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IAP Antagonists Target cIAP1 to Induce TNFα-Dependent Apoptosis

James E. Vince,[1] W. Wei-Lynn Wong,[1,8] Nufail Khan,[1,8] Rebecca Feltham,[1,8] Diep Chau,[1] Afsar U. Ahmed,[1] Christopher A. Benetatos,[2] Srinivas K. Chunduru,[2] Stephen M. Condon,[2] Mark McKinlay,[2] Robert Brink,[3] Martin Leverkus,[4] Vinay Tergaonkar,[5] Pascal Schneider,[6] Bernard A. Callus,[1] Frank Koentgen,[7] David L. Vaux,[1] and John Silke[1,*]

[1]Department of Biochemistry, La Trobe University, Kingsbury Drive, Melbourne, VIC 3086, Australia
[2]TetraLogic Pharmaceuticals, 343 Phoenixville Pike, Malvern, PA 19355, USA
[3]Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, NSW 2010, Australia
[4]Laboratory for Experimental Dermatology, Department of Dermatology and Venerology, Otto-von-Guericke-University Magdeburg, Germany
[5]3-02B, IMCB, 61 Proteos, Biopolis Drive, Singapore 138673
[6]Department of Biochemistry, University of Lausanne, CH-1066 Epalinges, Switzerland
[7]Ozgene Pty Ltd P.O. Box 1128 Bentley DC, Western Australia 6983, Australia
[8]These authors contributed equally to this work.
*Correspondence: j.silke@latrobe.edu.au
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SUMMARY

XIAP prevents apoptosis by binding to and inhibiting caspases, and this inhibition can be relieved by IAP antagonists, such as Smac/DIABLO. IAP antagonist compounds (IACs) have therefore been designed to inhibit XIAP to kill tumor cells. Because XIAP inhibits postmitochondrial caspases, caspase 8 inhibitors should not block killing by IACs. Instead, we show that apoptosis caused by an IAC is blocked by the caspase 8 inhibitor crmA and that IAP antagonists activate NF-κB signaling via inhibtion of cIAP1. In sensitive tumor lines, IAP antagonist induced NF-κB-stimulated production of TNFα that killed cells in an autocrine fashion. Inhibition of NF-κB reduced TNFα production, and blocking NF-κB activation or TNFα allowed tumor cells to survive IAC-induced apoptosis. Cells treated with an IAC, or those in which cIAP1 was deleted, became sensitive to apoptosis induced by exogenous TNFα, suggesting novel uses of these compounds in treating cancer.

INTRODUCTION

Inhibitor-of-apoptosis proteins (IAPs) are characterized by the presence of one or more baculoviral IAP repeats (BIRs). The BIR domains of XIAP, cIAP1, and cIAP2 bind to caspases, the key effector proteases of apoptosis, and XIAP has been shown to be a potent inhibitor of caspase 9 and caspase 3. However, cIAP1 and cIAP2 were originally identified by their association with TNF-R2 via TRAF1 and TRAF2 (Rothe et al., 1995; Uren et al., 1996), and although they might bind to caspases 7 and 9, they cannot directly inhibit their proteolytic activity (Eckelman and Salvesen, 2006; Tenev et al., 2005). It has therefore been suggested that they might regulate apoptosis indirectly, by influencing signaling pathways elicited by the TNF receptor superfamily (Varfolomeev et al., 2006; Fotin-Mleczek et al., 2002; Li et al., 2002; Samuel et al., 2006).

The physiological role of IAPs has been studied in vivo by the creation of genetic knockout mice. Mice lacking either XIAP or cIAP1 display no phenotypic abnormalities and no functional deficiencies in apoptosis initiated by a variety of stimuli (Conze et al., 2005; Harlin et al., 2001). Similarly, although cIAP2 knockout mice display an increased resistance to LPS-induced endotoxic shock; cells derived from cIAP2 knockout mice undergo normal cell death in response to a variety of apoptotic-inducing agents (Conte et al., 2006). Hence, cIAP1, cIAP2, and XIAP appear to be dispensable for gross mouse development and the apoptotic program.

Although seemingly unimportant for normal development, overexpression of cIAP1, cIAP2, or XIAP has been implicated in tumor cell survival (Hunter et al., 2007), and recent studies have demonstrated that genetic amplification of cIAP1 can both promote tumorigenesis and sustain tumor growth in a mouse model of liver cancer (Zender et al., 2006). How cIAP1 functions to sustain tumor growth and prevent apoptosis, however, remains unclear.

Natural IAP antagonists include Grim in *Drosophila* and Smac/DIABLO in mammals. These proteins have been shown to bind to the same groove in the BIRs of XIAP as caspases and can thereby antagonize XIAP's anticaspase activity in vitro (Chai et al., 2000; Srinivasula et al., 2001). Compounds have been designed to mimic the IAP antagonists and prevent XIAP from inhibiting caspases, thereby

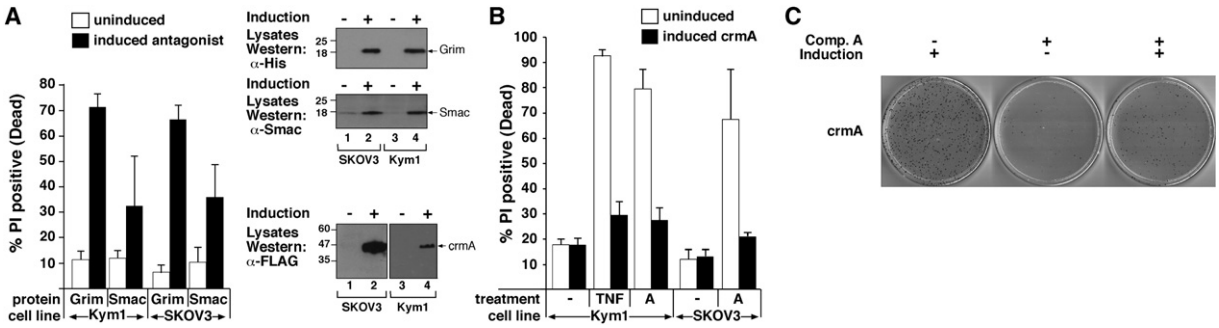

Figure 1. Compound A, a "Smac-Mimetic" IAP Antagonist, Induces Death of Tumor Cells that Can Be Blocked by crmA

(A) Inducible IAP antagonists kill compound A-sensitive cells. Ubiquitin Grim or ubiquitin Smac fusions were expressed in inducible stable lines in Kym1 and SKOV3 cells for 24 hr (Kym1) or 48 hr (SKOV3), and cell death was analyzed by propidium iodide (PI) staining and flow cytometry. Western blots depicting induction of Grim or Smac in Kym1 and SKOV3 cells are shown.

(B) Stable Kym1 or SKOV3 cell lines expressing inducible crmA were induced for 24 hr; this was followed by addition of 5 nM (Kym1) or 500 nM (SKOV3) of compound A or TNFα (Kym1). Cell death was assessed by PI staining and flow cytometry 24 hr (Kym1) and 48 hr (SKOV3) after compound A addition. Unless stated otherwise, these concentrations of compound and time points were used throughout. As in (A), bars depict the average of four independent experiments; error bars are SEM. Western blots depicting crmA induction in SKOV3 and Kym1 cells are shown.

(C) Inducible expression of crmA provides long-term clonagenic survival to compound A treatment in Kym1 cell lines.

causing cancer cells to undergo apoptosis (Li et al., 2004; Schimmer et al., 2004).

In this paper, we show that, surprisingly, the primary target of IAP antagonists is cIAP1 and that removal of cIAP1 through genetic deletion or IAC application (1) activates NF-κB signaling that induces TNFα production and (2) kills sensitive tumor cells through enhanced TNF-R1 death-receptor signaling and caspase 8 activation. These results unveil how cIAP1 might functionally contribute toward tumor cell survival.

RESULTS

Synthetic or Natural IAP Antagonists Induce Tumor Cell Death that Can Be Blocked by an Inhibitor of Caspase 8

We used the crystal structure of four amino acids of Smac/DIABLO bound to the BIR3 of XIAP to develop a small molecule IAP antagonist, compound A (Figure S1 in the Supplemental Data available online), with a binding affinity of 0.003 and 0.001 μM to XIAP and cIAP1 BIR3 domains, respectively. Compound A, an IAP antagonist compound (IAC), can compete with Smac for binding to IAPs and also for caspase 9 binding and interacts specifically with the BIR2 and BIR3 domains of cIAP1, cIAP2, and XIAP (data not shown). We tested several cell lines for their sensitivity to this molecule as a single agent. Significant killing of Kym1, OVCAR4, and SKOV3 lines was observed with doses of 5 nM and 500 nM of compound A, respectively, but not with a negative control, compound B (data not shown).

Although we have reported that neither cytoplasmic Smac or Grim are capable of killing HEK293 cells when stably expressed (Silke et al., 2004), we reasoned that if compound A killed sensitive cells by antagonizing IAPs, then inducible expression of either of these natural IAP antagonists should be sufficient to kill cells sensitive to IAP antagonists. By using a tamoxifen-inducible lentiviral system, we inducibly expressed these natural IAP antagonists and showed that induction of mature cytosolic forms of Smac or Grim was sufficient to cause apoptosis of SKOV3 and Kym1 cells (Figure 1A). Therefore inhibition of IAPs alone, by either a synthetic (compound A) or naturally occurring (Smac or Grim) IAP antagonist, is sufficient to cause apoptosis in a number of human tumor cell lines.

To exclude the involvement of a death-receptor signaling pathway in IAP-antagonist-induced cell death, we tested whether the caspase 8 inhibitor crmA could block cell death. Surprisingly, crmA was able to prevent apoptosis caused by compound A in both transient (Figure 1B) and clonagenic assays (Figure 1C), suggesting that activation of a death-receptor pathway is the mechanism by which IAP antagonists induce apoptosis and that cIAP1 and cIAP2, which are known to interact with TNF-R1 (Shu et al., 1996), might be the key targets of IAP antagonists.

IAC or Natural IAP Antagonists Induce Loss of cIAP1 in a Proteasomal-Dependent Manner

It has been reported that Smac is able to induce degradation of cIAP1 in transient transfection assays (Yang and Du, 2004). We therefore tested the ability of our IAC to induce degradation of wild-type and mutant cIAP1 (see Schematic, Figure 2A) by using a similar transient transfection assay. Compound A efficiently depleted transfected wild-type cIAP1 (Figure 2A, lanes 1 and 2) and cIAP1 mutants that had single-point mutations in individual BIRs (Figure 2A, lanes 3–8). However mutation of key residues in cIAP1's BIR 2 and BIR 3 domains that prevent IAC binding completely abrogated compound A-induced degradation (Figure 2A, lanes 9 and 10). IAC-induced depletion of endogenous cIAP1 was extremely rapid, occurring within 5 min (Figure 2B), and is unlikely to be

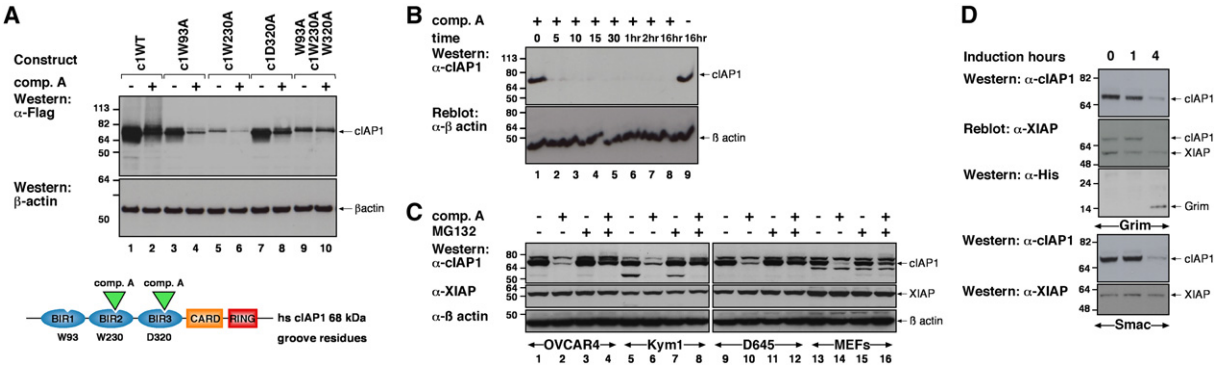

Figure 2. Compound A, a "Smac-Mimetic" IAP Antagonist, Induces Rapid Degradation of cIAP1 in a Binding- and Proteasomal-Dependent Manner

(A) Cells were transiently transfected with Flag-tagged cIAP1 constructs that contain point mutations in the groove residues required for IAP-antagonist binding (see schematic). Transfections were split into two, and cells were treated with or without compound A.

(B) IAP antagonist induces rapid degradation of cIAP1. Cells were treated with compound A for the indicated period of time and lysed immediately in 8 M urea, run on an SDS/PAGE gel, and western blotted and probed with anti-cIAP1.

(C) Compound A-induced degradation of cIAP1 is blocked by the proteasome inhibitor MG132 in multiple cell lines.

(D) Natural IAP antagonists induce degradation of cIAP1. Stable cell lines described in Figure 1 were induced for the indicated time, and western blots were probed with the indicated antibodies.

a postlysis artifact or involve the relocalization of cIAP1 to a detergent-insoluble compartment because cIAP1 depletion was observed in cell lysates prepared with 8 M urea (Figure 2B).

Smac-induced degradation of cIAP1 has been shown to be proteasome dependent (Yang and Du, 2004), and consistent with this, IAC-induced depletion of cIAP1 could be blocked by the proteasome inhibitor MG132 (Figure 2C) in both IAC-resistant (D645, MEF) and -sensitive (OVCAR4, Kym1) cell lines. It is noteworthy that during the course of these assays, there was no detectable loss of XIAP (Figure 2C), consistent with observations made with Smac itself (Yang and Du, 2004). Similar to IACs, the expression of cytosolic forms of Grim and Smac also induced rapid degradation of endogenous cIAP1 but not XIAP (Figure 2D), demonstrating a similar mode of action for both synthetic and natural IAP antagonists.

Loss of cIAP1 Enhances RIP1 Binding to TNF-R1, Stabilizes NIK, and Activates Canonical and Noncanonical NF-κB Signaling

Because death-receptor activation was involved in IAP-antagonist killing, we determined whether compound A affected TNF-R1 complex formation by using Fc-TNFα to pull down endogenous TNF-R1 and associated proteins in the presence or absence of compound A (Figure 3A). Because compound A induces the degradation of cIAP1 (Figures 2 and 3B), we transfected cells with its ΔC6 variant (lacking the c-terminal 6 residues), which does not degrade in response to compound A. Surprisingly, endogenous RIP1 was recruited to TNF-R1 in the absence of TNF ligand binding within half an hour of treatment with compound A (Figure 3A, lane 2).

To determine whether RIP1 binding upon compound A treatment was due to cIAP1 inhibition (and not XIAP), we examined TNF-R1 complexes in WT, cIAP1$^{-/-}$, or XIAP$^{-/-}$ SV40 large T immortalized MEFs. In contrast to D645 cells, we observed some RIP1 binding at time 0 in our MEF cell lines (Figure 3B, lanes 1, 4, 7, and 10). Consistent with our observations in D645 cells, enhanced RIP1 binding to TNF-R1 was also observed in compound A-treated wild-type MEFs (Figure 3B; compare lanes 5 and 6 with lanes 2 and 3). Significantly, we also observed prolonged RIP1 binding in untreated cIAP1 knockout, but not in XIAP knock-out MEFs, (Figure 3B), showing that it is loss of cIAP1 that leads to increased RIP1 recruitment to TNF-R1.

Because RIP1 binding to TNF-R1 is known to result in the activation of NF-κB, we tested the ability of compound A to induce NF-κB signaling. Consistent with the rapid degradation of cIAP1 induced by compound A (Figure 2B) and the rapid recruitment of RIP1 to TNF-R1 (Figures 3A and 3B), western blots showed that NF-κB p65 became phosphorylated 5–15 min after addition of compound A (Figure 3C). Phosphorylation of p65 oscillated, consistent with previously described reports of receptor-mediated activation of NF-κB (Werner et al., 2005).

We also examined markers for the activation of the noncanonical NF-κB pathway in an attempt to exclude its involvement. Remarkably, however, compound A induced NF-κB2 processing of p100 to the active p52 form (Figure 3D). Equally remarkably, this treatment also caused a significant stabilization of NIK, the kinase that is required to activate noncanonical NF-κB, which is usually undetectable in cells (He et al., 2006; Senftleben et al., 2001). Compound A-induced activation of noncanonical NF-κB was due to antagonism of cIAP1 because cIAP1 knockout cells (Figure 3D, lanes 3 and 4) showed constitutive noncanonical NF-κB activation that, like compound A treatment, resulted in increased levels of p52 and the stabilization of NIK. As previously reported for B cells (Grech et al.,

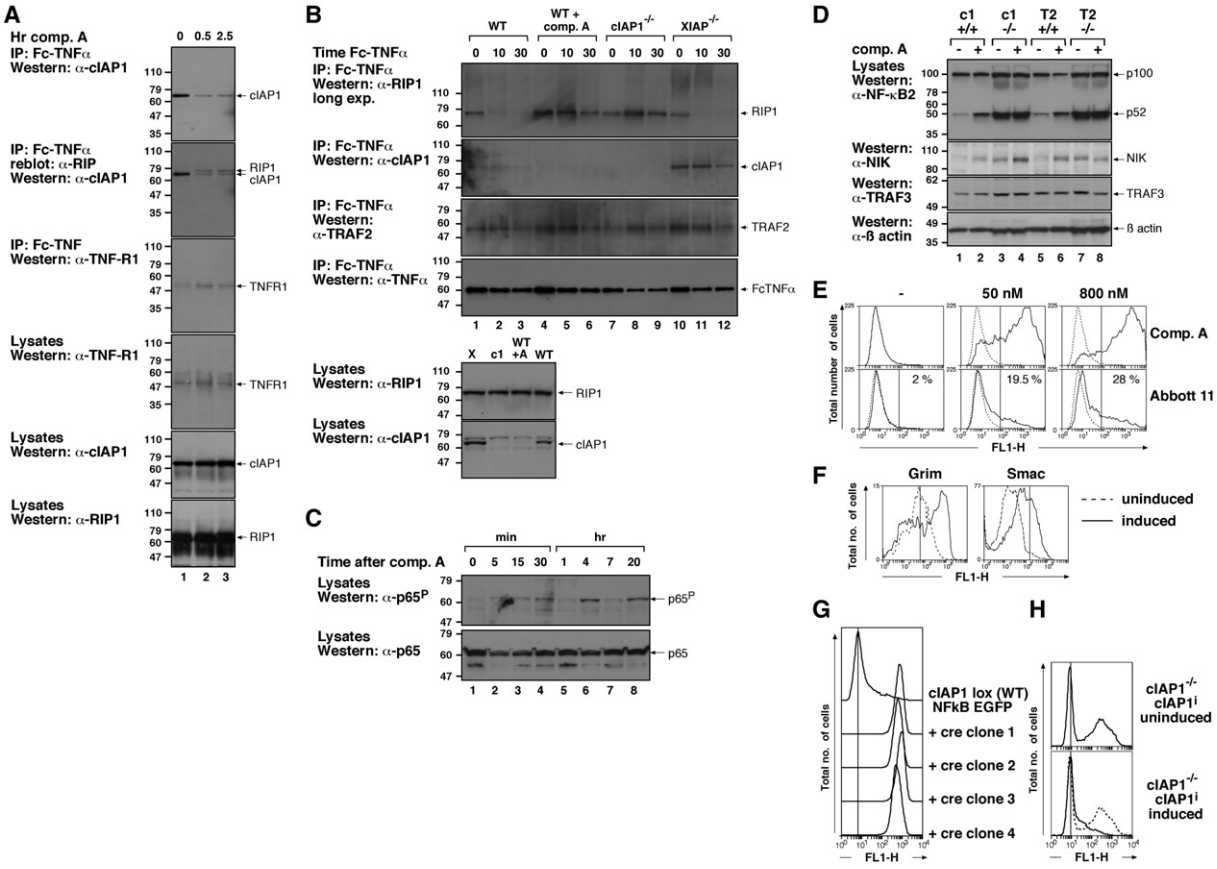

Figure 3. IAP Antagonists Activate NF-κB Signaling

(A) Endogenous RIP1 is recruited to TNF-R1 after addition of compound A. D645 cells transfected with ΔC6 cIAP1 were treated with compound A for the indicated time in hours, and endogenous TNF-R1 immunoprecipitated from DISC lysates with Fc-TNFα and bound proteins were examined by western blotting. Where indicated, blots were probed first with anti-cIAP1 and then reprobed with anti-RIP1.

(B) Endogenous RIP1 is retained on TNF-R1 after addition of compound A or spontaneously in cIAP1 knockouts. Wild-type MEFs (WT), wild-type MEFs treated with compound A (WT + A), XIAP knockout MEFs (XIAP$^{-/-}$), and cIAP1 knockout MEFs (cIAP1$^{-/-}$) were treated with Fc-TNFα for the indicated time in minutes, and endogenous TNF-R1 immunoprecipitated from DISC lysates.

(C) Compound A addition induces phosphorylation of p65. Cells were treated with compound A for the indicated period of time in minutes and hours, and lysates were probed for phospho-p65 and p65.

(D) Conditional knockin cIAP1 cells (c1$^{+/+}$), cIAP1 knockout MEFs (c1$^{-/-}$), conditional knockin TRAF2 cells (T2$^{+/+}$), and TRAF2 knockout MEFs (T2$^{-/-}$) were treated with or without compound A overnight and examined by western blotting.

(E) IAP antagonism by independent Smac-mimetic compounds induces NF-κB. NF-κB reporter cell lines were generated by infection with an NF-κB EGFP lentiviral vector, and the effect of compound A or Abbott 11 was monitored in multiple cell lines. Data from one stable 293T line are shown in which the dashed line represents basal FL1 fluorescence in unstimulated cells and the solid line represents FL1 24 hr after compound A addition. The experiment was repeated three times with additional doses not shown.

(F) Natural cytoplasmic IAP antagonists induce NF-κB. A 293 FlpIN clone was infected with the NF-κB EGFP reporter and then transfected with a pcDNA5 vector expressing ubiquitin-fused Smac or ubiquitin-fused Grim. Isogenic clones were selected according to the manufacturer's protocol and induced with doxycyclin. The fluorescence in the FL1 channel of uninduced (dashed line) and induced (solid line) cells was measured by flow cytometry.

(G) Genetic ablation of cIAP1 activates NF-κB. Transformed cIAP1 loxP knockin MEFs were infected with a lentiviral NF-κB EGFP reporter. A single clone was obtained and infected with cre recombinase. Basal NF-κB activity was measured by flow cytometry in the loxP knockin (WT) and four independent knockout MEF clones.

(H) cIAP1 complementation of cIAP1 knockout MEFs blocks spontaneous activation of NF-κB caused by loss of cIAP1. An immortalized cIAP1 knockout MEF line was infected with an inducible cIAP1 construct and then with the NF-κB EGFP reporter. The NF-κB EGFP reporter infected ?50% of the cells. We then induced the polyclonal population to express cIAP1 for 48 hr, and fluorescence in the FL1 channel was measured by flow cytometry.

2004), loss of TRAF2 in MEFs also resulted in constitutive noncanonical NF-κB activation that was comparable to the levels observed in cIAP1 knockout MEFs (Figure 3D, compare lanes 5 and 7).

Because compound A induced elements of both canonical (i.e., RIP1 recruitment to TNF-R1 and phosphorylation of p65) and noncanonical NF-κB signaling (i.e., NIK stabilization and p100 processing), we created an NF-κB

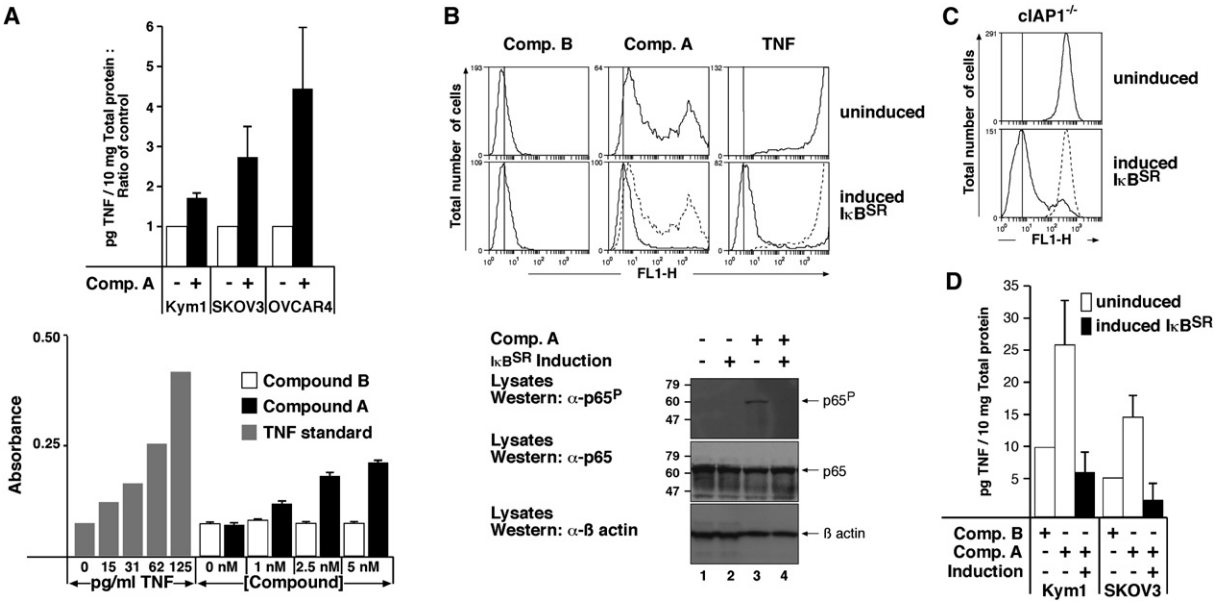

Figure 4. Loss of IAP Induces Production of TNFα in an NF-κB-Dependent Manner

(A) Compound A induces production of TNFα in drug-sensitive cell lines. As shown in the upper graph, Kym1 and SKOV3 and OVCAR4 cells were treated with compound A, and the increase in TNFα production from cell lysates was measured by ELISA. As shown in the lower graph, SKOV3 cells were treated with increasing doses of compound A as indicated, and TNFα production in cell culture supernatants was measured by ELISA.

(B) Inducible $I\kappa B^{SR}$ cell lines were generated and subsequently infected with an NF-κB GFP reporter virus; in the upper panel, 293T inducible stables were treated with compound A (500 nM) or TNFα in the presence or absence of induction of the $I\kappa B^{SR}$ and NF-κB activity measured by flow cytometry. In the lower panel, Kym1 cells inducibly expressing $I\kappa B^{SR}$ were established and treated with compound A (5 hr) in the presence or absence of induction, and lysates were probed with antibodies to p65 and phospho p65. Representative histograms are shown from three independent experiments.

(C) Inducible $I\kappa B^{SR}$ resets high basal level of NF-κB activation in cIAP1 knockout MEFs. cIAP1 knockout NF-κB EGFP MEFs were generated as described in Figure 1 and subsequently infected with inducible $I\kappa B^{SR}$. $I\kappa B^{SR}$ was induced overnight, and activation of NF-κB was measured by flow cytometry. The data was reproduced twice with the polyclonal inducible population.

(D) Inducible $I\kappa B^{SR}$ blocks compound A-induced production of TNFα in sensitive lines. Inducible $I\kappa B^{SR}$ cells were treated with compound A (8 hr Kym1 and 24 hr SKOV3), and TNFα levels were measured by ELISA as in (A). Error bars are SEM.

reporter 293T cell line by using a commercial lentiviral NF-κB reporter vector, in which increased GFP fluorescence indicates an increase in total NF-κB activity. Consistent with the results obtained with western blots, an increase in total NF-κB activity was observed upon addition of compound A or a previously described IAP antagonist, Abbott 11, in a dose-dependent manner (Figure 3E [Oost et al., 2004]). This activation of total NF-κB induced by compound A, but not the inactive compound B, was observed in several different cell lines including primary untransformed MEFs from various genetic backgrounds (e.g., Figure S2B and data not shown), C2CL2 cells (Figure S2A), and BT549, HT29, SKOV3, and OVCAR4 cells (data not shown).

As in the previous assays, the natural IAP antagonists, Grim or Smac (Figure 3F), mimicked the activity of the IACs and activated NF-κB, demonstrating that the NF-κB activation induced by IACs is likely to be mechanism based.

We also examined total levels of NF-κB activity in cIAP1 knockout MEFs by infecting cIAP1 knockin MEFs with the lentiviral NF-κB reporter (Figure 3G and Figure S3). One cIAP1 knockin NF-κB EGFP clone was selected to generate the parental line and was then infected with cre-expressing lentivirus to knock out the cIAP1 locus. Consistent with results obtained by western blotting (Figure 3D) and analysis of both synthetic and natural IAP antagonists (Figures 3E and 3F), all cIAP1-deleted clones showed spontaneous, high-level activation of NF-κB (Figure 3G).

To confirm that loss of cIAP1 was responsible for the increase in activation of NF-κB, we reintroduced inducible cIAP1 into cIAP1 knockout MEFs and subsequently infected them with the NF-κB EGFP reporter vector (Figure 3H). Induction of cIAP1 resulted in inhibition of constitutive NF-κB activity, confirming beyond doubt the role of cIAP1 in controlling NF-κB signaling (Figure 3H).

IAP Antagonists Induce TNFα in an NF-κB-Dependent Fashion

Although activation of NF-κB is usually thought to promote cell survival, there are certain situations in which activation of NF-κB is proapoptotic and might lead to the production of TNFα, TNF-R1, and FasL (Kasibhatla et al., 1998). We therefore examined whether compound A induced production of TNFα in cell lines that were sensitive to compound A. Cell lysates from all these lines contained detectable amounts of TNFα, and addition of compound A increased the amount of TNFα within 24 hr (Figure 4A,

top panel). Moreover, media supernatant collected from SKOV3 cells treated with increasing amounts of compound A demonstrated that TNFα was released from cells treated with compound A at doses as low as 1 nM (Figure 4A, bottom panel). Cell lines resistant to IAP-antagonist treatment did not produce detectable amounts TNFα in response to compound A (Figure S4 and data not shown), suggesting that compound A-induced TNFα production only occurs in compound A-sensitive cell lines.

To test whether NF-κB activation was required for TNFα production after treatment with IAP antagonists, we created an inducible $I\kappa B^{SR}$ (super repressor) lentiviral vector that expresses a well-described nondegradable form of IκB, and this form blocks translocation of NF-κB to the nucleus (Van Antwerp et al., 1996). As expected, expression of $I\kappa B^{SR}$ blocked the NF-κB activation observed when TNFα was applied (Figure 4B). Induction of $I\kappa B^{SR}$ also inhibited compound A-induced activation of NF-κB (Figure 4B, top panel) and prevented phosphorylation of p65 (Figure 4B, compare lanes 3 and 4). Expression of $I\kappa B^{SR}$ also reduced the high basal activation of NF-κB in $cIAP1^{-/-}$ MEFs to levels observed in the original parental line (Figures 4C and 3G). Significantly, $I\kappa B^{SR}$ prevented induction of TNFα by compound A in Kym1 and SKOV3 cells and reduced it to levels below those observed in untreated cells (Figure 4D), indicating that NF-κB activation in response to compound A is required to drive TNFα production.

IAP-Antagonist-Induced Cell Death Can Be Blocked by Inhibition of NF-κB or Blocking TNFα

Because compound A activated NF-κB, leading to production of TNFα, and because killing by this IAP antagonist could be blocked by crmA, we tested whether cell death caused by compound A required TNFα. To do so, we incubated IAP-antagonist-sensitive cells with compound A in the presence or absence of antibodies that blocked TNFα, TRAIL, or FasL activity. Consistent with a requirement for TNFα, apoptosis induced by compound A was completely inhibited by TNFα-blocking antibodies, but was not affected by either FasL- or TRAIL-blocking antibodies (Figure 5A and Figure S5A). As for crmA (Figure 1C), dnTNFR2, and $I\kappa B^{SR}$ (Figure 5D), blocking TNFα antibodies also provided clonagenic protection from compound A-induced death (Figure S5B). In addition, cell death caused by induction of Grim in Kym1 cells or Grim and Smac in SKOV3 cells was also attenuated by TNFα-blocking antibodies (Figure S5C), demonstrating that both natural and synthetic IAP antagonists kill tumor cells through TNFα.

To confirm that TNFα alone was required for compound A-induced killing, we created stable cell lines that inducibly expressed dominant negative (dn) receptors for several TNF family ligands (Figure 5B, inset). These dominant-negative receptors bear the extracellular domain of the receptor fused to the GPI anchor sequence of TRAIL-R3 and can therefore sequester the cognate ligand, but they are incapable of intracellular signaling (Bossen et al., 2006). Consistent with the previous experiments, dnTNF-R2, but not dnCD27 or dnTRAIL-R2, was able to completely protect OVCAR4 and SKOV3 cells and substantially protect Kym1 cells from compound A-induced apoptosis (Figure 5B).

We then determined whether induction of $I\kappa B^{SR}$ could inhibit compound A-induced cell death. This was a particularly demanding test because $I\kappa B^{SR}$ normally increases cell sensitivity to TNFα-induced death (Van Antwerp et al. [1996] and data not shown). Nevertheless, $I\kappa B^{SR}$ was able to reduce the number of Kym1 cells killed by compound A and almost completely protected SKOV3 cells (Figure 5C). Although we did not observe any protection from death caused by compound A in OVCAR4 cells expressing $I\kappa B^{SR}$ (Figure 5C), the lower levels of $I\kappa B^{SR}$ in this cell line (Figure 5C, inset) might have been insufficient to completely block TNFα production.

To demonstrate that blocking TNF receptor signaling allowed long-term clonagenic survival of cells treated with compound A, we induced expression of $I\kappa B^{SR}$ or dnTNF-R2 in Kym1 or SKOV3 cells, exposed them to compound A, and replated them. After 10 days, several hundred Kym1 colonies formed when $I\kappa B^{SR}$ or dnTNF-R2 were induced, and no colonies were observed in the absence of induction (Figure 5D). SKOV3 cells were also well protected by expression of these two proteins (Figure 5D). These results suggest that NF-κB activation caused by compound A is required to drive TNFα production that is required to kill these IAP-antagonist-sensitive tumor cells.

Brefeldin A Blocks Compound A-Induced Cell Death

To determine whether blocking TNFα secretory transport to the cell surface was sufficient for inhibition of compound A-induced death, we incubated Kym1 cells with Brefeldin A prior to addition of compound A (Figure 5E). Brefeldin A almost completely blocked cell death induced by compound A (Figure 5E), suggesting that TNFα trafficking to the cell surface is required for death-receptor engagement and apoptotic signaling. Significantly, Kym1 death induced by exogenous addition of TNFα or TRAIL was not altered in Brefeldin A-treated cells, making it unlikely that cell-surface death-receptor signaling was perturbed (Figure 5E). The cytotoxicity associated with Brefeldin A prevented us from testing SKOV3 and OVCAR4 cells in this assay.

Geldanamycin Blocks NF-κB Activation and Compound A-Induced Cell Death

The finding that the $I\kappa B^{SR}$ could block IAC-induced death was surprising because $I\kappa B^{SR}$ usually sensitizes cells to TNFα-induced death. Although it is not unprecedented that NF-κB can be proapoptotic (He et al., 2006; Kasibhatla et al., 1998), we nevertheless sought to further test the requirement for NF-κB in IAC-induced death in an independent manner. Geldanamycin is an HSP90 inhibitor that is cytotoxic to cancer cells and is currently in clinical trials (Nowakowski et al., 2006). Although there are many

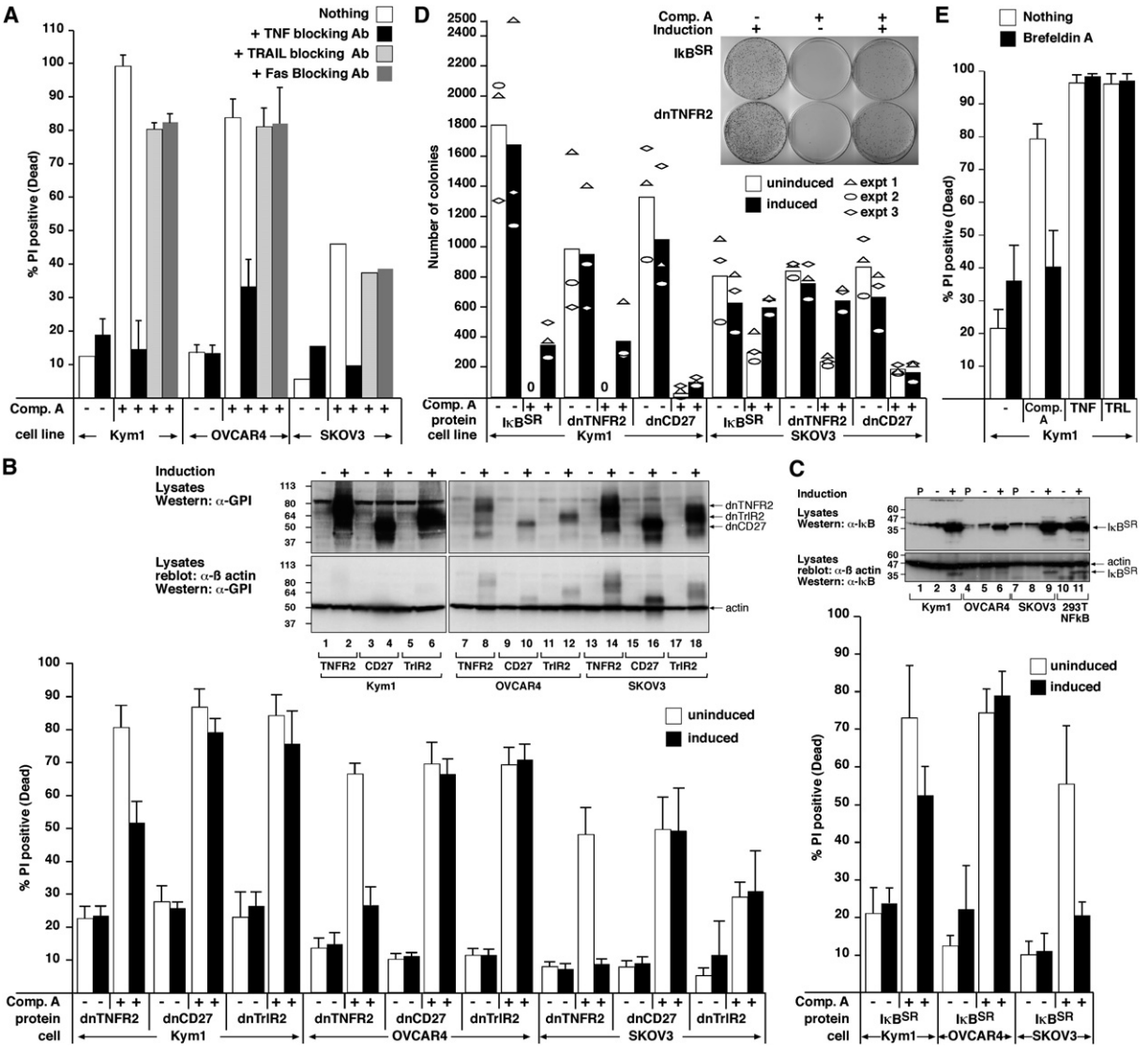

Figure 5. Compound A-Induced Autocrine TNFα Causes Death of Tumor Cells

(A) Compound A-induced death of tumor cells can be blocked by TNFα neutralizing antibody. A total of 10 μg/ml of the respective blocking antibody was added simultaneously with compound A. Cell death was assessed by PI staining and flow cytometry throughout. Experiments were repeated three times with Kym1 cells and OVCAR4 cells, and twice for SKOV3 cells.

(B) Compound A-induced death can be blocked by inducible dominant-negative TNFR2 (dnTNFR2). Cells were induced overnight prior to compound A addition. Duration and concentration of compound A treatment were the same as in (A). Experiments were repeated six times for dnTNFR2 and at least four times for dnCD27 and dnTRAILR2 (dnTrlR2) in each cell line. The inset shows expression of inducible constructs by western blotting. Anti-β-actin demonstrates equivalent protein loading in all lanes.

(C) Compound A-induced death can be blocked by inducible $I\kappa B^{SR}$. Induction conditions, duration, and concentration of drug treatment were the same as in (B). Experiments were repeated six to eight times for Kym1 s, four to six times for SKOV3, and three times for OVCAR4 cells. The inset shows expression of inducible constructs by western blotting. P; parental.

(D) Inducible expression of $I\kappa B^{SR}$ and dnTNFR2 provide long-term clonagenic survival to compound A in Kym1 and SKOV3 cell lines. Cells were treated as in (B) and (C), except that after treatment, compound A was washed off, and cells were replated and left to form colonies over 10 days. The average number of colonies was plotted as bars, and individual experimental values were also plotted. The picture panel shows a selection of Kym1 colony assays from a single experiment. Colonies were stained with crystal violet so that counting was facilitated.

(E) Kym1 cells were treated with compound A, TNFα, or TRAIL (TRL) overnight in the presence or absence of Brefeldin A, and cell death was measured by PI staining and flow cytometry. The average of three independent experiments was plotted. Error bars are SEM throughout.

client proteins that interact with HSP90, it is well established that HSP90 is a critical component of IKK complex function and downstream NF-κB signaling (Chen et al., 2002; Wang et al., 2006; Broemer et al., 2004). Inhibition of HSP90 by Geldanamycin inhibits the formation of IKK complexes and has been shown to inhibit activation of

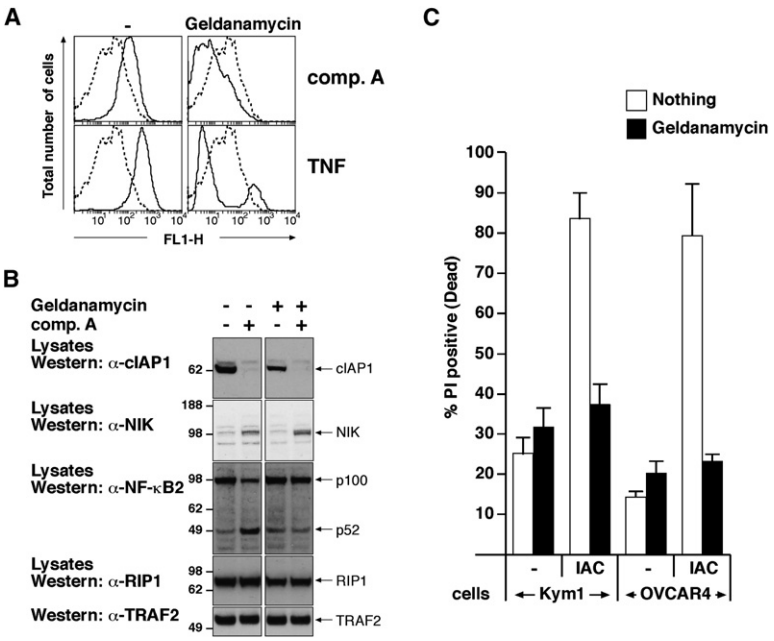

Figure 6. 17-(Allylamino)-17-Demethoxy-Geldanamycin Inhibits Compound A Induced NF-κB Activity and Prevents Compound A Killing

(A) Geldanamycin prevents compound A-induced NF-κB. Wild-type MEFs infected with lentivirus NF-κB EGFP reporter were pretreated with 500 nM of Geldanamycin [17-(Allylamino)-17-demethoxy-geldanamycin] for 2 hr; this was followed by addition of compound A or TNFα for a further 22 hr. NF-κB activation was measured by flow cytometry. Dotted-line histograms represent background fluorescence with no treatment. Histograms shown are representative of four experiments from three separate NF-κB EGFP clones.

(B) Geldanamycin blocks compound A-induced NF-κB2 processing. OVCAR4 cells were pretreated with Geldanamycin for 2 hr; this was followed by compound A stimulation for 5 hr, and cell lysates were analyzed by western blotting. Identical results were observed in Kym1 cells (not shown).

(C) Geldanamycin inhibits compound A-induced death. OVCAR4 and Kym1 cells were pretreated with Geldanamycin for 2 hr; this was followed by addition of compound A for a further 24 hr (Kym1) or 48 hr (OVCAR4). Cell death was assessed by PI staining and flow cytometry. Error bars are SEM of three independent experiments.

NF-κB from TNF-R1 (Chen et al., 2002). Consistent with these previous findings, we observed that IAC-induced activation of NF-κB was also completely blocked by pretreating NF-κB reporter cells with 500 nM of Geldanamycin (Figure 6A). Western-blot analysis of cells pretreated with Geldanamycin before compound A addition demonstrated that Geldanamycin does not block compound A-induced cIAP1 degradation or stabilization of NIK (Figure 6B). However, consistent with the inhibition of NF-κB observed by flow cytometry (Figure 6A), Geldanamycin completely blocked IAC-induced NF-κB2 processing of p100 into p52 (Figure 6B).

We therefore tested whether Geldanamycin could inhibit compound A-induced death. In line with the requirement for NF-κB activation in compound A-induced death, but nonetheless remarkably given the cytotoxic nature of this drug, Geldanamycin conferred complete protection from compound A killing of Kym1 and OVCAR4 cells (Figure 6C). These data further underscore the fact that IAC-induced activation of NF-κB is required for IAC-induced death.

IAP Antagonists Sensitize Tumor Cells to TNF-R1-Induced Death

TNFα alone is unable to cause apoptosis of most cell types, and cell death occurs only if protein translation is simultaneously inhibited. Addition of a translation inhibitor such as cycloheximide is thought to block TNFα-induced expression of prosurvival genes and hence skews TNF-R1 signaling in favor of caspase activation. Indeed, addition of exogenous TNFα does not induce death of SKOV3 and OVCAR4 cells (data not shown), even though they are killed by a TNFα-dependent mechanism after treatment with compound A. We therefore hypothesized that IAP antagonists might increase signaling by the TNF receptors constitutively, as well as in response to ligation by exogenously added ligand. According to this model, compound A allows spontaneous activation of NF-κB by TNF-R1, which in sensitive cell lines drives TNFα production. In addition, compound A also sensitizes cells to TNFα by removing the inhibitory effect of cIAP1 on TNF-R1 signaling.

To test this hypothesis, we examined whether treatment with compound A sensitized cells (both IAP antagonist resistant and sensitive) to exogenous TNFα application. Killing of compound A-sensitive OVCAR4 and SKOV3 cells was further increased by addition of TNFα (Figure 7A). As predicted from our model, cells that are resistant to compound A alone (D645, T98G, and MEFs) and that do not produce TNFα in response to compound A, despite the fact that compound A induced the degradation of cIAP1 (Figure 2C), were nevertheless greatly sensitized to induction of apoptosis by TNFα when they were treated with compound A (Figure 7A). However, compound A did not increase sensitivity of normal primary human HUVEC cells to TNFα (data not shown).

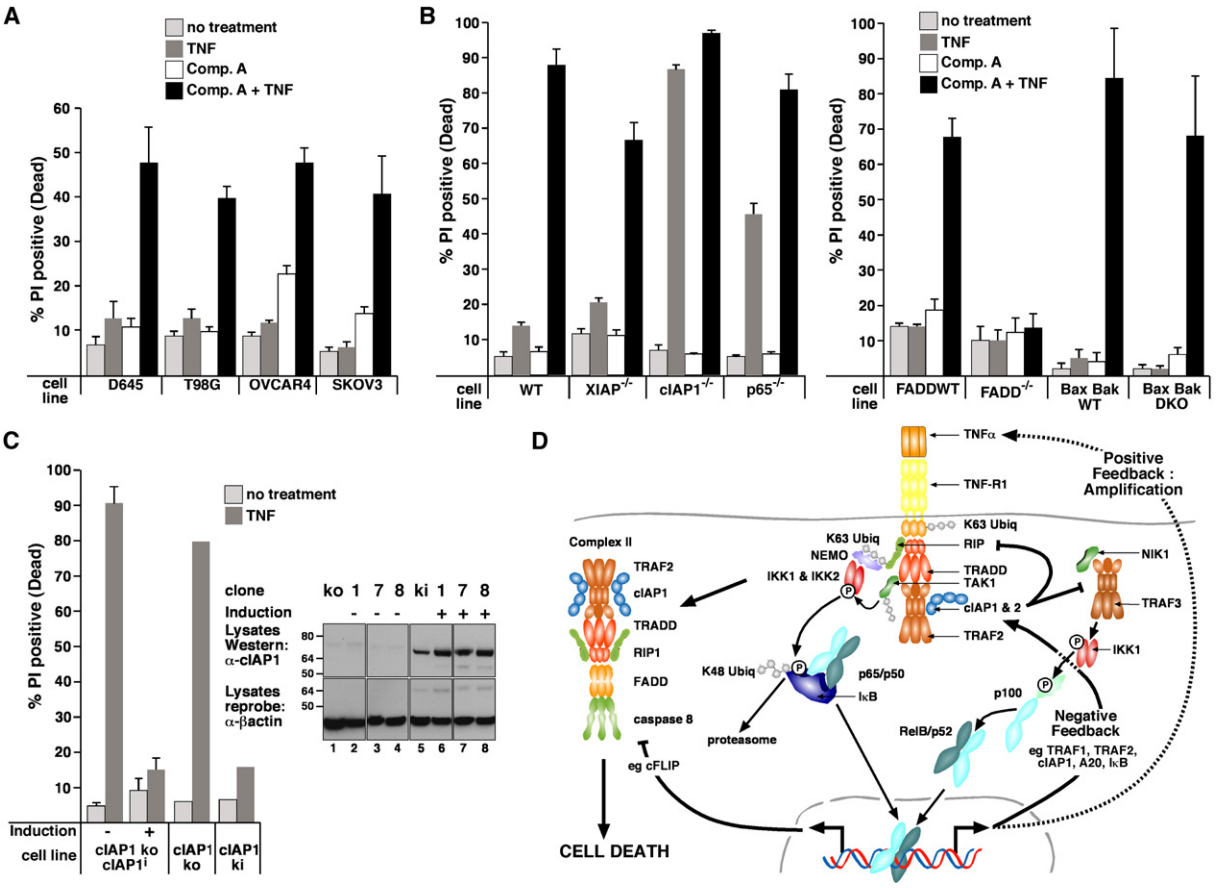

Figure 7. Compound A Targets cIAP1 to Sensitize Cells to TNFα

(A) The indicated tumor cell lines were treated with TNFα for 24 hr in the presence or absence of compound A (500nM), and cell death was measured by PI staining and flow cytometry.

(B) Loss of cIAP1 sensitizes MEFs to TNFα. Transformed knockout embryo MEFs and respective control wild-type MEFs were treated with TNFα for 24 hr in the presence or absence of compound A (500 nM), and cells stained for PI and analyzed by flow cytometry.

(C) Inducible cIAP1 blocks sensitivity of cIAP1 knockout cells to TNFα that is caused by loss of cIAP1. A cIAP1 knockout MEF line immortalized with SV40 large T was infected with an inducible cIAP1 construct. Three independent clones of cIAP1 knockout (ko) MEFs infected with inducible cIAP1 were tested for inducible expression of cIAP1 by western blotting. Lysates from MEFs generated from the knockin (ki) construct are shown to indicate normal levels of cIAP1. The inducible cIAP1 clones were left uninduced or induced for cIAP1 and then treated with TNFα for 24 hr, and cells stained by PI and analyzed by flow cytometry. Error bars are SEM throughout.

(D) TNFα binding to TNF-R1 causes recruitment of RIP and activation of the canonical NF-κB pathway. Loss or inhibition of cIAP1 causes constitutive recruitment of RIP and activation of NF-κB. The noncanonical pathway is also activated by loss or antagonism of cIAP1 function with an increase in levels of NIK1 and processing of p100. In IAC-sensitive cells, this activation of NF-κB causes an increase in levels of TNFα that kills these cells through enhanced apoptotic TNF-R1 signaling.

To confirm that the increased sensitivity to TNFα after compound A treatment was due to inhibition of cIAP1 and not XIAP, we tested the ability of TNFα to kill immortalized gene knockout MEF lines. Consistent with the above findings, immortalized wild-type MEFs were insensitive to TNFα alone but were significantly sensitized to TNFα when combined with compound A (Figure 7B). As expected, NF-κB p65 knockout MEFs could be killed by TNFα alone (Figure 7B). Strikingly, cIAP1 knockout MEFs were exquisitely sensitive to TNFα alone, whereas XIAP knockouts were not, even though the XIAP knockouts, like wild-type MEFs, can be sensitized to TNFα by addition of compound A. Compound A-induced sensitization to TNFα killing was clearly dependent upon caspase 8 activation because FADD knockout MEFs were completely resistant to the combination of TNFα and compound A (Figure 7B). Moreover, the Bax- and Bak-dependent apoptotic pathway was not required for death caused by compound A and TNFα because Bax/Bak double knockout MEFs were efficiently killed by compound A together with TNFα (Figure 7B).

To confirm that the sensitivity of transformed cIAP1 knockout MEFs to TNFα killing was due to the loss of cIAP1 alone, we complemented a knockout line with inducible cIAP1 and analyzed three clones. When cIAP1 was induced in the cIAP1 knockout cells, resistance to

TNFα was completely restored (Figure 7C). These results show that IAP antagonists are able to sensitize cells to TNFα killing by inhibiting cIAP1, and consequent perturbation of TNF-R1 signaling, and are consistent with reports that IAP antagonists increased sensitivity of HeLa cells to TNFα (Li et al., 2004).

DISCUSSION

We have developed a small molecule IAP antagonist that binds specifically to cIAP1, cIAP2, and XIAP. In this study, we show that synthetic and naturally occurring (Smac and Grim) IAP antagonists kill susceptible tumor cells through antagonism of cIAP1, resulting in NF-κB activation, which drives TNFα production and initiates tumor cell death through an enhanced susceptibility to TNF-R1 killing (Figure 7D). We demonstrate that blocking either NF-κB activation or TNFα signaling is sufficient to confer clonagenic survival to IAP-antagonist-compound-induced cell death.

cIAP1 knockout mice appear overtly normal, and primary cIAP1 null cells do not display any abnormalities in NF-κB signaling or sensitivity to TNFα-induced cell death (Conze et al. [2005] and unpublished data). Our work demonstrates that in immortalized cells, however, including human tumor cells and SV40 large T immortalized MEFs, cIAP1 plays a critical role in regulating both NF-κB and cell-death signaling emanating from TNF-R1. It will be important to work out why cIAP1 becomes so critical after immortalization or transformation, but our findings are consistent with observations that cIAP1 amplification is a frequent event in tumors and is required for tumorigenesis in vivo (Zender et al., 2006).

Smac has been shown to promote the degradation of cIAP1 and cIAP2 but not XIAP (Yang and Du, 2004). Somewhat surprisingly, our Smac mimetic, compound A, that mimics only the minimal groove-binding region of Smac, also promotes the rapid degradation of cIAP1 and leaves XIAP relatively untouched. This specificity of compound A for cIAP1 fits well with previous observations that Smac does not promote degradation of XIAP but antagonizes its ubiquitin ligase activity (Silke et al., 2004; Creagh et al., 2004). As expected from previous results with Smac, proteasome inhibitors can block compound A-induced degradation, suggesting a similar mechanism of action.

Antagonism or loss of cIAP1 induced spontaneous NF-κB signaling in both IAC-sensitive and -resistant cell lines. IAC-induced NF-κB signaling was not due to the induction of TNFα because (1) IAC resistant cells do not produce TNFα in response to compound A yet still show significant NF-κB activation and (2) neutralizing TNFα antibodies do not affect compound A-induced NF-κB activation (data not shown).

Loss of cIAP1, induced by IAP antagonist or in cIAP1 knockout MEFs, enhanced and prolonged RIP1 binding to TNF-R1. Similarly, in D645 cells, compound A induced the spontaneous recruitment of RIP1 to TNF-R1. It has been well documented that RIP1 binding to TNF-R1 induces canonical NF-κB through its ability to recruit the NEMO/IKK1/IKK2 complex. The enhanced RIP1 binding to TNF-R1 observed after IAC treatment is therefore consistent with a role for cIAP1 in canonical NF-κB activation, as is the observation that compound A induced the rapid phosphorylation of p65. Surprisingly though, antagonism, or loss, of cIAP1 also resulted in the stabilization of NIK and NF-κB2 p100 processing to p52, both markers of noncanonical NF-κB signaling. The strength of noncanonical NF-κB signaling upon loss of cIAP1 was comparable with the noncanonical NF-κB activity observed in TRAF2 knockout MEFs and B cells (Grech et al., 2004). Given that cIAP1 interacts with TNF receptors through TRAF2 and that TNFSF ligands can activate both canonical and noncanonical NF-κB pathways (Dejardin et al., 2002), these results imply that both components of a cIAP1/TRAF2 complex are required for inhibiting spontaneous NF-κB activation.

We detected compound A-induced TNFα only in compound A-sensitive cell lines and not in compound A-resistant lines. This implies that TNFα production in response to compound A is a prerequisite for tumor cell sensitivity to IAP-antagonist killing. Nevertheless, all cell lines examined so far, including primary or transformed, compound A-sensitive or -resistant lines, show substantial NF-κB activity, after IAP antagonism, that in sensitive cell lines is required for TNFα production. It is possible that the chromatin structure of the TNFα promoter in different cell lines influences the IAP-antagonist-induced production of TNFα, and it is noteworthy that the sensitive human tumor cells examined in this study already produce detectable levels of TNFα before addition of compound A. Although IAP antagonism might influence other signaling pathways apart from NF-κB, the fact that inhibition of NF-κB signaling blocked TNFα production and promoted tumor cell survival in response to compound A treatment strongly suggests that NF-κB signaling is the major pathway by which IAP antagonists kill tumor cells.

Although IAP antagonism does not induce apoptosis in all tumor cell types as a single agent, in all the cell lines we examined, compound A sensitized cells to TNFα-induced cell death. This suggests that in tumor cell lines, cIAP1 critically regulates TNF-R1 prosurvival and apoptotic signaling. It is possible that NF-κB activation that is the result of IAP antagonism or deletion of cIAP1 influences the nature of NF-κB signaling emanating from TNF-R1 and thereby sensitizes them to TNFα killing. Precedence for this proposal exists in a number of knockout cells, such as TRAF2 and TRAF3, that regulate activation of NF-κB from TNF receptors. In TRAF2 knockouts, signaling from TNF receptors changes from canonical to noncanonical and similarly in TRAF3 knockouts in which lack of inhibition of noncanonical NF-κB results in p100-dependent lethality in mice (Grech et al., 2004; He et al., 2006).

Amplification and enhanced levels of cIAP1 have been observed in several cancer types (Zender et al., 2006), and recent work has demonstrated that together with

other oncogenic events, amplification of the cIAP1 gene locus is required to both initiate and enhance liver cancer growth in a mouse model (Zender et al., 2006). Tumors might therefore develop that rely on cIAP1 to inhibit TNFα apoptosis signaling. By targeting cIAP1 with IAP-antagonist drugs, physiological TNF-receptor apoptotic signaling might be greatly augmented, resulting in enhanced tumor cell death.

EXPERIMENTAL PROCEDURES

Analysis of IAP-Antagonist Binding to XIAP BIR3 Domain

Binding constants (K_d) were measured with fluorescence polarization and the method of Nikolovska-Coleska et al. (2004) and were described in detail in the Supplemental Data.

Cell Culture, Transfections, and Infections

All cell lines were maintained at 37°C, 10% CO_2 in DMEM supplemented with 10% FBS and passaged twice weekly. Effectene (QIAGEN) was used for transient transfections according to the manufacturer's protocol. Flp In T-Rex 293 cells (Invitrogen) containing doxycycline-inducible HA ubiquitin Smac or HA ubiquitin Grim were generated according to the manufacturer's instructions.

To generate lentiviral particles, we transfected 293T cells with packaging constructs pCMV ðR8.2 and VSVg and the relevant lentiviral plasmid. The virus containing supernatants were harvested, filtered, and supplemented with Polybrene (12 μg/ml), and target cells were infected with virus supernatant. Successful infection was selected for with Puromycin (2–5 μg/ml) or Hygromycin B (100–500 μg/ml) or by screening for GFP flourescence. pF 5× UAS-inducible constructs were induced with 100 nM 4-hydroxy tamoxifen.

Constructs

pTRH1 NF-κB EGFP was purchased from System Biosciences (TR503PA-1). Cre-recombinase and SV40 large T antigen were cloned into the lentiviral vector pFU. Grim and Smac were cloned into pcDNA5 FRT TO HA ubiquitin construct (Silke et al., 2004). The inducible transcriptional activator Gal4ER^{T2}VP16 (GEV16) was cloned into pFU PGKHygro, and the genes dnTNF-R2, dnTRAIL-R2, or dnCD27 (Bossen et al., 2006), IκB^{SR} (Van Antwerp et al., 1996), HA ubiquitin Grim, HA ubiquitin Smac, and N-Flag crmA were cloned into pF 5× UAS SV40Puro. The complete sequence of all constructs can be obtained upon request.

Generation of MEFs

Wild-type and knockout MEFs were generated from E15 embryos in accordance with standard procedures and were infected with SV40 large T antigen expressing lentivirus. cIAP1 and TRAF2 conditional knockin MEFs were similarly generated from cIAP1 and TRAF2 LoxP/LoxP E15 embryos. To delete the gene, we infected the transformed MEFs with a cre-expressing lentivirus (pFU cre SV40Puro), and deletion was confirmed by PCR and western blotting. To generate NF-kB reporter cIAP1 MEFs, we first infected conditional knockout MEFs with pTRH1 NF-κB EGFP lentivirus, and a monoclonal cell line with low levels of basal EGFP was selected. This line was then infected with cre-expressing lentivirus, and several independent clones were selected.

Death Assays

Cells were seeded at ?40% confluency and were allowed to adhere for 12–16 hr. Compound A (5 nM Kym1 cells or 500 nM all other cell types), human Fc-TNFα (Bossen et al., 2006) (70 ng/ml), or human TRAIL ligand (50 ng/ml) were added to cells for 24 hr (Kym-1) or 48 hr (other cells), and cell death measured by propidium iodide (PI) staining and flow cytometry.

Western Blotting and Immunoprecipitations

Standard procedures for western blotting and imunoprecipitation were followed, and exact details are provided in the Supplemental Experimental Procedures. The primary antibodies used for western blotting are described completely with catalog numbers in Supplemental Data.

ELISA Assays

Cells were grown on 10 cm plates, harvested, washed, and lysed in 300 μl DISC buffer for 20 min on ice. A cell lysate was spun at 14,000 g, and the soluble material was collected. Soluble cell lysate was used for human or mouse TNF-α ELISA assays (R&D Systems, human, cat. HSTA00C, mouse, cat. #MTA00) according to the manufacturer's protocol. Protein from the cell lysate was quantified with the BCA assay (Pierce).

Colony Assays

Cells inducible for crmA, IκB^{SR}, dnTNF-R2, or dnCD27 were plated at equal densities on six-well plates, induced overnight, and treated with or without compound A for 24 (Kym1) or 48 hr (SKOV3). Alternatively, cells were treated with blocking TNFα antibody and with or without compound A. After treatment, cells were trypsinized and replated. Cells were grown for 10 days and fixed, and colonies were stained with crystal violet and counted.

Supplemental Data

Supplemental Data include five figures and are available online at http://www.cell.com/cgi/content/full/131/4/682/DC1/.

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