivated for 16 hours in serum-free medium had similar survival and apoptosis (data not shown), suggesting that the increased survival of circulating metastatic prostate carcinoma cells observed in suspension is specific and does not occur under serum withdrawal conditions.

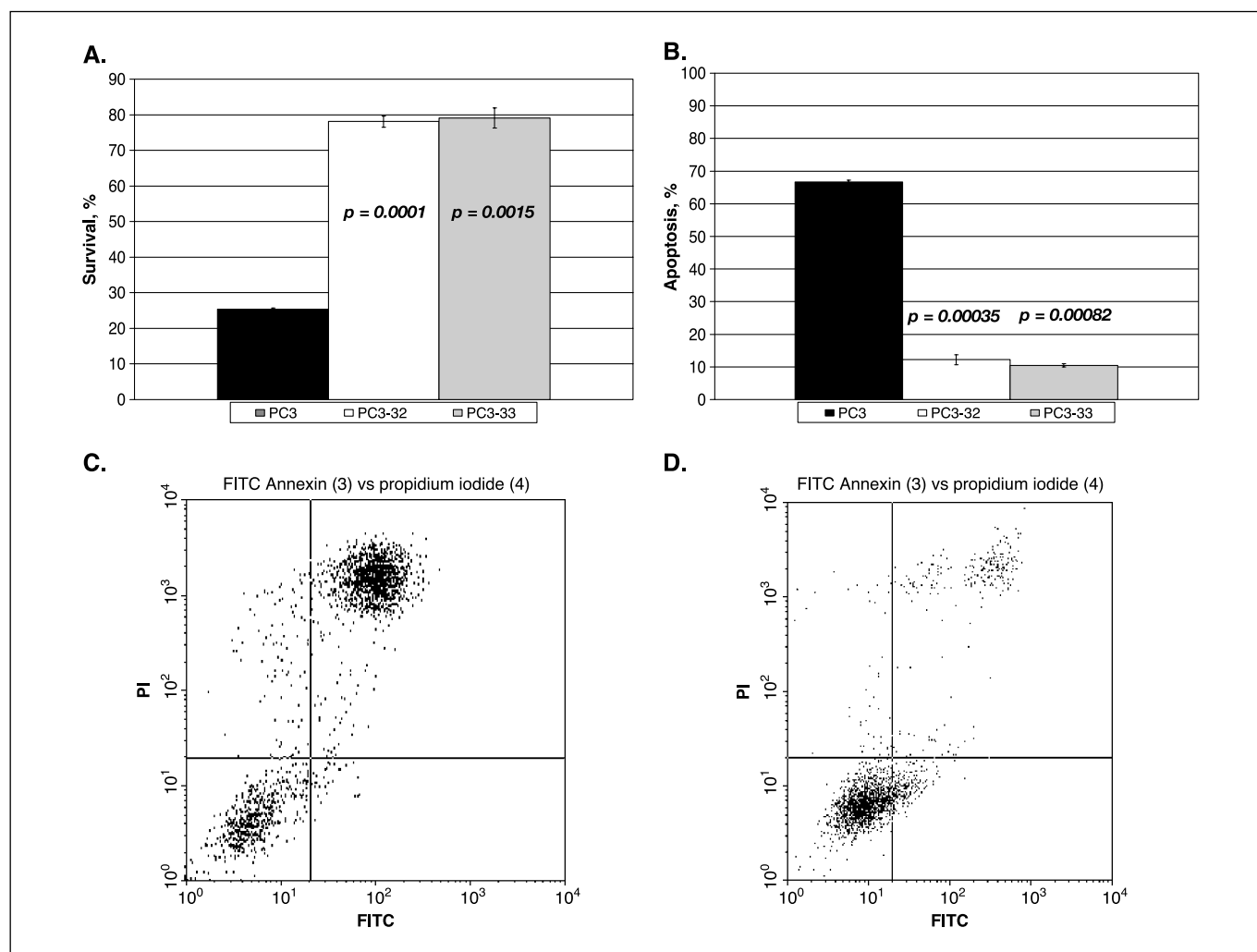


Figure 1. Increased resistance to anoikis of circulating metastatic human prostate carcinoma cells. *A*, increased survival of circulating metastatic cells subjected to nonadherent conditions. *B*, diminished apoptosis in circulating metastatic cells subjected to nonadherent conditions. *C*, scattergram of PC-3 parental prostate carcinoma cells in anoikis. *D*, scattergram of PC-3-32 circulating metastatic prostate cancer cells in anoikis. Parental PC-3 and two isolates of circulating metastatic cells (PC-3-32 and PC-3-33) were cultivated under nonadherent conditions for 16 hours and monitored for survival and apoptosis as described in Materials and Methods. Representative results of one of four independent experiments are shown.

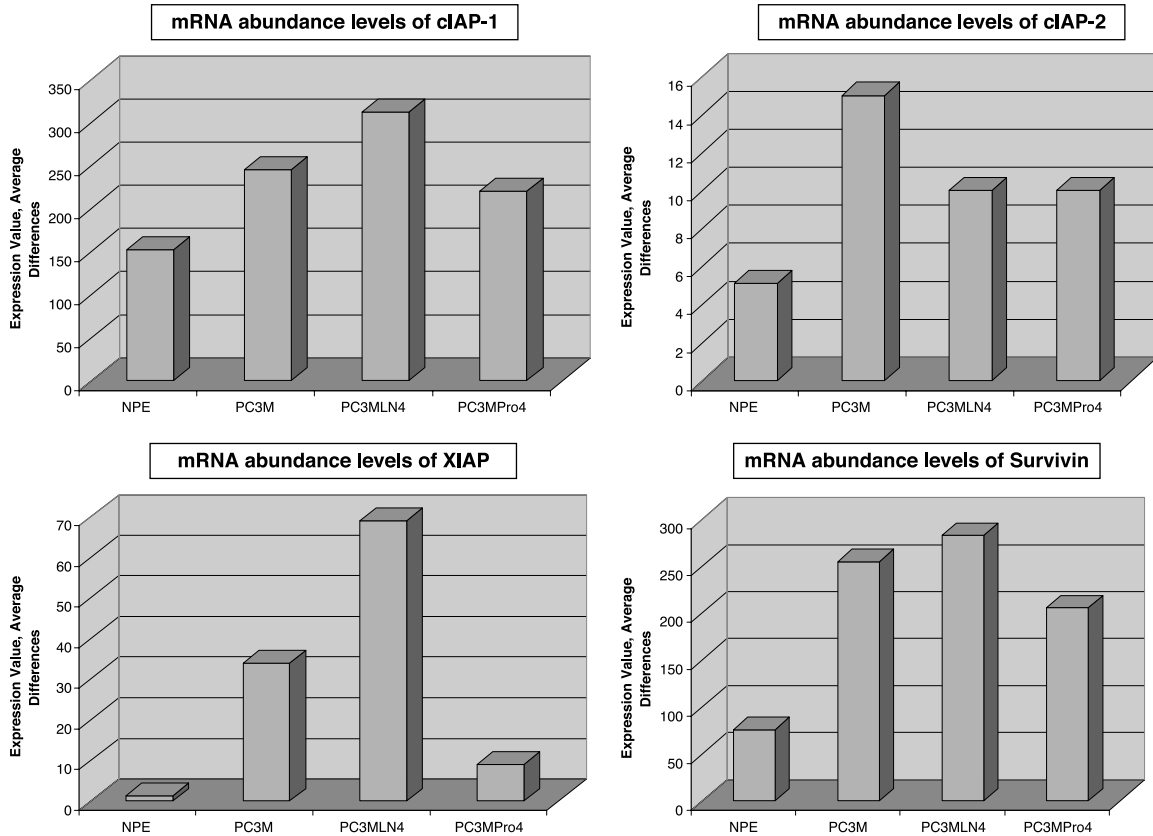
Increased Expression of Inhibitor of Apoptosis Proteins in Circulating Metastatic Prostate Carcinoma Cells. Gene expression microarray analysis revealed that the mRNA expression level of several IAPs is consistently elevated in several established human PC cell lines with elevating metastatic potential including cIAP-1, cIAP-2, XIAP, and survivin (ref. 42; Fig. 2A). We compared the protein expression levels of four IAP family members in the parental PC-3 cell line and circulating

metastatic cells. Western blot analysis indicated that the expression of XIAP, survivin, and cIAP-2 is elevated in the circulating metastatic cells (Fig. 2B). The expression of cIAP-1 seems similar in the two cell types (Fig. 2A). These results indicate that increased IAP expression is readily detectable in the circulating metastatic prostate carcinoma cells.

Increased XIAP Expression Renders Prostate Cancer Cells More Resistant to Anoikis. We then analyzed whether increased

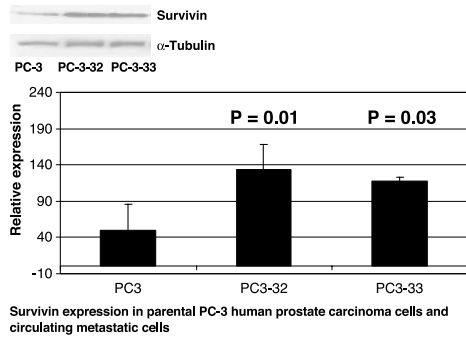
Figure 2. *A*, gene expression microarray analysis reveals that mRNA abundance levels of *cIAP-1*, *cIAP-2*, *XIAP*, and *survivin* are higher in human prostate carcinoma metastatic variants compared with the normal prostate epithelial cells (*NPE*, first column). Note that the levels of expression of *cIAP-1*, *XIAP*, and *survivin* seem to increase more significantly in the highly metastatic prostate cancer variant PC-3MLN4 (third column) compared with less metastatic PC-3MPro4 prostate carcinoma cells (last column). *PC-3M*, parental; *PC-3MLN4*, highly metastatic; *PC-3MPro4*, less metastatic (see ref. 42 for details). *B*, increased IAP expression in circulating metastatic human prostate carcinoma cells. *i*, survivin expression in parental PC-3 cells and circulating metastatic cells. *ii*, cIAP-2 expression in parental PC-3 human prostate carcinoma cells and circulating metastatic cells. *iii*, XIAP expression in parental PC-3 human prostate carcinoma cells and circulating metastatic cells. *iv*, cIAP-1 expression in parental PC-3 human prostate carcinoma cells and circulating metastatic cells. Western blot and densitometry analyses of IAP expression were carried out in subconfluent adherent cultures (~70% confluence) of corresponding cell variants as described in Materials and Methods. The differences in the expression of XIAP, survivin, and cIAP-2 between PC-3 parental cells and circulating metastatic PC-3-32 and PC-3-33 cells were statistically significant ($P \leq 0.05$). There were no significant differences in expression between two isolates of the circulating metastatic cells; for example, for survivin, $P = 0.11$ between PC-3-32 and PC-3-33 cells.

A.

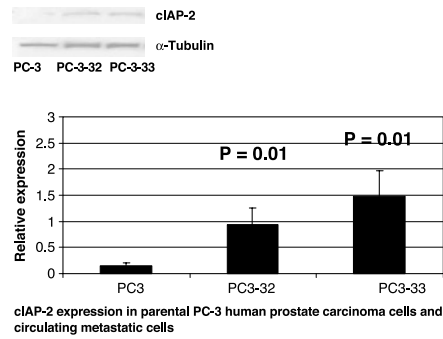


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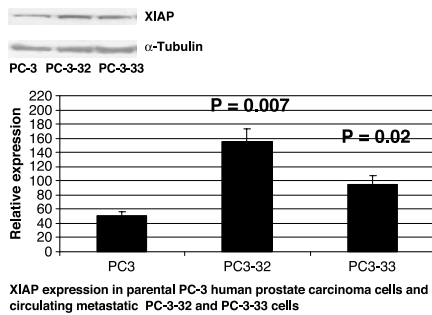
(i)



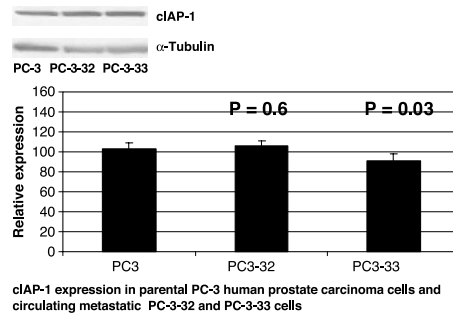
(ii)



(iii)



(iv)



XIAP expression would promote survival and diminish apoptosis in human prostate carcinoma cells subjected to nonadherent conditions. In these experiments, we transiently transfected anoikis-sensitive parental PC-3 cells (Fig. 1) with a *XIAP*-encoded plasmid or mock-plasmid (control), then transferred the resulting transformants to suspension culture. Transfection efficacy was controlled by cotransfection of a *GFP*-expressing plasmid. Cultures with similar levels of *GFP*-expressing cells (~40-45%) were subjected to biological assay. In the experiments presented in Fig. 3, parental PC-3 cells were transiently transfected with *XIAP*-expressing or control plasmids, subjected to detachment conditions, and monitored for survival and apoptosis. As shown in Fig. 3A, the survival of mock-transfected PC-3 cells is 10%. However, the survival of *XIAP*-transfected PC-3 cells is 18% ($P = 0.0082$; two-tailed Student's *t* test), thus demonstrating an XIAP-mediated increase in survival. Conversely, *XIAP*-transfected PC-3 cells had a decreased percentage of apoptosis compared with mock-transfected cells (25% versus 35%, respectively; $P = 0.0206$; Fig. 3B). In this experiment, the early apoptotic cells were scored whereas in the experiment in Fig. 1B the late apoptotic cells were scored (68%), accounting for the lower percentage of apoptotic cells scored in Fig. 3B. The percentage of late apoptotic cells in nontransfected PC-3 cells cultured in adherent conditions is 5% to 7%. The survival abilities and sensitivity to anoikis most likely reflect the conditions of each type of experiment. In all instances, the required controls were carried out to account for nonspecific effects of experimental protocols and reagents (Figs. 1 and 3).

Survival of mock-transfected circulating metastatic PC-3-32 cells under nonadherent conditions was higher compared with mock-transfected parental PC-3 cells (38% versus 10% survival, respectively, data not shown), consistent with our observation that circulating metastatic human prostate carcinoma cells exhibit increased resistance to anoikis.

These results suggest that increased XIAP expression in the circulating metastatic prostate carcinoma cells promotes increased anchorage independence, correlating with increased resistance to anoikis and elevated metastatic potential.

siRNA-Mediated XIAP Silencing Increases Sensitivity to Anoikis of Circulating Metastatic Prostate Carcinoma Cells.

In the next set of experiments, prostate carcinoma cells isolated from the circulation of the orthotopic models were transfected with *XIAP* siRNA or control luciferase siRNA and continuously monitored for growth, viability, and mRNA expression levels for several IAP family members (Fig. 4). Quantitative reverse transcription-PCR analysis showed that *XIAP* siRNA caused >60% reduction in *XIAP* mRNA expression levels in PC-3-32 circulating metastatic cells (Fig. 4A), without significant changes in the mRNA expression levels of other IAP family members (Fig. 4B). In adherent cultures no effects on cell growth and viability by *XIAP* siRNA was observed (data not shown).

siRNA-treated circulating metastatic cells were subjected to cell detachment and then measured for viability and apoptosis. siRNA-mediated diminished expression of *XIAP* mRNA caused decreased viability (Fig. 4C) and enhanced apoptosis (Fig. 4D) during suspension culture of PC-3-32 circulating metastatic cells. As shown in Fig. 4C, the survival of control luciferase siRNA-transfected PC-3-32 cells was 34%, whereas survival of *XIAP* siRNA-transfected PC-3-32 cells with diminished level of endogenous *XIAP* mRNA expression was significantly reduced to 21% survival ($P = 0.0003$). Thus, the siRNA-mediated decrease in the

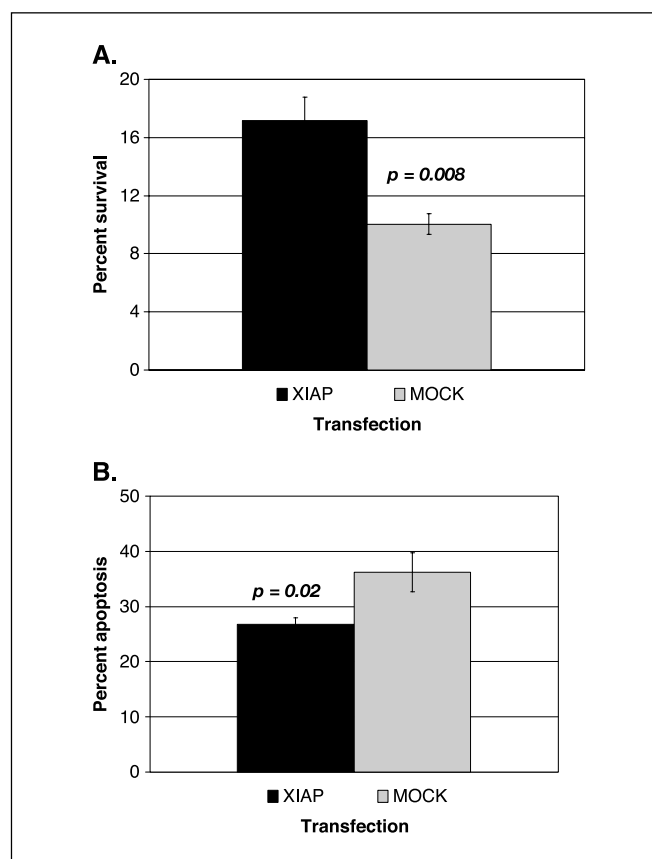


Figure 3. Transient XIAP transfection increases resistance to anoikis of human prostate carcinoma cells. *A*, transient XIAP transfection increases survival of parental human prostate carcinoma cells subjected to nonadherent conditions compared with mock-transfected controls. *B*, transient XIAP transfection decreases apoptosis in human prostate carcinoma cells subjected to nonadherent conditions compared with mock-transfected controls. Parental PC-3 cells were transiently transfected with *XIAP*-expressing or mock plasmids, subjected to nonadherent conditions, and monitored for survival and apoptosis as described in Materials and Methods. Transfection efficiency in each culture was assessed using cotransfection of a *GFP*-expressing plasmid and counting green fluorescent cells in control and experimental cultures (see Materials and Methods for details).

level of endogenous *XIAP* in PC-3-32 cells correlates with diminished sensitivity to anoikis. These results are consistent with the results of transfection of *XIAP* and support the hypothesis that *XIAP* plays a role in anoikis resistance and metastasis.

Inhibitor of Apoptosis Suppressor XIAP Enhances Sensitivity to Anoikis of Circulating Metastatic Prostate Carcinoma Cells and Reduces Their Metastatic Potential. We subjected circulating prostate carcinoma cells to nonadherent conditions after treatment with either of the two active small-molecule XIAP inhibitors (1396-12 or 1396-34) or with an inactive structural analogue (C28). Each compound was used at a concentration of 7 $\mu\text{mol/L}$, which was noncytotoxic in adherent culture. In contrast, the XIAP antagonists at this concentration decreased the viability of PC-3-32 and PC-3-33 circulating metastatic prostate carcinoma cells growing in suspension (Fig. 5A and B) and increased their sensitivity to anoikis (Fig. 5C and D). This effect seems specific, as treatment of cells with the inactive structural analogue did not influence cell viability and did not increase their sensitivity to anoikis (Fig. 5E).

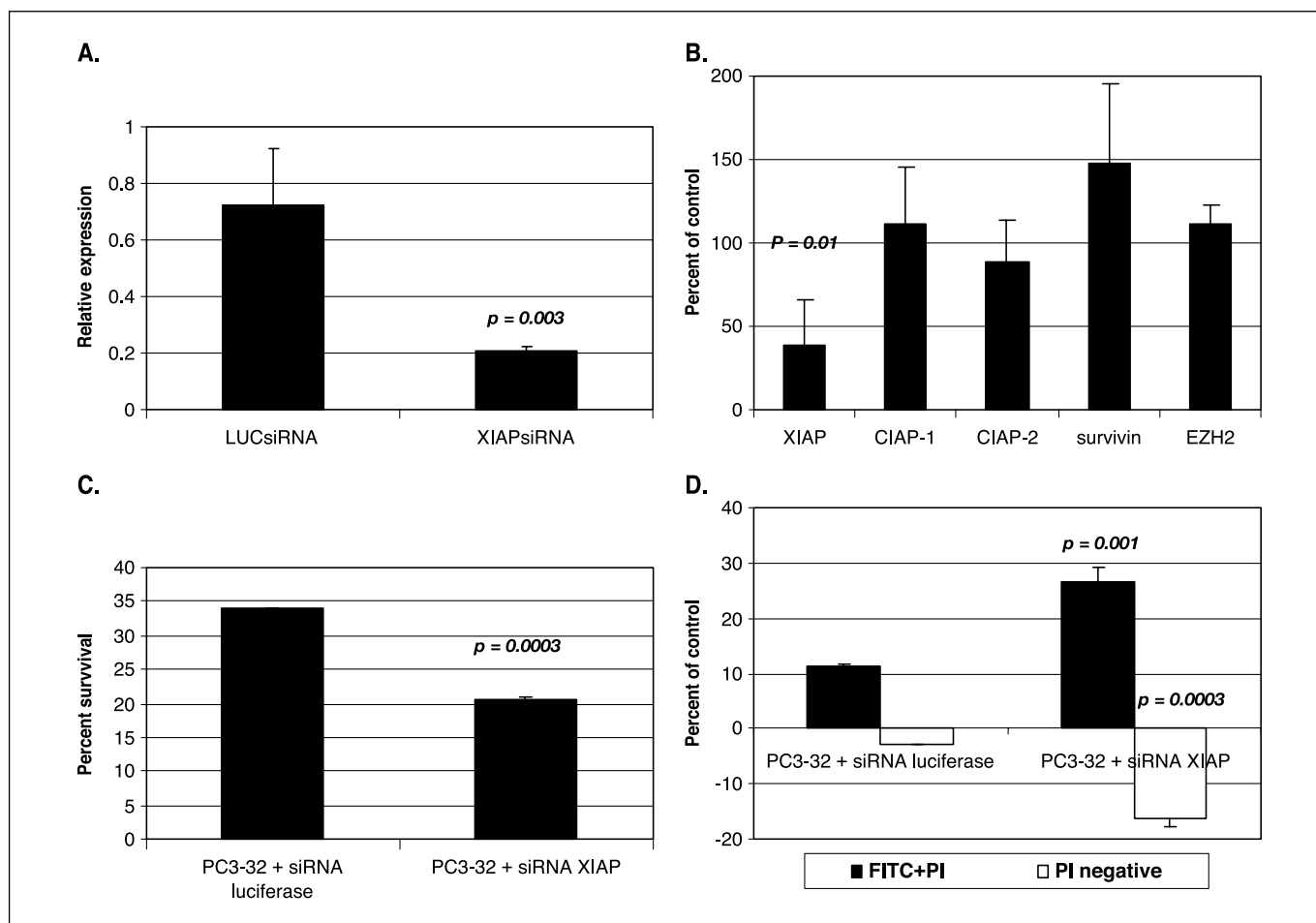


Figure 4. siRNA-mediated XIAP silencing increases sensitivity to anoikis of circulating metastatic human prostate carcinoma cells. *A*, siRNA-mediated reduction of XIAP mRNA expression in PC-3-32 circulating metastatic cells compared with control siRNA-treated cells. *B*, specificity of XIAP siRNA-mediated gene silencing in PC-3-32 circulating metastatic cells. *C*, siRNA-mediated XIAP silencing diminishes survival of circulating metastatic cells subjected to nonadherent conditions compared with control siRNA-treated cells. *D*, XIAP silencing increases sensitivity to anoikis of PC-3-32 circulating metastatic cells compared with control siRNA-treated cells. PC-3-32 circulating metastatic cells were subjected to siRNA-mediated XIAP silencing as described in Materials and Methods. Control cultures were treated with the luciferase siRNA (*LUCsiRNA*). Control and experimental cultures were monitored for survival and apoptosis during growth in suspension.

The XIAP inhibitors were then tested in an orthotopic model implanted with previously isolated GFP-expressing PC-3-GFP circulating metastatic prostate cancer cells. The mice were continuously treated for 2 weeks with i.p. daily injections of compounds (12.5 mg/kg) and monitored for tumor growth and metastasis by fluorescence imaging after 4 weeks. No effect on tumor take and primary tumor growth was observed by direct measurements of tumor growth kinetics and tumor weights (data not shown). However, significantly reduced tumor spread outside the prostate compared with the control (untreated) animals was visualized by GFP imaging (Fig. 6). The mean imaged total tumor pixel areas were, respectively, 2.3- and 2.9-fold smaller in the animals treated with XIAP inhibitor 1396-12 [pixel area (mean \pm SD), 670.0 \pm 39.9; $P = 0.04$] and XIAP inhibitor 1396-34 [pixel area (mean \pm SD), 537.7 \pm 8.6; $P = 0.04$] compared with the control group [pixel area (mean \pm SD), 1560.3 \pm 342.6]. In contrast, no statistically significant changes in the total tumor pixel area were detected in the vehicle-treated group compared with the control group [pixel area (mean \pm SD), 1336.0 \pm 599.9; $P = 0.15$]. These results indicate that the therapeutic effect of the XIAP antagonists was primarily on metastatic spread. The results suggest the metastatic inhibition

was possibly effected by diminishing the survival of prostate carcinoma cells in the circulation. Our data sets the stage for large-scale mouse studies of the antimetastatic and survival activity of the XIAP inhibitors.

Previous studies have shown that phenylurea-based XIAP inhibitors block interactions of downstream effector caspases with XIAP and induce apoptosis in target cells through a caspase-dependent, Bcl-2/Bax-independent mechanism (39, 43). Recent experimental evidence suggests that formation of an IAP-XIAP complex (in particular, survivin-XIAP complex) increases XIAP stability and causes synergistic inhibition of apoptosis (44). These data imply that small-molecule XIAP inhibitors may have a complex mechanism of action *in vivo*. In prostate cancer cells the increased expression levels of several IAPs have been shown (ref. 38; this study). The biological role of these seemingly redundant prosurvival antiapoptotic genes is not well understood. Further studies would be required to assess the relative contribution of other IAP family members such as cIAP-2 and survivin to the anoikis resistance of metastatic human prostate carcinoma cells.

Increased XIAP expression is not likely to be the only factor contributing to anoikis resistance of metastatic cancer cells

(45–48). Other genes may also participate in anoikis resistance, which in turn may confer increased metastatic potential. For example, it has been recently shown that the neurotrophic receptor TrkB confers such anoikis resistance and high metastatic potential (49).

Conclusion

We have shown that circulating metastatic human prostate carcinoma cells isolated from orthotopic models of prostate cancer are anoikis resistant compared with the parental cells comprising the bulk of the primary tumor. Gene expression

and transfection studies suggest that the increased expression of apoptosis suppressor *XIAP* plays an important role in anoikis resistance and in survival of circulating human prostate carcinoma cells, thereby promoting metastasis. The *XIAP* siRNA and small-molecule inhibitor efficacy studies also suggest that XIAP is involved in anoikis resistance and therefore is an attractive target for antimetastatic drug design. The small-molecule XIAP inhibitors were previously shown to significantly inhibit the growth of s.c. human prostate carcinoma xenografts in mice (39) with little toxicity to normal tissues. We extended these early observations to show that circulating metastatic human prostate carcinoma cells are

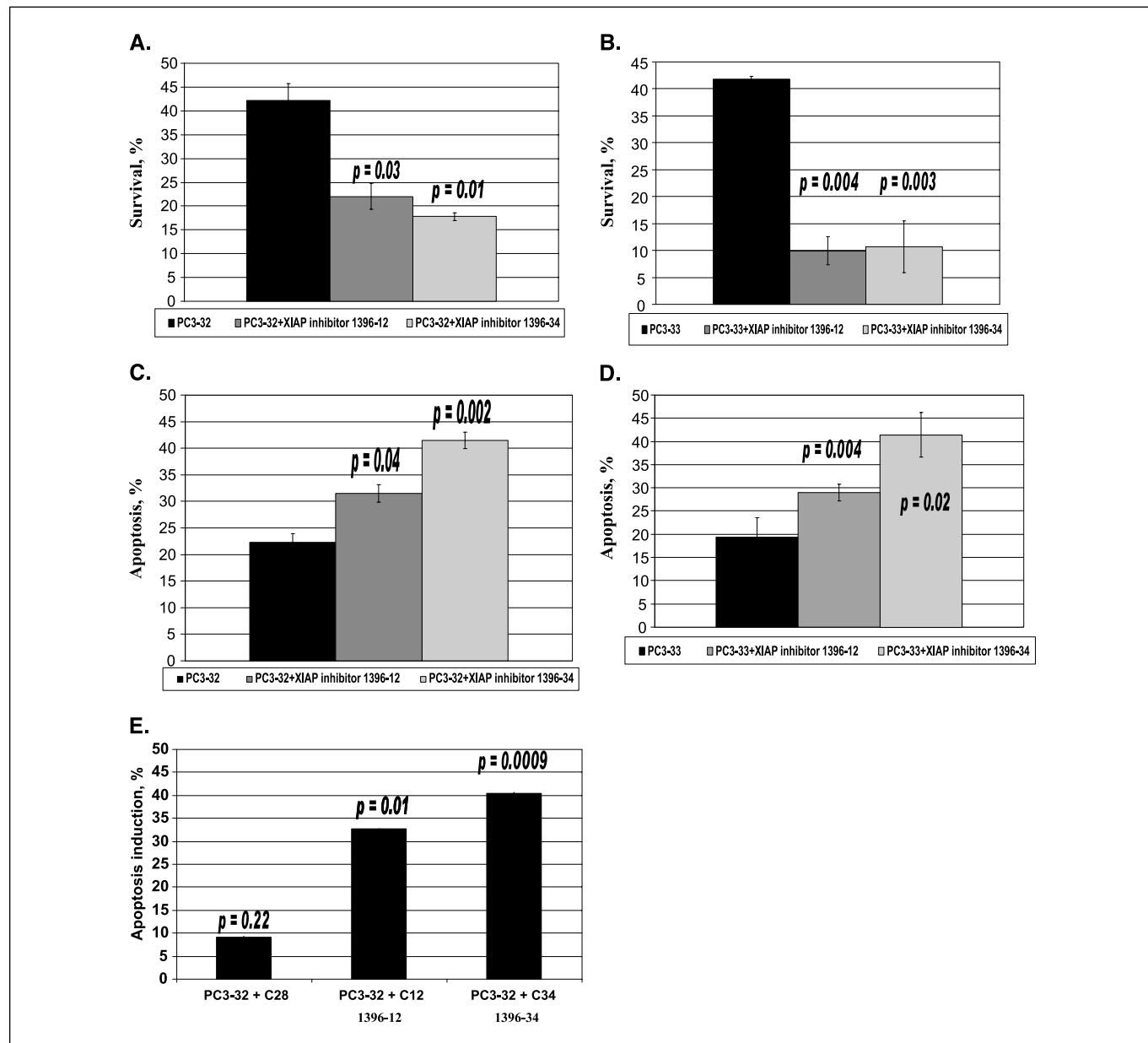


Figure 5. XIAP inhibitors enhance sensitivity to anoikis of circulating metastatic human prostate carcinoma cells. *A*, XIAP inhibitors 1396-12 and 1396-34 diminish survival of PC-3-32 circulating metastatic cells subjected to nonadherent conditions. *B*, XIAP inhibitors 1396-12 and 1396-34 diminish survival of PC-3-33 circulating metastatic cells subjected to nonadherent conditions. *C*, XIAP inhibitors 1396-12 and 1396-34 enhance apoptosis of PC-3-32 circulating metastatic cells subjected to nonadherent conditions. *D*, XIAP inhibitors 1396-12 and 1396-34 enhance apoptosis of PC-3-33 circulating metastatic cells subjected to nonadherent conditions. *E*, nonactive structural analogue C28 of XIAP inhibitors failed to enhance apoptosis of PC-3-32 circulating metastatic cells subjected to nonadherent conditions compared with active XIAP inhibitors 1396-12 and 1396-34.

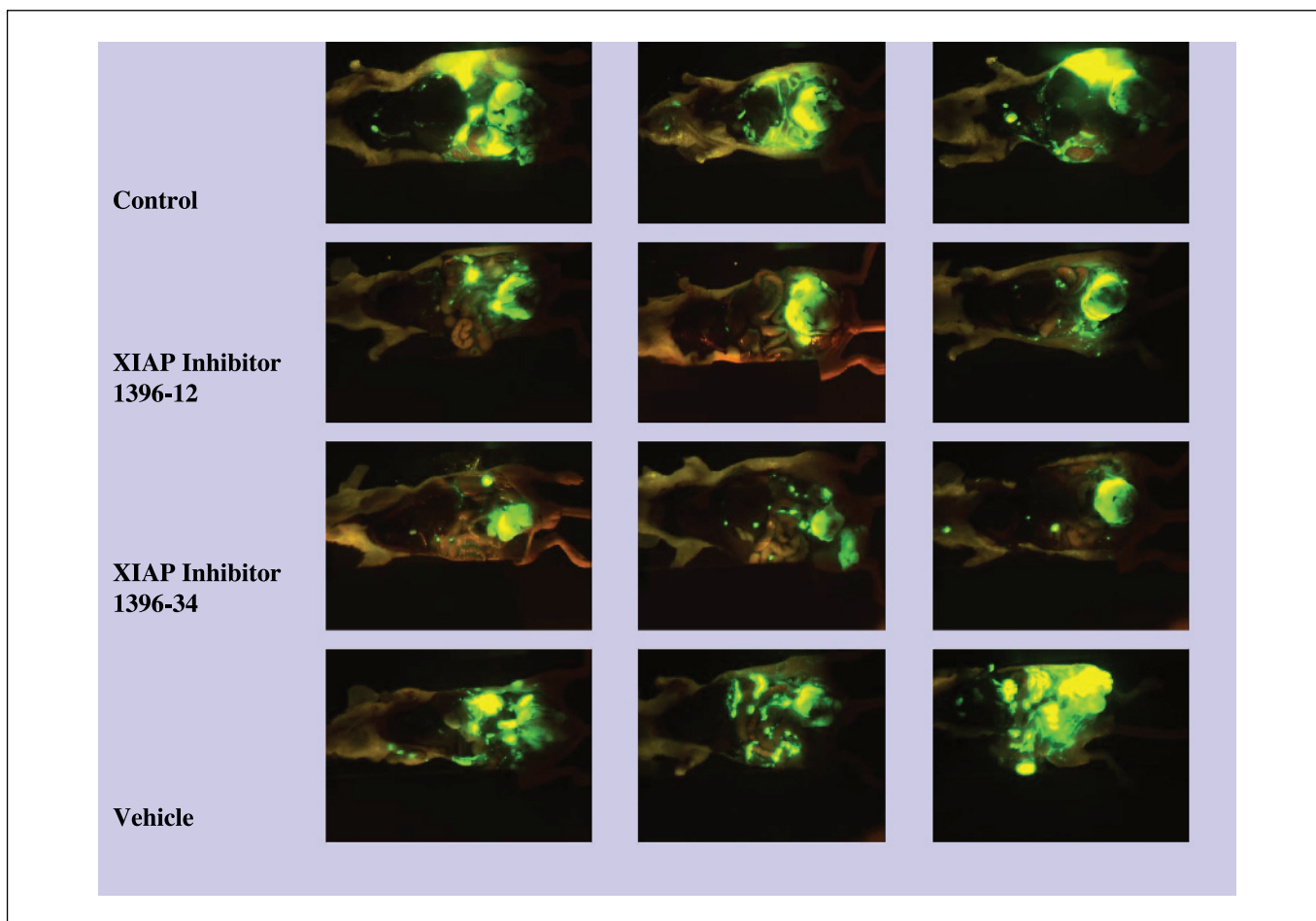


Figure 6. Effect of XIAP inhibitors on prostate cancer metastasis in an orthotopic fluorescent PC-3-GFP nude mouse model. Tumor-bearing mice were continuously treated for 2 weeks with daily i.p. injections of 1396-12 and 1396-34 (both at 12.5 mg/kg). Four weeks after tumor cell implantation in the prostate, the animals were sacrificed and subjected to postmortem GFP imaging. Fluorescent images of control (*top*), XIAP inhibitor 1396-12–treated (*middle top*), XIAP inhibitor 1396-34–treated (*middle bottom*), and vehicle-treated (*bottom*) animals are shown. Note visible reduction of extent of tumor spread (*areas of green fluorescence*) in XIAP inhibitor–treated animals. See text for details.

sensitive to these molecules. The antimetastatic activity of the small-molecule XIAP inhibitors is suggested by our data. These results indicate the potential clinical utility of this class of compounds for treatment of metastatic prostate cancer. Future studies will use the GFP-expressing circulating metastatic cells generated in orthotopic models of prostate cancer to further study the role and genetic control of anoikis resistance in metastasis.

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