Gefitinib Reverses TRAIL Resistance in Human Bladder Cancer Cell Lines via Inhibition of AKT-Mediated X-Linked Inhibitor of Apoptosis Protein Expression

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Abstract

In a previous study, we found that the small-molecule epidermal growth factor receptor (EGFR) inhibitor gefitinib (ZD1839, Iressa) blocked cell proliferation at biologically relevant concentrations in approximately one third (6 of 17) of human bladder cancer cell lines examined. Here, we studied the effects of gefitinib on apoptosis in a representative subset of the same panel of cells. The drug had modest effects on DNA fragmentation as a single agent at concentrations that produced strong growth inhibition (≤1 μmol/L) and also failed to promote apoptosis induced by conventional chemotherapeutic agents (gemcitabine and paclitaxel). However, gefitinib did interact with recombinant human tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) to induce high levels of apoptosis in gefitinib-responsive but not gefitinib-unresponsive lines. The molecular mechanisms involved down-regulation of active AKT and X-linked inhibitor of apoptosis protein (XIAP) expression and were mimicked by chemical inhibitors of the phosphatidylinositol 3-kinase/AKT pathway but not of the mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase/ERK pathway. Furthermore, direct small interfering RNA–mediated knockdown of AKT resulted in down-regulation of XIAP and TRAIL sensitization, and knockdown of XIAP itself was sufficient to reverse TRAIL resistance. Together, our results show that EGFR pathway activation limits TRAIL-induced apoptosis via an AKT- and XIAP-dependent mechanism in EGFR-dependent human bladder cancer cells, providing the conceptual framework for a further evaluation of the combination in relevant preclinical *in vivo* models. [Cancer Res 2007;67(4):1430–5]

Introduction

Gefitinib (also known as ZD1839 or Iressa) is an orally active, selective epidermal growth factor receptor (EGFR) tyrosine kinase antagonist that inhibits tumor cell growth at nanomolar concentrations in responsive cell lines *in vitro* (1, 2). Although it displayed promising activity in preclinical models (2), it produced disappointing results in large-scale clinical trials involving more than 60,000 patients: response rates in non–small cell lung cancer (NSCLC) hovered ~10% and response rates in other types of cancer were equivalent (3–5). Approximately 10% of NSCLC tumors from patients in the United States contain mutant EGFRs that may render the cells that express them "addicted" to EGFR-mediated signaling for cell survival (6, 7), but these mutations do not seem to accumulate to any significant degree in other solid malignancies (8, 9). On the other hand, the EGFR regulates downstream signal transduction pathways [i.e., extracellular signal-regulated kinase (ERK) and AKT activation] that could limit apoptosis induced by conventional or investigational cytotoxic agents, and it is likely that the effects of EGFR inhibitors on these pathways can be exploited in EGFR-dependent tumors in combination regimens (2). Therefore, we designed the present study to directly test the effects of EGFR inhibition on cell death induced by clinically relevant cytotoxic agents that are either currently being evaluated or could be evaluated in EGFR inhibitor-based clinical trials in patients with bladder cancer. Our data reveal that gefitinib is a particularly effective tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)-sensitizing agent in EGFR-dependent cells via its ability to inhibit AKT activation and down-regulate X-linked inhibitor of apoptosis protein (XIAP) expression.

Materials and Methods

Cell culture and reagents. The 253J B-V cells were derived by orthotopic "recycling" as described preciously (10). The KU7 cells were provided by Dr. William Benedict (Department of Genitourinary Medical Oncology, University of Texas M. D. Anderson Cancer Center, Houston, TX). All other cell lines were provided by H. Barton Grossman (Department of Urology, University of Texas M. D. Anderson Cancer Center). None of the cell lines contain activating Ras mutations.

The antibodies used for immunoblotting were from the following sources: rabbit anti–total AKT, rabbit anti–phosphorylated AKT (Ser^{473}), rabbit anti–total mitogen-activated protein kinase (MAPK), mouse anti–phosphorylated MAPK (Thr^{202}-Tyr^{204}), mouse anti–cleaved caspase-8 (clone IC12), and rabbit anti–cleaved caspase-3 (clone 8G10) were from Cell Signaling Technology (Beverly, MA); rabbit anti-human actin (Sigma, St. Louis, MO); anti-human/mouse XIAP (clone 117320, R&D Systems, Minneapolis, MN); mouse anti-human p27 (clone 57, BD PharMingen, San Diego, CA); and secondary antibodies (horseradish peroxidase–conjugated sheep anti-mouse or donkey anti-rabbit; Amersham Biosciences, Piscataway, NJ).

Gefitinib, Gemzar (gemcitabine hydrochloride; Eli Lilly and Company, Indianapolis, IN), and paclitaxel (Bristol-Myers Squibb, New York, NY) were purchased from the University of Texas M. D. Anderson pharmacy. Wortmannin, PD98059, LY294002, and U0126 were purchased from Calbiochem (La Jolla, CA). XIAP small interfering RNA (siRNA; SMARTpool BIRC4), p27 siRNA (SMARTpool cdk-N-1B), AKT1 siRNA (SMARTpool), and siRNA Nonspecific Control IV were purchased from Dharmacon (Lafayette, CO), and siIMPORTER was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

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Quantification of DNA fragmentation. DNA fragmentation was measured by propidium iodide staining and fluorescence-activated cell sorting (FACS) analysis (Beckman Coulter, Mountain View, CA) as described previously (8).

Immunoblot analysis for p27 and XIAP. Total cell lysates were prepared using a 1% Triton X-100 buffer as described previously (8). Expression of p27 and XIAP was evaluated by 12% SDS-PAGE and immunoblotting (11).

Two-color analyses of intracellular p27 expression and DNA fragmentation. Two-color intracellular staining for p27 and DNA fragmentation was done as described previously (12). p27 was detected using an Alexa Fluor 488–conjugated secondary antibody (Molecular Probes, Eugene, OR).

siRNA-mediated silencing of p27, XIAP, or AKT. Cells were transiently transfected with specific or nonspecific siRNA constructs for 48 h according to the manufacturer's protocols. The constructs were all used at 200 nmol/L. Liposome-mediated transfection was accomplished with the siIMPORTER reagent according to the manufacturer's instructions. The efficiency of silencing was verified in each experiment by immunoblotting.

Results and Discussion

Effects of gefitinib on apoptosis. To assess the direct effects of gefitinib on apoptosis, we incubated four gefitinib-sensitive (253J B-V, UM-UM-4, UM-UC-5, and UM-UC-10) and three gefitinib-insensitive (KU7, UM-UC-2, and UM-UC-14) cell lines (13) with 1 or 10 μmol/L of gefitinib and measured DNA fragmentation 48 h later by propidium iodide staining and FACS analysis. Gefitinib induced concentration-dependent increases in all of the EGFR-dependent cells, whereas it had no effects in the EGFR-insensitive cells (Fig. 1*A*). We also characterized the effects of gefitinib on apoptosis induced by paclitaxel or gemcitabine in representative gefitinib-sensitive (253J B-V) and gefitinib-resistant (UM-UC-3) cells using concentrations of gemcitabine or paclitaxel that were found to be optimal in preliminary studies (data not shown). Gefitinib had no effect on gemcitabine-induced apoptosis in either of the cell lines, and it actually antagonized the proapoptotic effects of paclitaxel in the gefitinib-sensitive cells in a schedule-dependent manner (i.e., when cells were preincubated for 24 h with gefitinib before they were exposed to paclitaxel; Fig. 1*B*).

The proteasome inhibitor bortezomib (Velcade) sensitizes bladder cancer cells to TRAIL via a mechanism that involves p21-dependent inhibition of cyclin-dependent kinase (cdk) 2 activity (14). Gefitinib also arrests EGFR-dependent cells in G_1 via mechanisms that involve p27-dependent down-regulation of cdk2 activity (8). Therefore, we speculated that gefitinib might promote TRAIL-induced apoptosis in human bladder cancer cells via p27-dependent G_1 arrest. To test this hypothesis, we exposed three gefitinib-sensitive (253J B-V, UM-UC-5, and UM-UC-10) and three gefitinib-insensitive (KU7, UM-UC-3, and UM-UC-14) cells to 1 μmol/L gefitinib in the presence or absence of 25 ng/mL recombinant human TRAIL (rhTRAIL) and measured DNA fragmentation by propidium iodide/FACS 24 h later. Gefitinib promoted TRAIL-induced apoptosis in all three gefitinib-sensitive lines but had no effect on the gefitinib-insensitive cells (Fig. 1*C*).

Roles of the ERKs and AKT in TRAIL sensitization. Gefitinib down-regulates constitutive phosphorylation of the ERKs and AKT in EGFR-dependent bladder cancer cells but has no effect on ERK or AKT activation in the resistant cells (13, 15). To determine whether inhibition of one or both pathways was required to promote TRAIL sensitivity, we incubated 253J B-V cells with optimal concentrations of small-molecule inhibitors of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway (wortmannin), the MAPK/ERK kinase (MEK)/ERK pathway (U0126), or both in the presence or absence of rhTRAIL and measured apoptosis 24 h later by propidium iodide/FACS. Neither compound nor other structurally distinct inhibitors of the pathways (PD98059 and LY294002; data not shown) induced much DNA fragmentation at this time point at concentrations that completely blocked constitutive and EGF-induced ERK and AKT phosphorylation (Fig. 2*A*; data not shown). However, like gefitinib, wortmannin interacted synergistically with TRAIL to induce high levels of apoptosis, whereas U0126 had minimal effects on cell death (Fig. 2*A*). Exposure to the combination of wortmannin plus U0126 produced the same degree of TRAIL sensitization as wortmannin alone, confirming that ERK inhibition played a minor role in TRAIL sensitization in these cells (Fig. 2*A*). Wortmannin promoted TRAIL-induced apoptosis in all of the cell lines we examined ($n = 7$), including two of the cell lines that were unresponsive to gefitinib (UM-UC-3 and KU7; data not shown).

As we previously implicated, cdk2 inhibition in the TRAIL sensitization observed in cells exposed to the proteasome inhibitor bortezomib (14), we investigated whether p27 accumulation might play a similar role in 253J B-V cells exposed to gefitinib. Gefitinib or U0126, but not wortmannin, induced significant accumulation of p27 protein (Fig. 2*B*), strongly suggesting that p27 accumulation was driven via ERK pathway inhibition. Bortezomib also increased p27 expression presumably by preventing proteasome-mediated degradation of the protein (Fig. 2*B*). Exposure to TRAIL actually reduced p27 expression, effects that were most pronounced in cells exposed to TRAIL plus either gefitinib or wortmannin and were displaying the most cell death (Fig. 2*B*). Moreover, simultaneous analysis of p27 expression and apoptosis by two-color flow cytometry showed that the subdiploid (apoptotic) cells exhibited low levels of p27 fluorescence, whereas viable cells displayed high levels of p27 fluorescence (Fig. 2*C*). Finally, direct knockdown of p27 by RNA interference (RNAi) had little effect on gefitinib plus TRAIL–induced apoptosis (Fig. 2*D*). Thus, in 253J B-V cells, TRAIL sensitivity seems to be driven primarily via inhibition of the PI3K/AKT pathway, whereas p27 accumulation is driven primarily via inhibition of the MEK/ERK pathway.

Effects of AKT inhibition on XIAP expression. The caspase-3/caspase-7/caspase-9 antagonist XIAP inhibits TRAIL-induced apoptosis and is a very attractive therapeutic target (16). XIAP expression may be regulated via AKT-dependent mechanism(s) (17, 18). Therefore, we characterized the effects of gefitinib, wortmannin, and U0126 on XIAP expression in 253J B-V cells. Gefitinib and wortmannin, but not U0126, inhibited XIAP protein expression in the cells (Fig. 3*A*). These effects were not associated with changes in XIAP mRNA levels as measured by RNase protection assay (data not shown), strongly suggesting that down-regulation occurred at the translational or post-translational level (17, 18). RNAi-mediated knockdown of XIAP expression did not directly induce much cell death (Fig. 3*B*). However, silencing XIAP produced marked TRAIL sensitization (Fig. 3*B*), confirming that XIAP promoted TRAIL resistance in the cells.

Finally, to more directly determine whether AKT was the target of gefitinib action, we transduced 253J B-V cells with an adenoviral construct encoding a constitutively active (myristoylated) form of AKT or a control construct and measured their effects on gefitinib-mediated TRAIL sensitization. DNA fragmentation levels

were inhibited by at least 50% in the AKT-transduced cells relative to controls (data not shown). To determine whether the effects of gefitinib on AKT accounted for the down-regulation of XIAP observed, we transfected 253J B-V cells with an AKT-specific siRNA construct or an off-target control and measured XIAP expression and TRAIL-induced DNA fragmentation. AKT1 was the predominant AKT isoform expressed by the 253J B-V cells (data not shown). Silencing AKT1 resulted in marked down-regulation of XIAP (Fig. 3*C*) and increased TRAIL sensitivity in the 253J B-V cells to levels that were equivalent to those observed in cells exposed to gefitinib or wortmannin (Fig. 3*D*). Together, these results establish that AKT inhibition is required for gefitinib-induced TRAIL sensitization and that the effects of AKT inhibition involve down-regulation of XIAP in the 253J B-V cells.

Previous preclinical studies and clinical trials have investigated the tumor growth-inhibitory effects of combinations of EGFR antagonists and conventional chemotherapy (particularly taxanes; refs. 2, 19). Whereas the preclinical studies consistently showed that combination therapy was more effective than therapy with either EGFR antagonists or conventional agents alone, the clinical trials failed to show that combination therapy provided benefit beyond what was observed with the conventional agents alone (19).

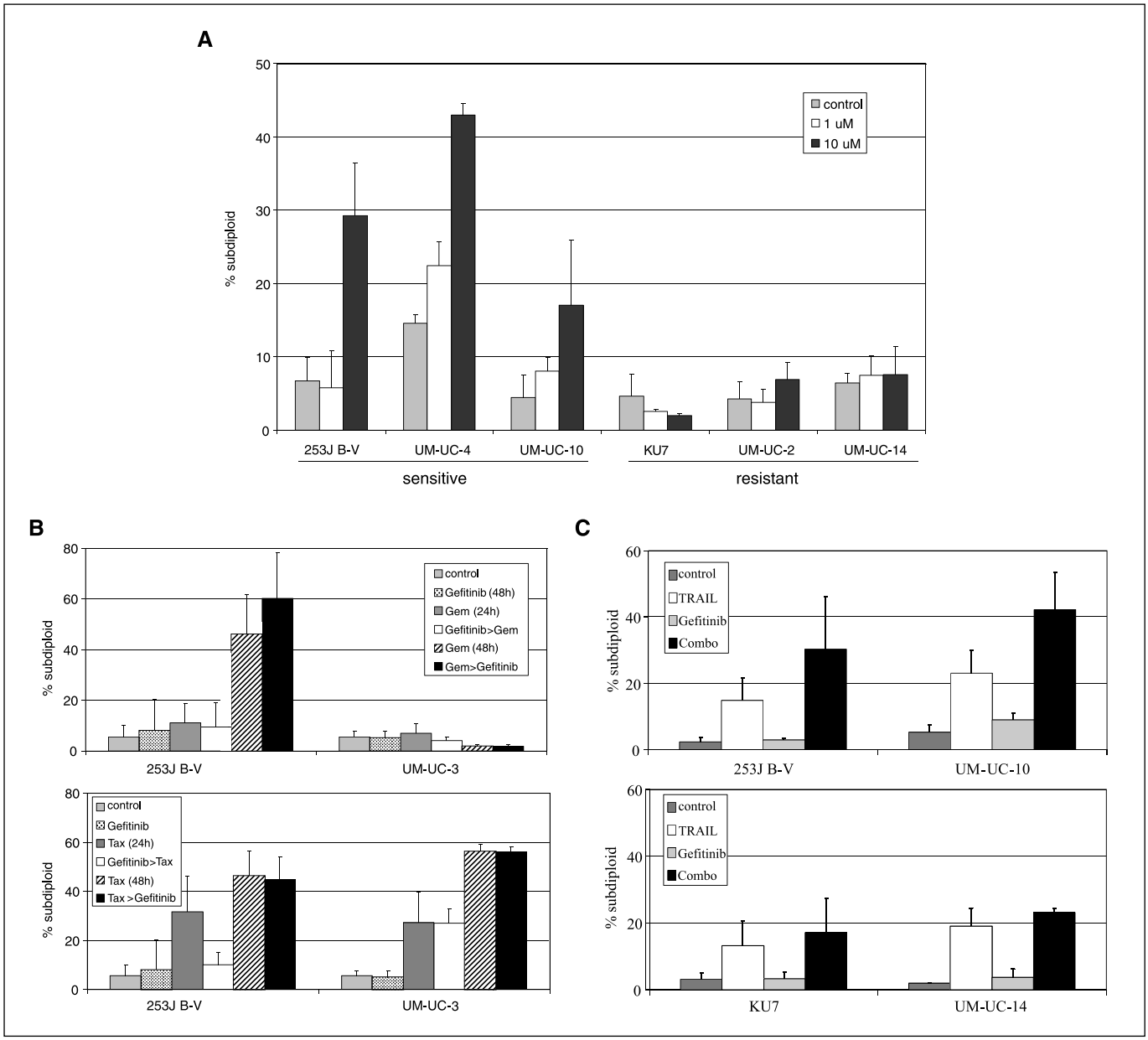

Figure 1. Effects of gefitinib on apoptosis. *A,* effects of single-agent gefitinib. Cells were incubated with or without 1 and 10 μmol/L of gefitinib for 48 h in serum-free medium. DNA fragmentation was measured by propidium iodide/FACS. *Columns,* mean ($n = 3$); *bars,* SD. *B,* effects of gefitinib plus gemcitabine (*Gem*) or paclitaxel. Cells were incubated with or without 1 μmol/L gefitinib for 24 h in the presence or absence of the indicated concentrations of gemcitabine or docetaxel for 48 h, and DNA fragmentation was measured by propidium iodide/FACS. *Columns,* mean ($n = 3$); *bars,* SD. *C,* effects of gefitinib plus rhTRAIL. Cells were incubated with or without 1 μmol/L gefitinib in the presence or absence of the indicated concentrations of TRAIL for 24 h, and DNA fragmentation was measured by propidium iodide/FACS. *Columns,* mean ($n = 3$); *bars,* SD.

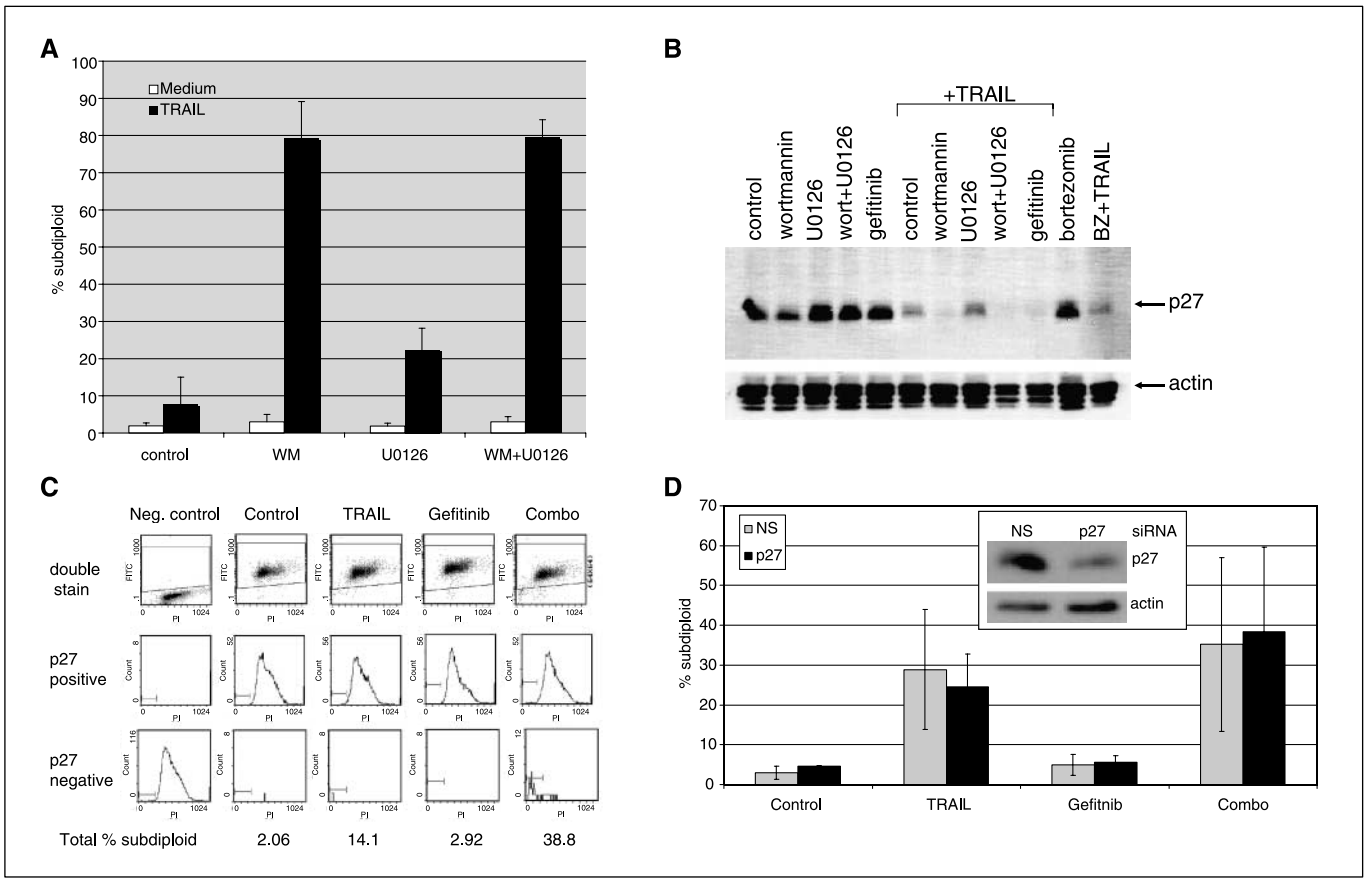

Figure 2. Effects of wortmannin and U0126 on p27 expression and apoptosis. *A,* effects of wortmannin (*WM*) and U0126 on TRAIL-induced apoptosis. 253J B-V cells were incubated for 24 h in serum-free medium with 2 μmol/L wortmannin, 10 μmol/L U0126, or both with or without 25 ng/mL TRAIL, and DNA fragmentation was measured by propidium iodide/FACS. All combinations were statistically significant compared with single agents alone ($P < 0.03$). *Columns,* mean ($n = 3$); *bars,* SD. *B,* effects of wortmannin, U0126, and gefitinib on $p27^{kip1}$ protein expression. Cells were incubated for 24 h with or without 1 μmol/L gefitinib, 1 μmol/L wortmannin, 10 μmol/L U0126, 10 nmol/L bortezomib (*BZ*), or wortmannin plus U0126 in the presence or absence of 25 ng/mL TRAIL for 24 h in serum-free medium, and $p27^{kip1}$ protein levels were analyzed in total cell lysates by immunoblotting. *C,* quantitative analysis of p27 expression in apoptotic cells. 253J B-V cells were incubated with 1 μmol/L gefitinib, 25 ng/mL TRAIL, or both for 12 h in serum-free medium, and p27 expression and DNA fragmentation were measured by two-color FACS as described in Materials and Methods. Representative histograms. Subdiploid cells are indicated by the gates (*left side of X axes*). Note that the only significant subdiploid peaks are found in the p27-negative cells. *PI,* propidium iodide. *D,* effect of p27 silencing on apoptosis induced by gefitinib plus TRAIL. 253J B-V cells were transiently transfected with a p27-specific siRNA construct or an off-target control construct (against luciferase) for 48 h. The cells were then incubated with 1 μmol/L gefitinib, 25 ng/mL TRAIL, or both for 24 h in serum-free medium, and DNA fragmentation was analyzed by propidium iodide/FACS. *Inset,* efficient p27 silencing was confirmed by immunoblotting. *Columns,* mean ($n = 3$); *bars,* SD.

Problems with patient selection undoubtedly plagued these studies (just as they did earlier single-agent trials) because gefitinib-responsive tumors were not identified prospectively. Nonetheless, our results and data from other groups show that gefitinib does not always enhance chemotherapy-induced apoptosis, and in some cases, it actually seems to interfere with apoptosis in gefitinib-sensitive cells (20, 21). In retrospect, this is not surprising given that gefitinib induces cell cycle arrest in responsive cells, and this G_1 arrest would not be expected to promote the cytotoxic effects of agents that seem to act preferentially within S or M phase (12). On the other hand, the combination of gefitinib plus rhTRAIL produces strong additive effects in EGFR-dependent, TRAIL-sensitive cells and synergistic effects in EGFR-dependent, TRAIL-insensitive cells. We are currently evaluating the effects of combination therapy with TRAIL plus gefitinib in orthotopic EGFR-dependent and EGFR-independent tumors to determine whether these promising effects can be reproduced without toxicity *in vivo*. Preliminary toxicity studies have shown that systemic combination therapy with gefitinib (100 mg/kg) plus rhTRAIL (10 mg/kg) is very well tolerated.[4]

Although our previous work with bortezomib strongly suggested that tumor cells arrested in G_1 might be sensitized to TRAIL via accumulation of the cdk inhibitor p21 (14), in the current study, we were able to completely distinguish the effects of gefitinib on TRAIL sensitization from the effects of the drug on p27. Indeed, cells exposed to TRAIL plus gefitinib expressed lower levels of p27 than did cells exposed to gefitinib alone, and two-color FACS revealed that p27 expression was restricted to viable cells. It is likely that the effects of AKT inhibition on the TRAIL pathway are complex and involve more than XIAP down-regulation. Indeed, inhibitors of the AKT target glycogen synthase kinase-3β (15) promote further increases in TRAIL sensitivity in cells exposed to gefitinib.

One major disadvantage associated with using EGFR antagonists as TRAIL-sensitizing agents in bladder cancer is that tumor cell EGFR dependency must be established to ensure optimal therapeutic outcome. In recent work, we showed that expression

[4] M. Shrader, unpublished data.

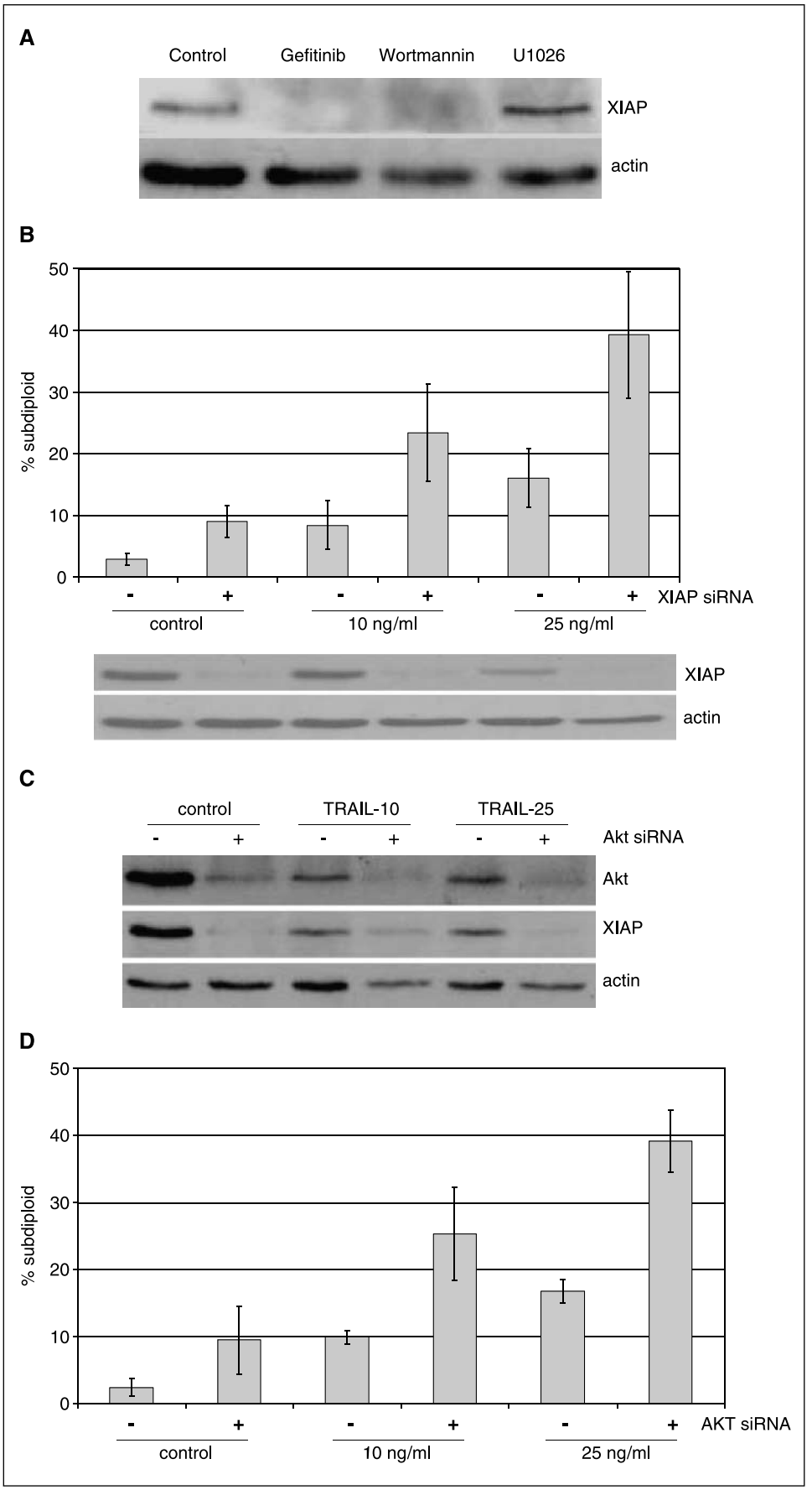

Figure 3. Role of XIAP in TRAIL resistance. *A,* effects of chemical inhibitors on XIAP expression. 253J B-V cells were exposed to gefitinib, wortmannin (1 μmol/L), or U0126 (10 μmol/L) for 24 h in serum-free medium, and XIAP expression was measured in total lysates by immunoblotting as described in Materials and Methods. *B,* effects of XIAP silencing on TRAIL-induced apoptosis. 253J B-V cells were transiently transfected with an XIAP-specific siRNA construct or an off-target control construct (against luciferase) for 48 h. The cells were then incubated with the indicated concentrations of rhTRAIL for 24 h in serum-free medium, and DNA fragmentation was analyzed by propidium iodide/FACS. *Bottom,* efficient XIAP silencing was confirmed by immunoblotting. *Columns,* mean (n = 3); *bars,* SD. *C,* effects of silencing AKT1 on XIAP expression. 253J B-V cells were transiently transfected with an AKT1-specific siRNA construct or an off-target control construct (against luciferase) for 48 h. The cells were then incubated for an additional 24 h, and AKT1 and XIAP expressions were measured by immunoblotting. *D,* effects of silencing AKT1 on TRAIL-induced 253J B-V cells were transiently transfected with an AKT1-specific siRNA construct or an off-target control construct (against luciferase) for 48 h. The cells were then incubated with 10 and 25 ng/mL of rhTRAIL for 24 h in serum-free medium, and DNA fragmentation was analyzed by propidium iodide/FACS. *Columns,* mean (n = 3); *bars,* SD.

of two markers of epithelial to mesenchymal transition (E-cadherin and vimentin) loosely correlates with gefitinib sensitivity and resistance, respectively, but this correlation is not perfect (13).[5] Thus, one would expect that agents that target TRAIL resistance mechanisms downstream of the EGFR would display broader activity that may preclude the need to define EGFR dependency prospectively. Consistent with this notion, we have observed that AKT inhibitors or chemical XIAP antagonists (SMAC mimetics) promote marked TRAIL sensitivity in almost all of the cell lines in our panel.[6,7] In ongoing work, we are comparing the effects of gefitinib, an AKT inhibitor, and a SMAC mimetic on TRAIL-induced apoptosis, tumor growth inhibition, and systemic toxicity in mice bearing 253J B-V xenografts.

[5] P. Black, et al., in preparation.
[6] Unpublished observations.
[7] M. Shrader and A. Metwalli, unpublished observations.

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