X Chromosome-linked Inhibitor of Apoptosis Regulates Cell Death Induction by Proapoptotic Receptor Agonists

Received for publication, July 2, 2009, and in revised form, September 25, 2009. Published, JBC Papers in Press, October 23, 2009, DOI 10.1074/jbc.M109.040139

Eugene Varfolomeev1, Bruno Alcicke1, J. Michael Elliott4, Kerry Zobel6, Kristina West3, Harvey Wong3, Justin M. Scheer4, Avi Ashkenazi**, Stephen E. Gould5, Wayne J. Fairbrother1, and Domagoj Vucic1,2

From the Departments of 1Protein Engineering, 4Translational Oncology, 5Protein Chemistry, 1Drug Metabolism and Pharmacokinetics, and 2**Molecular Oncology, Genentech, Inc., South San Francisco, California 94080

Proapoptotic receptor agonists cause cellular demise through the activation of the extrinsic and intrinsic apoptotic pathways. Inhibitor of apoptosis (IAP) proteins block apoptosis induced by diverse stimuli. Here, we demonstrate that IAP antagonists in combination with Fas ligand (FasL) or the death receptor 5 (DR5) agonist antibody synergistically stimulate death in cancer cells and inhibit tumor growth. Single-agent activity of IAP antagonists relies on tumor necrosis factor-α signaling. By contrast, blockade of tumor necrosis factor-α does not affect the synergistic activity of IAP antagonists with FasL or DR5 agonist antibody. In most cancer cells, proapoptotic receptor agonist-induced cell death depends on amplifying the apoptotic signal via caspase-8-mediated activation of Bid and subsequent activation of the caspase-9-dependent mitochondrial apoptotic pathway. In the investigated cancer cell lines, induction of apoptosis by FasL or DR5 agonist antibody can be inhibited by knockdown of Bid. However, knockdown of X chromosome-linked IAP (XIAP) or antagonism of XIAP allows FasL or DR5 agonist antibody to induce activation of effector caspases efficiently without the need for mitochondrial amplification of the apoptotic signal and thus rescues the effect of Bid knockdown in these cells.

Apoptosis, or programmed cell death, is a genetically regulated process with critical roles in development and homeostasis in metazoans (1). Deficient apoptosis leads to the absence of normal cell death and contributes to the development and progression of human cancers (2). Apoptotic cell death can be initiated through the engagement of cell surface proapoptotic receptors by their specific ligands or by changes in internal cellular integrity (3, 4). Both of these pathways converge at the activation of caspases, cysteine-dependent aspartyl-specific proteases that comprise the effector arm of apoptotic cell death (5, 6). The intrinsic or mitochondrial pathway is initiated by developmental cues or cellular stress signals. These signals activate Bcl-2 homology 3 (BH3) proteins, leading to neutralization of the antiapoptotic proteins, such as Bcl-2, Bcl-xL, or Mcl-1, activation of proapoptotic proteins Bax and Bak, and subsequent disruption of mitochondrial membrane potential (7). The resulting release of cytochrome c from the mitochondria into the cytoplasm leads to Apaf-1-mediated caspase-9 activation and consequent activation of effector caspases-3 and -7 and culminates in cell death.

The extrinsic apoptotic pathway is triggered when proapoptotic receptors such as Fas or death receptor 5 (DR5) are engaged by their respective ligands, resulting in recruitment of the adaptor protein FADD and the apical caspases 8- or 10 (3). Incorporation of these caspases into the receptor-associated death-inducing signaling complex causes their autoactivation and leads to ensuing activation of effector caspases-3 and -7. In most cell types (type II cells), amplification of extrinsic pathway signaling through caspase-8-mediated activation of the BH3-only protein Bid is critical for efficient execution of apoptosis (8, 9); in type I cells direct activation of effector caspases by caspase-8 is sufficient. Bid plays an important role in a number of cellular pathways including regulation of Fas- and TNFR1-mediated hepatocellular injury (9–13). In addition to stimulation by their respective ligands, proapoptotic receptors can be engaged by agonistic antibodies (14). DR5 agonist antibody (PRO95780) binds DR5 tightly and selectively, triggering apoptosis in various types of cancer cells and inhibiting tumor xenograft growth in vivo (15, 16).

IAP proteins represent the ultimate line of defense against cellular suicide by regulating caspase activity and preventing caspase activation (17). c-IAP1 and c-IAP2 are components of TNF receptor (TNFR) complexes where they modulate apoptotic signaling and caspase-8 activation (18–20). X chromosome-linked IAP (XIAP) is the only true endogenous inhibitor of caspases because other IAP proteins exhibit weak binding to and inhibition of caspases (21). XIAP inhibits weak caspases-3 and -7 using the linker region between its baculoviral IAP-repeat (BIR) domain 1 (BIR1) and BIR2 as well as the BIR2 domain, whereas inhibition of caspase-9 relies on the binding of the BIR3 domain to an N-terminal

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 51–58.

1 To whom correspondence may be addressed: Dept. of Protein Engineering, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080. Tel.: 650-225-6372; Fax: 650-225-6127; E-mail: fairbro@gene.com.
2 To whom correspondence may be addressed: Dept. of Protein Engineering, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080. Tel.: 650-225-8839; Fax: 650-225-6127; E-mail: domagoj@gene.com.

3 The abbreviations used are: BH3, Bcl-2 homology 3; BIR, baculoviral IAP-repeat; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; DR5, death receptor 5; FasL, Fas ligand; IAP, inhibitor of apoptosis; XIAP, X chromosome-linked IAP; PARA, proapoptotic receptor agonist; siRNA, small interfering RNA; SMAC, second mitochondrial activator of caspases; TNF, tumor necrosis factor; TNFR, TNF receptor.
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IAP-binding motif of partially processed caspase-9 (21, 22). Caspase inhibition by XIAP is blocked by second mitochondrial activator of caspases (SMAC) (23, 24). During induction of apoptosis, SMAC undergoes proteolytic processing, resulting in its release from mitochondria into the cytoplasm where it can bind to and antagonize the BIR2 and BIR3 domains of XIAP via an exposed IAP-binding motif (23, 24). IAP-mediated inhibition of cell death and promotion of survival signaling pathways are important for tumor maintenance and therapeutic resistance to anticancer agents. These properties distinguish IAP proteins as attractive targets for anticancer therapeutic intervention (25). Efforts to identify small molecule antagonists of IAPs have led to the discovery of a number of IAP antagonistic compounds that possess antiapoptotic activity both in vitro and in vivo (26, 27). These IAP antagonists induce cell death that depends on TNF signaling, stimulation of NF-κB pathways, and caspase activation (28–31).

Here, we demonstrate that combination of IAP antagonists and proapoptotic receptor agonists (PARAs), namely Fas ligand (FasL) or DR5 agonist antibody, synergistically activates apoptosis in cancer cells and inhibits tumor growth in vivo. Contrary to its essential role in IAP antagonist-induced cell death, TNFα is not required for synergistic activity of PARAs and IAP antagonists. In type II cells, FasL- or DR5 agonist antibody-induced apoptosis is efficiently inhibited by down-regulation of Bid. However, down-regulation of XIAP or antagonism of XIAP by small molecules rescues the effect of Bid knockdown in these cells by allowing activation of effector caspases via a type I-like mechanism, thus enabling efficient PARA-induced apoptosis. These data provide a mechanistic foundation for investigating combinations of IAP antagonists and proapoptotic receptor agonists as a novel cancer therapeutic approach.

EXPERIMENTAL PROCEDURES

Cell Lines, Reagents, Western Blot Analyses, and Transfections—UACC62 human melanoma cells, HT1080 human fibrosarcoma cells, and SW620 and HT29 human colorectal adenocarcinoma cells were obtained from ATCC. MDA-MB-231-X1.1 (GFP-expressing variant of MDA-MB-231 cells) human breast carcinoma cells were established in Genentech. All cell lines were grown in 50:50 Dulbecco’s modified Eagle’s and FK12 medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Human recombinant soluble TNFα, FasL, DR5 agonist antibody (PRO95780), and TNFR1-Fc were from Genentech, as was BV6 (15). The primary antibodies against c-IAP1 were purchased from R&D (affinity-purified goat antibody). Anti-Bid antibodies were purchased from BD. Anti-caspase-3 and anti-caspase-9 antibodies were from Cell Signaling Technology. Anti-XIAP antibody was from Sigma. Anti-tubulin antibody was from ICN Biomedicals. Western blot analyses were performed as described previously (30, 32). Bid-specific (pairs 6 and 7), c-IAP1-specific (pairs 12 and 13), and XIAP-specific (pairs 14, 15, 16, 17) ON-TARGET plus siRNA oligonucleotides were purchased from Dharmacon. Sequences of c-IAP2-specific siRNA oligonucleotides were designed by using the Dharmacon siDESIGN Center (Dharmacon Research, Inc., Lafayette, CO) software and synthesized at Genentech. The following siRNA pairs were used for c-IAP2 gene knockdown experiments: 5’TCTAACAACAA-GATCATTTGAtt-3’ and 5’TCAATGATCTTGTGTTAGAtt-3’; 5’ATTCGCTACAGTCCATGTrt-3’ and 5’CATGTGAAC-TGTTACGAAATtt-3’. Cells were transfected with siRNA duplexes as described previously (30, 32) using siRNA MAX reagent (Invitrogen).

Viability Assays—Cells (1–1.5 × 10⁴/well) were seeded into 96-well dishes. 8–12 h later, the media were changed, and cells were treated as indicated in the figure legends. Cell viability was measured by neutral red uptake as described previously (33). DR5 agonist antibody (PRO95780) was used in combination with anti-Fc antibody at 1:1 ratio as described previously (15). Statistical significance was determined using Student’s t test.

Tumor Xenograft Study—All procedures involving animals were performed in accordance with the guidelines of the Genentech Institutional Animal Care and Use Committee.
generate tumors, 100 μl of a single-cell suspension containing 5 × 10^6 MDA-MB-231-X1.1 cells was injected subcutaneously into the right thoracic flanks of female C.B-17 SCID.bg mice (Charles River Laboratories, Hollister, CA) aged 9–19 weeks. Tumor volume was calculated using the mean diameter measured with vernier calipers using the formula $v = \frac{1}{2}ab^2$, where $a$ and $b$ are the largest and smallest perpendicular tumor diameters, respectively. Treatment was initiated once tumors reached a size of 100–200 mm^3. DR5 agonist antibody (3 mg/kg) or vehicle (15% hydroxypropyl-β-cyclodextrin and 20 mM succinic acid) was administered intravenously every 4 days for a total of six treatments (q4d × 6) starting on day 1. Both agents were delivered in a volume of 100 μl to weight-matched animals. Tumor volumes and body weights were measured twice weekly until the end of the study.

Caspase Activity Assay—Recombinant caspase-3, caspase-7, and XIAP BIR2-BIR3 were cloned, expressed, and purified as described previously (30, 34, 35). XIAP BIR2-BIR3 titrations were performed on 300 pm caspase-3 and caspase-7 to determine IC_{50} and the range of inhibition. XIAP BIR2-BIR3 was used at 10 nM, which resulted in ~95% inhibition for both caspase-3 and caspase-7. 10-fold excess concentrations of BV6 and BV6 were made in 100% dimethyl sulfoxide prior to addition in the assay. If the BV compounds were not used, dimethyl sulfoxide was added in their place. The assay buffer used for all other components, and dilutions consisted of 50 mM Hepes (pH 7.5), 50 mM KCl, 0.1% CHAPS, and 10 mM dithiothreitol. The XIAP BIR2-BIR3, BV compounds, and caspase were added to a 96-well plate (Corning 3686) and allowed to sit at room temperature for 1 h. The substrate ac-DEVD-AFC was added with a final concentration of 25 μM, and the plate was kinetically read at 37 °C for 30 min in a Tecan Safire II with an excitation of 365 nm and an emission of 495 nm. The data were normalized by assigning 100% activity to the controls with 300 pM enzyme alone and 0% activity to the controls with 300 pM caspases and 10 nM XIAP BIR2-BIR3 but no IAP antagonists. Caspase-3/7, caspase-8, and caspase-9 activity assays in cultured cells were performed according to manufacturer’s instructions (Promega) as described previously (32).

RESULTS

IAP Antagonists and Proapoptotic Receptor Agonists Synergistically Activate Apoptosis in Cancer Cells and Inhibit Tumor Growth in Vivo—IAP antagonists have been shown to stimulate apoptosis alone or in combination with proapoptotic TNF family ligands (26, 27). To elucidate the mechanism of combined
apoptotic activity of IAP antagonists and proapoptotic receptor agonists, we examined the activity of the IAP antagonist BV6 in combination with FasL or the DR5 agonist antibody, in cultured cancer cells and in a tumor xenograft model. Treatment of several cancer cell lines, with the exception of A2058 cells, with increasing amounts of BV6 did not cause a decrease in cell viability (Fig. 1, upper panels, and supplemental Fig. S1). All of the examined cell lines were found to be relatively resistant to DR5 agonist antibody treatment as well (Fig. 1, upper panels, and supplemental Fig. S1). However, cotreatment with BV6 and DR5 agonist antibody resulted in a marked reduction in cellular viability (Fig. 1, upper panels, and supplemental Fig. S1). Although not as striking as with DR5 agonist antibody, BV6 demonstrated synergy with FasL in stimulating apoptosis in cells treated with the combination (Fig. 1, lower panels). Apoptotic signaling by PARAs is initiated by the activation of apical caspase-8 through autoproteolytic processing (3, 36). Activated caspase-8 cleaves and activates downstream effector caspases-3 and -7 and can trigger activation of the mitochondrial pathway, via processing of Bid, leading to the activation and processing of another apical caspase, caspase-9. To investigate caspase activation in IAP antagonist- and DR5 agonist antibody- or FasL-treated cells, we examined protein levels of full-length and processed forms of caspases. Although we could not detect caspase activation by BV6 alone, both DR5 agonist antibody and FasL stimulated caspase processing in treated cells (Fig. 2, A and B). Although combination of DR5 agonist antibody or FasL with BV6 did not alter the extent of caspase-8 activation, processing of caspase-9 and caspase-3 triggered by proapoptotic receptor agonists was enhanced by BV6 addition (Fig. 2, A and B). The caspase activation was quantified using enzymatic activity measurements. In agreement with Western blot analyses, addition of BV6 did not significantly affect DR5 agonist antibody- or FasL-stimulated activation of caspase-8 (Fig. 2C). However, combination of IAP antagonist BV6 and FasL or DR5 agonist antibody prominently enhanced PARA-induced activation of caspase-9 and caspases-3/7 (Fig. 2, D and E). These data demonstrate the benefit of combination of BV6 and DR5 agonist antibody or FasL for the activation of caspases-3/7 and caspase-9, but not for caspase-8, suggesting that IAP antagonist-mediated enhancement of FasL- and DR5 agonist antibody-induced caspase activation occurs following initial caspase-8 activation.

Next, we examined the antitumor activity of the IAP antagonist BV6 and DR5 agonist antibody in a mouse xenograft model. BV6 and DR5 agonist antibody synergistically induced apoptosis and caspase-3 activation in MDA-MB-231-X1.1 cells (Fig. 3A and supplemental Fig. S2). To determine the effect of BV6 and DR5 agonist antibody on inhibition of tumor growth, mice bearing established MDA-MB-231-X1.1 tumors were treated with DR5 agonist antibody or BV6 alone or with a combination of the two agents (Fig. 3B). Both BV6 and DR5 agonist antibody caused significant inhibition of tumor growth (Fig. 3B). Nevertheless, combination of both agents resulted in almost complete inhibition of tumor growth (Fig. 3B). Notably, none of the regimens used in this study caused adverse effects in the animals as reflected by minimal body weight changes (supplemental Fig. S3). These data demonstrate a strong synergistic combination of IAP antagonists and PARAs that results in efficient promotion of apoptosis in cultured cancer cells and inhibition of tumor growth in vivo.

Proapoptotic Activity of IAP Antagonists and DR5 Agonist Antibody or FasL Does Not Depend on TNFα—Cell death induction by IAP antagonists depends on TNFα-mediated signaling and de novo protein synthesis (28–31). If the observed synergistic proapoptotic activity of IAP antagonists and FasL or DR5 agonist antibody also depends on TNFα-mediated signaling, then cotreatment with TNFR1-Fc should enhance the survival of cells exposed to the combination of these agents. However, addition of TNFR1-Fc did not affect the viability of cells treated with the combination of IAP antagonist and FasL or DR5 agonist antibody (Fig. 4A and supplemental Fig. S4A). In an analogous fashion, cotreatment with the protein synthesis inhibitor, cycloheximide, did not protect the cells from this apoptotic insult (Fig. 4B and supplemental Fig. S4B). At the same time, and in agreement with earlier reports, TNFR1-Fc fusion protein afforded protection from BV6-plus-TNFα-in-
duced cell death (supplemental Fig. S4c). Together, these results demonstrate that the synergistic proapoptotic activity of IAP antagonists and FasL or DR5 agonist antibody does not require TNFα-mediated signaling or de novo protein synthesis.

Neutralization of XIAP Bypasses the Requirement for Amplification of Proapoptotic Receptor Agonist-induced Apoptotic Signaling—We have demonstrated that combination of BV6 and DR5 agonist antibody or FasL enhances caspase-3 activation without a need for TNFα-mediated signaling (Figs. 1 and 4). XIAP is a prominent inhibitor of caspases-3 and -7, and BV6 abrogates XIAP-mediated inhibition of caspase-3 and -7 in a concentration-dependent manner (Fig. 5, A and B, and supplemental Fig. S5). At the same time, the enantiomer of BV6 (which does not bind to XIAP), BVE6 (29), did not promote any significant caspase activation, thus confirming the specificity of BV6 activity (Fig. 5, A and B). These findings prompted us to investigate the role of XIAP in DR5 agonist antibody- and FasL-induced apoptosis. Down-regulation of XIAP expression synergizes with PARAs to induce efficient apoptosis in otherwise resistant cancer cells.

Disruption of XIAP antagonism rescues the effect of Bid knockdown in these cells and allows efficient PARA-induced effector caspase activation by effectively bypassing the requirement for mitochondrial amplification of the apoptotic signal.

**DISCUSSION**

Efforts to translate fundamental understanding of the apoptotic pathways into novel cancer therapeutics have yielded several promising approaches that are currently undergoing evaluation. IAP proteins are expressed at elevated levels in many tumor types (37). In addition, IAP-mediated inhibition of caspase-8-mediated cleavage of the BH3-only Bcl-2 family member Bid (8). Indeed, treatment of cells with DR5 agonist antibody or FasL induced caspase-8 activation and concomitant processing of Bid (supplemental Fig. 7a). At the same time, down-regulation of Bid with Bid-specific siRNA oligonucleotides afforded significant protection from DR5 agonist antibody- or FasL-induced cell death (Fig. 6 and supplemental Fig. 7b). Having established the critical role of Bid in DR5 agonist antibody- and FasL-stimulated apoptosis, we next explored the importance of XIAP in these apoptotic signaling pathways. siRNA-mediated down-regulation of XIAP enabled efficient apoptosis following treatment with DR5 agonist antibody or FasL in the presence or absence of Bid-specific siRNA oligonucleotides (Fig. 7 and supplemental Fig. 7c). In a similar fashion, IAP antagonist BV6 also synergized with DR5 agonist antibody or FasL in promoting efficient cell death irrespective of Bid knockdown (Fig. 7 and supplemental Fig. 7c).
of cell death and promotion of survival signaling pathways distinguish IAP proteins as attractive targets for anticancer therapeutic intervention (26). Another promising approach is based on engagement of the proapoptotic receptors DR4 and/or DR5 (38). DR4 and DR5 appear to be expressed widely in tumor tissues, and the DR5 agonist antibody has demonstrated antitumor activity in vitro and in vivo and is currently undergoing clinical investigation (15, 16, 38). Combination of these two anticancer strategies has shown marked proapoptotic activity, thus validating this potential therapeutic approach (39, 40). However, questions remain regarding the mechanism of the synergistic interaction observed between IAP antagonists and PARAs. In the present study, we have identified critical mechanistic aspects of...
this tumor growth-inhibiting combination that contribute to our understanding of proapoptotic receptor signaling and its regulation by IAP proteins.

IAP antagonists induce caspase-8 activation and cell death in a subset of cancer cell lines in a TNFα-dependent fashion (28–31). This process also requires de novo production of TNFα. The present study establishes that combination of IAP antagonists and PARAs synergistically cause apoptosis independently of TNFα-mediated signaling or protein synthesis. In addition, we found that IAP antagonists do not enhance caspase-8 activation by FasL or DR5 agonist antibody; rather, they enhance caspase-3 and caspase-9 activation by these PARAs. Therefore, the observed synergistic activity probably involves reversal of XIAP-mediated inhibition of the caspases-3, -7, and -9. As a result, down-regulation of XIAP protein expression greatly enhances FasL- and DR5 agonist antibody-induced apoptosis.

Fas and other proapoptotic TNFR family members are important in regulating development and homeostasis and have critical roles in immune responses (8). These receptors promote apoptosis by activating caspase-8 through the assembly of the death-inducing signaling complex. In some cells (type I cells), this apoptotic signal suffices for efficient activation of the effector caspases-3 and -7, without the need for XIAP antagonism, resulting in cell death. However, in most cell lines (type II cells), this signal needs to be amplified to accomplish robust apoptosis. This amplification requires caspase-8-dependent cleavage of Bid, which induces the release of cytochrome c and endogenous XIAP antagonists, such as SMAC, from the mitochondria. Our study identifies a requirement for XIAP antagonism as a key component of death receptor-stimulated apoptotic signaling. How XIAP influences the apoptosis mechanism between different cell types is not entirely clear, although its expression levels may be one of several determining factors. The levels of XIAP that will effectively block caspase-8-mediated effector caspase activation potentially also depend on the degree of caspase-8 activation as well as the levels of caspase-3. Thus, the interplay between caspase activation and the ability of XIAP to inhibit caspase activity probably differs between different cell types. Neutralization of XIAP through down-regulation of its expression or by small molecule IAP antagonists effectively bypasses the need for mitochondrial amplification of the apoptotic signal by enabling prominent activation of the effector caspases, resulting in efficient death in cancer cell...
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lines that are otherwise resistant to FasL- or DR5 agonist antibody-induced apoptosis.

In summary, the study reported here provides a mechanistic explanation for the observed synergistic combination of IAP antagonists and FasL or DR5 agonist antibody and confirms that XIAP-mediated inhibition of the effector caspases is an important determinant of proapoptotic receptor-mediated signaling.

Acknowledgments—We thank Kurt Deshayes, John Flygare, Scott Marsters, Kim Newton, and the Oligonucleotide Synthesis facility for providing help with insightful discussions, suggestions, and reagents.

Addendum—While this article was under review, Jost et al. (41) published a study on the role of XIAP in apoptotic signaling. Jost et al. (41) used XIAP- and Bid-knock-out mice to demonstrate that XIAP is a critical discriminator between type I and type II Fas-induced apoptotic signaling. Our study complements the Jost et al. report by providing evidence for the importance of XIAP in FasL- and DR5 agonist antibody-stimulated apoptosis in cancer cells and by demonstrating that TNFα-mediated signaling does not play a significant role in these apoptotic events.

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