A Smac Mimetic Rescue Screen Reveals Roles for Inhibitor of Apoptosis Proteins in Tumor Necrosis Factor-α Signaling

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Abstract

Smac mimetic compounds targeting the inhibitor of apoptosis proteins (IAP) baculoviral IAP repeat-3 domain are presumed to reduce the threshold for apoptotic cell death by alleviating caspase-9 repression. We explored this tenet in an unbiased manner by searching for small interfering RNAs that are able to confer resistance to the Smac mimetic compound LBW242. Among the screening hits were multiple components of the tumor necrosis factor α (TNFα) signaling pathway as well as X-linked inhibitor of apoptosis (XIAP) itself. Here, we show that in a subset of highly sensitive tumor cell lines, activity of LBW242 is dependent on TNFα signaling. Mechanistic studies indicate that in this context, XIAP is a positive modulator of TNFα induction whereas cellular inhibitor of apoptosis protein 1 negatively regulates TNFα-mediated apoptosis. [Cancer Res 2007;67(24):11493–8]

Introduction

Inhibitor of apoptosis proteins (IAP) are overexpressed in human cancers where they are thought to antagonize apoptotic cell death by interacting with and repressing caspase-3, caspase-7, and caspase-9. The interaction between the baculoviral IAP repeat-3 (BIR3) domain of X-linked inhibitor of apoptosis (XIAP) and caspase-9 is of therapeutic interest because this interaction is inhibited by the NH_2-terminal seven-amino-acid residues of Smac, a naturally occurring antagonist of IAPs (1). Moreover, the hydrophobic and hydrogen bond interactions anchoring the amino terminus of Smac into the BIR3 binding groove are colinear within a four-residue motif (AVPI; ref. 1). We and others have generated small-molecule Smac mimetics anticipating efficacy in cancer based on reinstitution of apoptotic signaling (2–4). The available data suggest that there is significant similarity in the structural and biochemical activities of the current generation of Smac mimetic small molecules (2, 3, 5). As these compounds enter human clinical trials, it is critical that we understand the mechanism of activity of such molecules and develop hypotheses for selecting the appropriate patients or tumor types. Toward this end, a series of studies were done to explore the mechanism through which a Smac mimetic compound, LBW242 (Supplementary Fig. S1), induces apoptosis in tumor cells.

Materials and Methods

Cell culture. Cell lines were purchased from American Type Culture Collection and cultured in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum, penicillin, and streptomycin. Recombinant human tumor necrosis factor α (TNFα), TNF-related apoptosis-inducing ligand (TRAIL), and soluble TRAIL receptor (sTRAIL-R) were from R&D Systems. For rescue experiments, cells were pretreated with 1 μg/mL sTRAIL-R, 1 μg/mL etanercept (Amgen/Wyeth), or 1 μg/mL anti-TNFα antibody (BD PharMingen) 30 min before treatment with LBW242. To evaluate whether degradation of cellular inhibitor of apoptosis protein 1 (cIAP1) was proteosome dependent, cells were pretreated for 2 h with the proteosome inhibitor MG132 (6 μmol/L; Calbiochem). The stable nuclear factor-κB (NF-κB) reporter variant of A549 cells was established with pNFκB-luc (Panomics) and maintained in 100 μg/mL hygromycin B. Luciferase was quantified using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). Cell viability was measured using CellTiter-Glo (Promega).

RNA interference reagents. For transient knockdown, small interfering RNAs (siRNA) were obtained from Dharmacon Technologies. Stable knockdown of XIAP in SKOV-3 cells (XIAP-KD) was achieved by retroviral transduction of a shRNA using the pCGll vector (core sequence, 5′-gtcattactttcaagcaaa-3′).

RNA interference screen. The apoptosis siRNA library was composed of 318 siRNA Smart pool reagents targeting apoptosis-related genes (Dharmacon) as 6.25 pmol of lyophilized siRNA per well in 96-well plates. Dharmafect2 at 0.28 μL (Dharmacon) diluted in 25-μL DCCR (Dharmacon) was added per well. MKSTYX (Dharmacon) and glyceraldehyde-3-phosphate dehydrogenase (Dharmacon) siRNAs were added to additional wells, serving as positive and negative controls, respectively. SKOV-3 cells (6,000 per well) were plated on the siRNA/lipid reagent complexes (6). After 24 h, cells were treated with LBW242 (20 μmol/L) or vehicle for 72 h and cell viability was measured as described above.

Validation of RNA interference screen results. SKOV-3 cells were transfected with original siRNA pools and four individual siRNA duplexes for selected genes, and viability after LBW242 treatment was assessed as above [RelA, green fluorescent protein (GFP), XIAP-1, XIAP-2, XIAP-3, XIAP-4, and XIAP-sp used at 50 nmol/L final concentration; all from Dharmacon]. Knockdown of mRNA was assessed by reverse transcription-PCR (RT-PCR). mRNA was isolated using the RNeasy96 kit (Qiagen), treated with DNase 1, and cDNA was generated using the High Capacity cDNA Archive kit (Applied Biosystems). The cDNA was quantified using custom-designed primers (Sigma Genosys) and Syber Green on an Applied Biosystems 7900HT. Changes in TNFα mRNA following compound treatment were normalized to β-actin and calculated as described (7).

Western blotting. Primary antibodies for immunoblot analyses were anti-cIAP1 (R&D systems), anti-XIAP (BD Biosciences), anti–proliferating cell nuclear antigen (Santa Cruz Biotechnology), and anti–β-actin (Sigma).

Cytokine analysis. TNFα protein was measured using a multiplexed sandwich ELISA at Endogen Searchlight services (Pierce).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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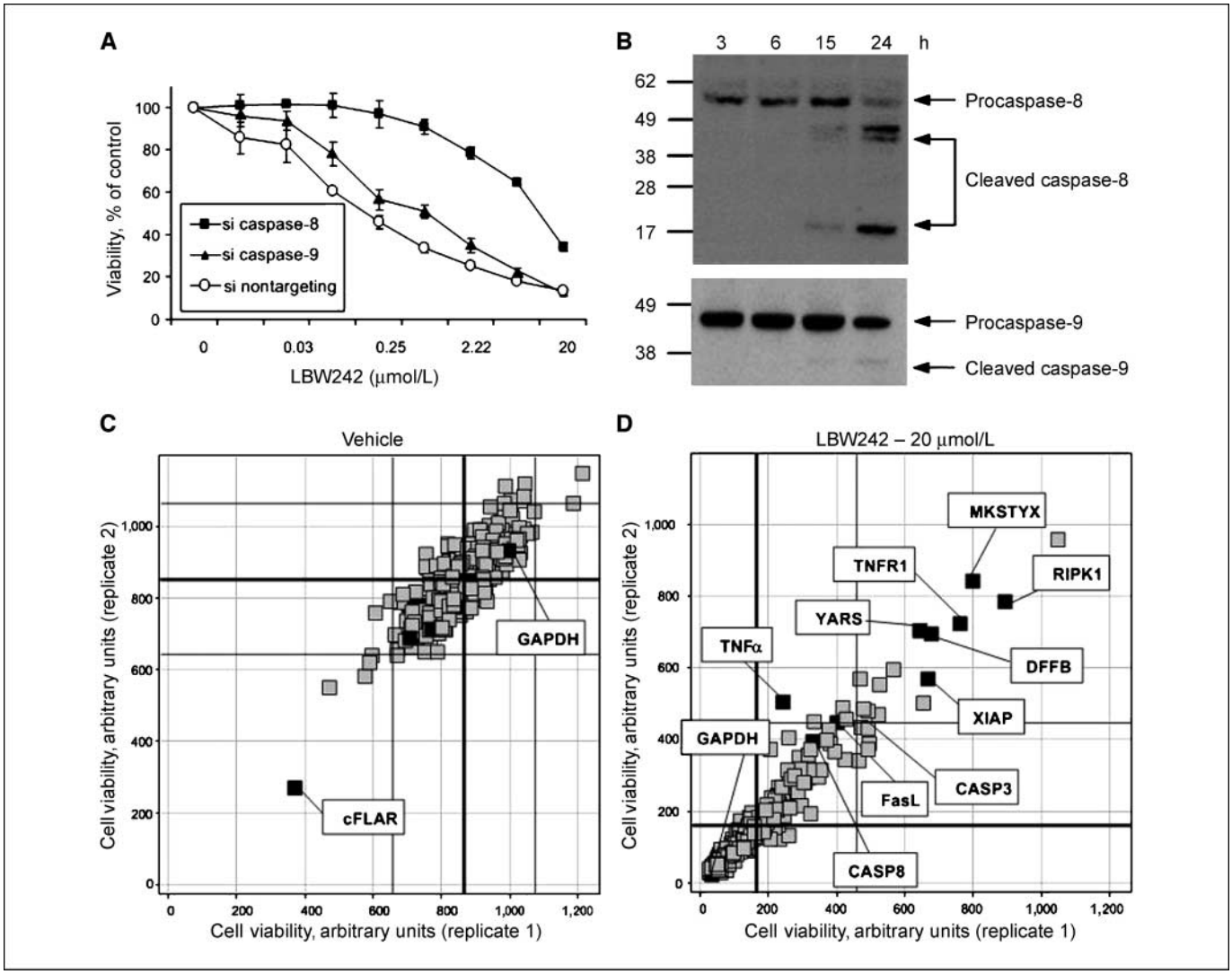

Figure 1. Response to Smac mimetic, LBW242, requires extrinsic apoptosis signaling genes. *A,* RNAi knockdown of caspase-8 (■; 83% mRNA knockdown; data not shown) but not caspase-9 (▲; 86% mRNA knockdown; data not shown) profoundly rescued SKOV-3 cells from LBW242-induced cell killing compared with cells transfected with nontargeting siRNA (○). Shown is SKOV-3 cell viability after LBW242 treatment (48 h) relative to untreated cells (viability, percent of control). *B,* caspase-8 (*top*) and caspase-9 (*bottom*) cleavage was assessed by Western blot following treatment with 500 nmol/L LBW242 for the indicated time points. *C* and *D,* to identify additional siRNAs that rescue from LBW242-induced cell death, cells were transfected with a collection of 319 siRNA pools targeting an apoptosis-related gene set. Changes in growth of cells in replicate wells following treatment (72 h) with either vehicle (*C*) or LBW242 (20 μmol/L; *D*) were assessed (cell viability, arbitrary units). Plate means and 2 SDs are indicated with bold and fine lines, respectively.

Results

It is presumed that Smac mimetics activate cell death by releasing caspase-9 from repression mediated by the BIR3 domain of XIAP. We and others have observed that the SKOV-3 ovarian carcinoma and MDA-MB-231 breast carcinoma cell lines are highly sensitive (IC_{50}, <1 μmol/L) to Smac mimetics (2, 3), yet the SKOV-3 ovarian carcinoma cell line is reported to have one or more defects in the intrinsic apoptosis signaling arm (8). LBW242 is a Smac mimetic compound (Supplementary Fig. S1) with IC_{50} values of 200 and 5 nmol/L versus XIAP and cIAP1, respectively, as measured in a Smac peptide competitive ELISA (9). By multiple criteria including poly(ADP-ribose) polymerase cleavage, DNA fragmentation, and rescue with zVAD, LBW242 was shown to induce apoptosis *in vitro* (9). Interestingly, RNA interference (RNAi) knockdown of caspase-9 had minimal effect on the response of SKOV-3 cells to LBW242, whereas RNAi knockdown of caspase-8 profoundly rescued SKOV-3 cells from LBW242-mediated cell killing (Fig. 1*A*). Western blot analyses confirmed that LBW242 treatment of SKOV-3 cells resulted in far more complete conversion of caspase-8 to its activated form than caspase-9 (Fig. 1*B*). Although IAPs do not directly inhibit caspase-8 (10), these results suggest that the extrinsic apoptosis signaling arm is equally, if not more, important than the intrinsic apoptosis signaling arm in Smac mimetic–mediated cell killing.

To elucidate the mechanism of action underlying the response to LBW242, we carried out a synthetic rescue screen in which siRNAs targeting 319 apoptosis-related genes were assayed for the ability to restore cell viability during exposure to LBW242. SKOV-3 cells were plated in 96-well plates and transfected with arrayed siRNA pools. Twenty-four hours later, replicate wells for each siRNA pool were treated with vehicle (DMSO) or LBW242 (20 μmol/L, representing the LD_{90}) and incubated for 72 h. With the exception of CFLAR,

no siRNA pools significantly affected viability in vehicle-treated wells (Fig. 1*C*; Supplementary Table S1). Treatment with LBW242 resulted in significant cell killing, largely unaffected by the majority of siRNAs tested (compare Fig. 1*C* and *D*). MKSTYX, a phosphatase-like protein required for cell death induced by a broad range of stimuli including Taxol, cisplatin, and etoposide (11), served as a positive control for the screen. In addition to MKSTYX, a handful of additional siRNA pools permitted survival equivalent to that observed in untreated cells (Fig. 1*D*).

Consistent with earlier observations, siRNAs targeting caspase-8 but not caspase-9 rescued SKOV-3 cells from LBW242 treatment (Fig. 1*D*). Knockdown of 35 candidate genes rescued viability as well as, or better than, caspase-8, many by >2 SDs above the plate mean. These candidate genes were subjected to further validation. Hits were considered valid when knockdown of the target mRNA (>70%) by two or more individual siRNAs was concordant with the ability to confer cell viability in the presence of LBW242. Ten genes met this criterion (Supplementary Figs. S2–S4). Unexpectedly, hits included multiple members of the TNFα signaling pathway (TNFα, TNFRI, and RIPK1) and XIAP, the presumed target of LBW242.

To determine whether Smac mimetic cellular activity was linked to TNFα signaling, we investigated whether TNFα expression was induced by LBW242 treatment in either sensitive or insensitive tumor cell lines. SKOV-3, MDA-MB-231, SK-MEL5, HCT116, A549, and C32 represent a range of sensitivities to LBW242 from most to least sensitive (Fig. 2*A*). LBW242 treatment induced TNFα mRNA expression 30- and 50-fold above background in SKOV-3 and MDA-MB-231 cells, respectively (Fig. 2*B*). In contrast, TNFα expression was induced <4-fold in three LBW242 insensitive (IC_{50}, >25 μmol/L) tumor cell lines (HCT116, A549, and C32). SK-MEL5, a cell line with moderate sensitivity (IC_{50}, 1 μmol/L), displayed ~10-fold TNFα induction and thus had an intermediate phenotype. TNFα induction was unaffected by the addition of zVAD-fmk (data not shown), suggesting that this event occurred before the onset of caspase activation. No TNFα induction was observed in any cell lines using LCJ787, a structurally related (Supplementary Fig. S1)

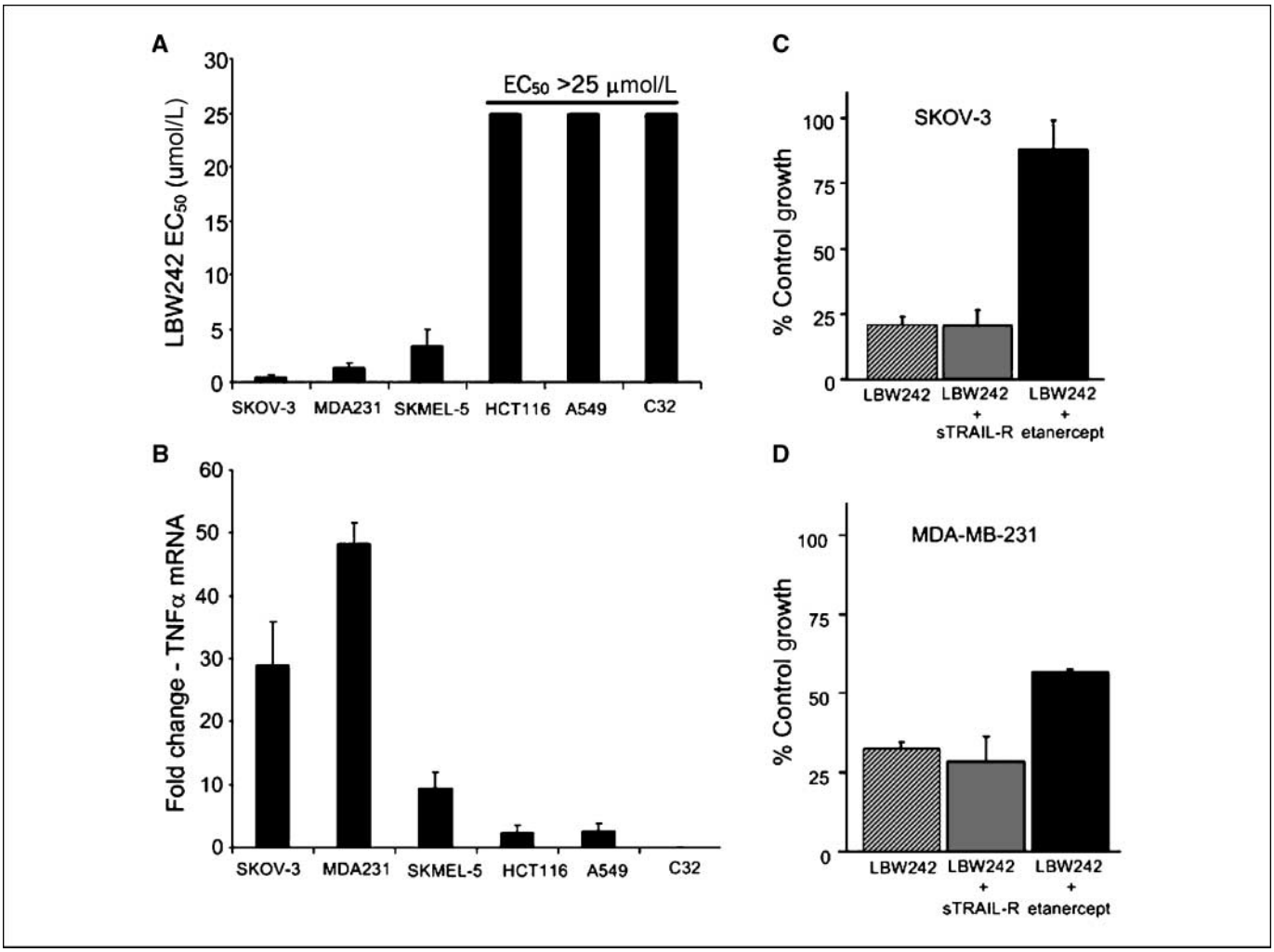

Figure 2. Sensitivity to Smac mimetic, LBW242, correlates with TNFα induction. *A,* cancer cell viability was measured following treatment with LBW242 (72 h), and LBW242 EC_{50} values (mean, $n = 3$) were plotted. *B,* TNFα mRNA expression following LBW242 treatment (6 and 24 h) was measured by RT-PCR. TNFα mRNA induction was determined by comparing LBW242-treated and vehicle-treated cells in three independent experiments and the maximum mean fold change is plotted. TNFα is required for Smac mimetic (LBW242)–induced cell death. SKOV-3 (*C*) and MDA-MB-231 (*D*) cancer cell lines were treated with LBW242 alone (0.1 and 1.0 μmol/L, respectively), LBW242 plus sTRAIL-R (1 μg/mL), or LBW242 plus TNFα-neutralizing agent, etanercept (1 μg/mL). *Columns,* mean percent cell growth relative to vehicle-treated cells ($n = 3$).

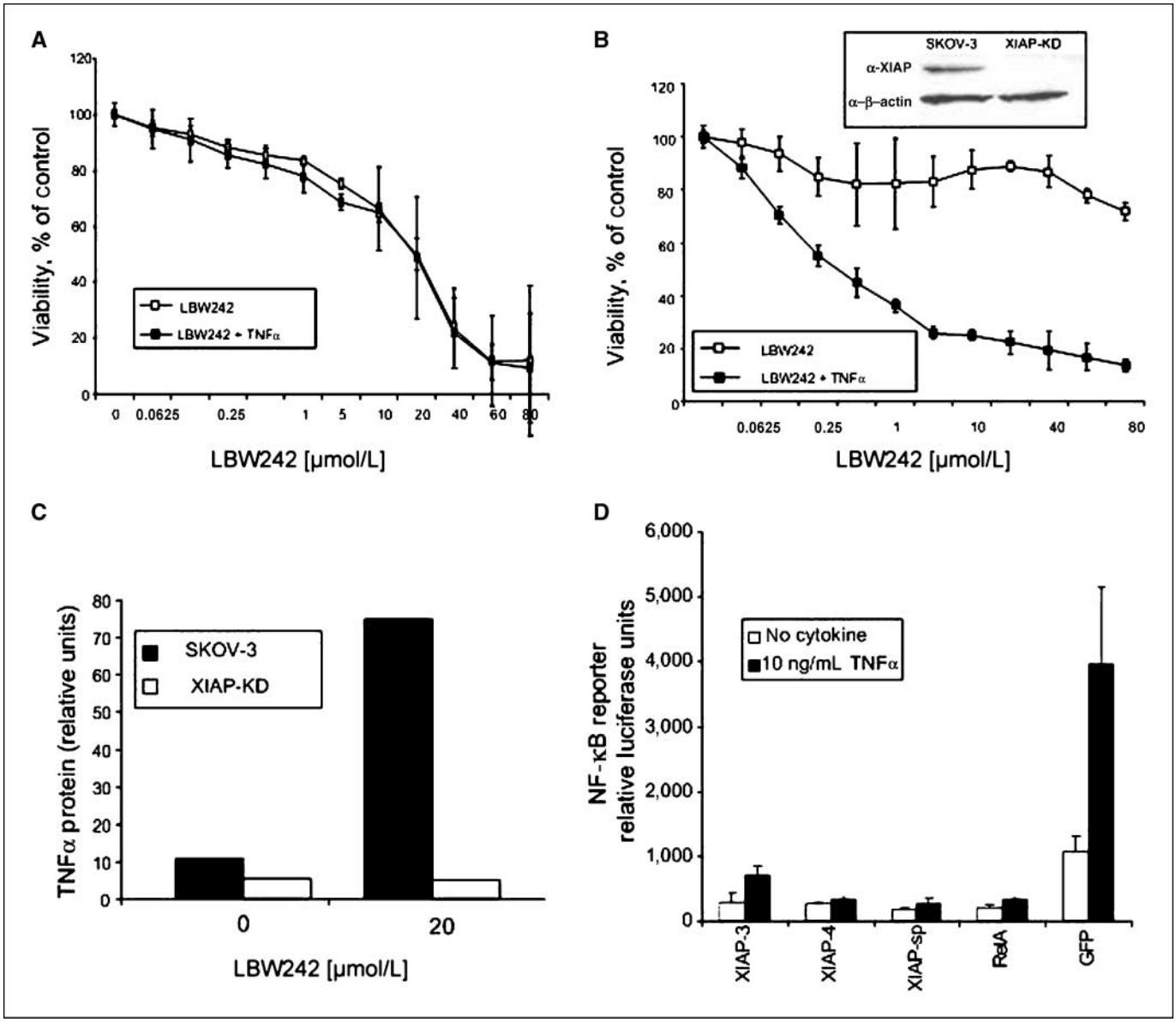

Figure 3. LBW242 fails to kill SKOV-3 cells lacking XIAP but profoundly sensitizes them to TNFα. SKOV-3 (*A*) and XIAP-KD (*B*) cells were treated with LBW242 in the presence (■) or absence (□) of 10 ng/mL TNFα. Shown is cell viability at 96 h (viability, percent of control). Western blot indicates loss of XIAP protein in XIAP-KD cells (*B, inset*). *C,* TNFα induction in SKOV-3 cells requires XIAP. SKOV-3 (*solid columns*) and XIAP-KD (*open columns*) cells were treated with vehicle or LBW242 (20 μmol/L) for 24 h, and secreted TNFα protein (*C*) was measured. *D,* A549 cells stably expressing a NF-κB-luciferase reporter were transfected with the siRNAs indicated; luciferase was quantified following 72 h culture in the presence (*solid columns*) or absence (*open columns*) of 10 ng/mL TNFα.

but non–IAP-binding analogue (data not shown). These data strongly suggest that sensitivity to the Smac mimetic LBW242 is linked to compound-mediated induction of TNFα.

TNFα is paradoxically able to initiate both survival signaling, via NF-κB, and apoptosis signaling, via caspase-8 (12). To determine whether nascent TNFα protein production was required for initiating apoptosis, SKOV-3 cells were treated with LBW242 in the presence or absence of the TNFα antagonist etanercept. Etanercept inhibited LBW242-induced cell killing in all three sensitive cell lines (Fig. 2*C* and *D*, and data not shown). Similar data were obtained using a neutralizing monoclonal antibody recognizing TNFα (Supplementary Fig. S6). This effect was specific for TNFα-neutralizing agents because sTRAIL-R did not affect LBW242 action in any of the sensitive lines tested (Fig. 2*C* and *D*). These results indicate that Smac mimetic–induced cell death requires induction of TNFα expression and activation of the extrinsic apoptosis pathway.

Although XIAP was thought to be the primary target of Smac mimetics, it was one of the top siRNA rescue hits identified in our screen, a result that was completely unanticipated. To further explore the role of XIAP in mediating sensitivity to Smac mimetics, a cell line was derived from SKOV-3 (XIAP-KD) in which XIAP levels were stably reduced with a shRNA (Fig. 3*B, inset*). Consistent with the results from the rescue screen, XIAP-KD cells were resistant to LBW242 (compare Fig. 3*A* and *B*).

Because we observed that TNFα seems to be required for Smac mimetic–induced cell death, we hypothesized that XIAP might be required for TNFα production. To explore this possibility, we

measured TNFα protein in SKOV-3 and XIAP-KD cells in the presence and absence of LBW242 (Fig. 3*C*). Indeed, loss of XIAP not only disrupted LBW242-triggered TNFα induction but also led to decreased basal TNFα levels. XIAP seems to regulate TNFα induction at the transcriptional level because LBW242-mediated induction of TNFα mRNA was ablated in the XIAP-KD cells (Supplementary Fig. S5).

Because TNFα is a known transcriptional target of NF-κB, we next asked whether XIAP was required for transcriptional activation of a NF-κB reporter. A549 cells stably expressing a NF-κB-luciferase reporter were stimulated with TNFα following transfection with siRNAs against RelA, GFP, or XIAP. As expected, knockdown of the NF-κB subunit RelA, but not GFP, significantly diminished TNFα-stimulated reporter activity (Fig. 3*D*). Like RelA, knockdown of XIAP abrogated NF-κB transcriptional activation (Fig. 3*D*). Together with recent findings from Lu et al. (13), these data show a requirement for XIAP in modulating certain NF-κB transcriptional programs.

If loss of XIAP expression conferred resistance to LBW242 because XIAP was required for compound-induced TNFα production, one would predict that XIAP-KD cells would be resensitized to LBW242 by the addition of exogenous TNFα. As shown in Fig. 3*A* and *B*, respectively, both SKOV-3 and XIAP-KD cells were resistant to exogenous TNFα alone. Addition of exogenous TNFα did not further increase the sensitivity of SKOV-3 cells (Fig. 3*A*) but dramatically sensitized XIAP-KD cells to LBW242 (Fig. 3*B*). The dose-dependent TNFα-hypersensitizing effect of LBW242 in cells lacking XIAP argued against the notion that XIAP is the principal target of Smac mimetics and raised the possibility that an alternative protein, which normally represses TNFα-mediated apoptosis, is targeted.

cIAP1 has been reported to negatively regulate TNFα-mediated apoptosis (14–16). Because a peptide derived from Smac was shown to facilitate proteosome-mediated degradation of cIAP1 (17), we asked whether the Smac mimetic LBW242 might behave similarly. Indeed, LBW242 treatment resulted in the disappearance of cIAP1 protein but not XIAP (Fig. 4*A*). This effect required binding of LBW242 to the BIR3 domain of cIAP1 because LCJ787, a nonbinding analogue (Supplementary Fig. S1), was inactive in this assay (Fig. 4*A*). Loss of cIAP1 protein occurred through a proteosome-mediated process because preincubation with MG132 completely inhibited degradation (Fig. 4*A*). To determine whether cIAP1 loss was requisite for TNFα–mediated cell death in the absence of LBW242, siRNA-mediated knockdowns of cIAP1 or GFP were done in XIAP-KD cells. Knockdown of cIAP1 had almost no effect on the viability of XIAP-KD cells in the absence of TNFα (Fig. 4*B*). In contrast, loss of cIAP1 in the presence of exogenous TNFα resulted in apoptosis similar to that observed following LBW242 treatment. This TNFα protective role seems to be specific for cIAP1 because knockdown of cIAP2 in the same system did not sensitize to TNFα (data not shown). Because knockdown of cIAP1 expression combined with exogenous TNFα phenocopied LBW242 treatment, we propose that cIAP1 loss is necessary, but not sufficient, for compound action. Together, the aggregate data argue for a model in which Smac mimetics act by eliminating cIAP1, an event that is lethal when accompanied by an XIAP-dependent increase in TNFα production.

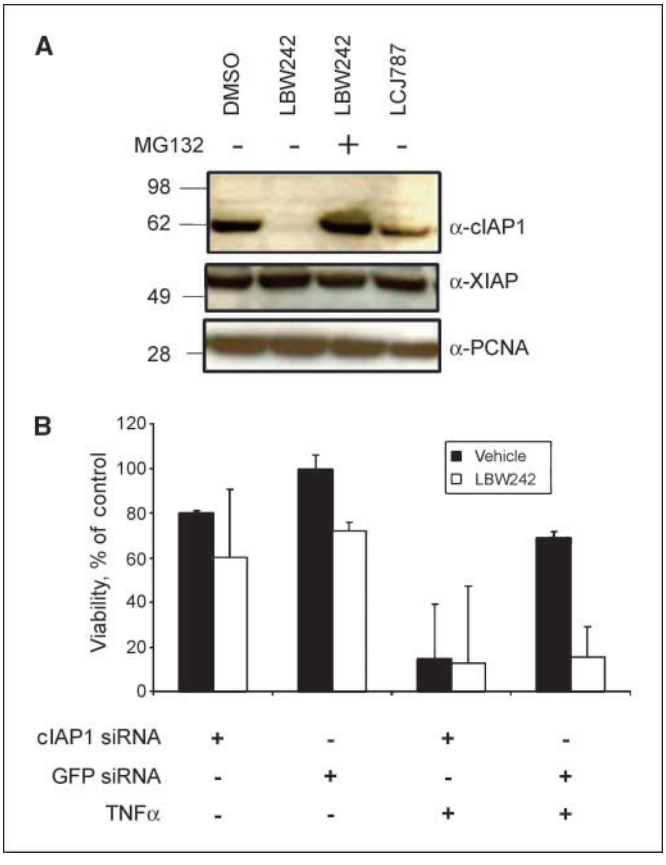

Figure 4. LBW242-mediated elimination of cIAP1 is requisite for TNFα-induced cell death. *A,* Western blot showing cIAP1 and XIAP proteins in SKOV-3 cells treated for 30 min with LBW242 (1 μmol/L), LCJ787 (1 μmol/L; an inactive analogue of LBW242), or vehicle (DMSO) with or without pretreatment (2 h) using proteosome inhibitor MG132 (6 μmol/L). *B,* XIAP-KD cells were transfected with siRNAs targeting cIAP1 (76% mRNA knockdown; Supplementary Fig. S7) or GFP (nontargeting negative control) and treated with vehicle (*solid columns*) or 20 μmol/L LBW242 (*open columns*) for 72 h and/or 10 ng/mL TNFα for 24 h. Cell viability was measured at 96 h and plotted relative to vehicle-treated cells transfected with GFP siRNA (viability, percent of control).

Discussion

Our data support a mechanistic model in which XIAP and cIAP1 play distinct roles in mediating cellular response to Smac mimetics. XIAP participates in a signaling cascade that positively regulates TNFα transcription, whereas cIAP1 acts to protect cells from TNFα-mediated apoptosis. Smac mimetic–mediated destruction of cIAP1 renders the cells sensitive to TNFα. Whereas TNFα is clearly a critical driver of Smac mimetic–induced killing, we cannot rule out whether additional cytokines may be involved.

We speculate that the requirement for XIAP in LBW242-mediated killing of SKOV-3 cells stems from the role of XIAP in NF-κB signaling and TNFα induction. Our observation that XIAP was required for activation of a NF-κB reporter as well as for transcription of endogenous TNFα is in agreement with the data of Lu et al. (13), which showed that the XIAP-TAB1 complex positively regulates TAK1 kinase activity. This functionality seems to be conserved evolutionarily because the *Drosophila* IAP homologue DIAP2 is required for Imd signaling, a TNFα-like pathway that modulates innate immunity (18).

Whereas the role of cIAP1 seems to be analogous to the caspase-8 inhibitor CFLAR, the mechanism through which cIAP1 antagonizes TNFα-mediated apoptosis remains unclear. cIAP1 does not directly inhibit either caspase-3, caspase-7, or caspase-8 (10, 19).

cIAP1 has been mapped to the TNFα death-induced signaling complex (DISC) via a direct interaction with TNF receptor–associated factor 2 (20). Other than cIAP1 itself, we have not observed significant changes in the total cellular levels of other DISC components following treatment with LBW242 (data not shown). It remains possible that through the destruction of cIAP1, LBW242 modulates the constellation of proteins recruited to the DISC and consequently affects its function.

Because TNFα induction was not observed when cIAP1 degradation was inhibited by MG132 (data not shown), we suspect that cIAP1 loss is necessary for TNFα induction. This does not seem to be sufficient, however, because cIAP1 elimination, but not TNF induction, occurs in IAP antagonist–resistant cells (data not shown).

In summary, we have identified unanticipated roles for cIAP1 and XIAP in TNFα signaling and unveiled cIAP1 as the primary target of the Smac mimetic class of small molecules in the monotherapy setting. This work suggests that Smac mimetic drugs may have particular benefit in tumors in which TNFα levels are known to be elevated. It is important to note that in chemotherapy-Smac mimetic combination settings, elimination of XIAP BIR3-mediated repression of caspase-9 may provide a TNFα-independent benefit.

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