XIAP is member of the IAP family of anti-apoptotic proteins and is known for its ability to bind and suppress caspase family cell death proteases. A phenylurea series of chemical inhibitors of XIAP was recently generated by our laboratories (Schimmer, A. D., Welsh, K., Pinilla, C., Bonneau, M., Wang, Z., Pedersen, I. M., Scott, F. L., Glinsky, G. V., Scudiero, D. A., Sausville, E., Salvesen, G., Nefzi, A., Ostresh, J. M., Houghten, R. A., and Reed, J. C. (2004) Cancer Cell 5, 25–35). We examined the mechanisms of action of these chemical compounds using biochemical, molecular biological, and genetic methods. Active phenylurea-based compounds dissociated effector protease caspase-3 but not initiator protease caspase-9 from XIAP in vitro and restored caspase-3 enzymatic activity. When applied to tumor cell lines in culture, active phenylurea-based compounds induced apoptosis in a rapid, concentration-dependent manner, associated with activation of cellular caspases. Apoptosis induced by active phenylurea-based compounds was blocked by chemical inhibitors of caspases, with inhibitors of downstream effector caspases displaying more effective suppression than inhibitors of upstream initiator caspases. Phenylurea-based XIAP antagonists induced apoptosis (defined by annexin V staining) prior to mitochondrial membrane depolarization, in contrast to cytotoxic anticancer drugs. Consistent with these findings, apoptosis induced by phenylurea-based compounds was not altered by genetic alterations in the expression of Bcl-2 family proteins that control mitochondria-dependent cell death pathways, including overexpression of anti-apoptotic proteins Bcl-2 or Bcl-X, and genetic ablation of pro-apoptotic proteins Bax and Bak. Conversely, conditional overexpression of an active fragment of XIAP or genetic ablation of XIAP expression altered the apoptosis dose-response of the compounds. Altogether, these findings indicate that phenylurea-based XIAP antagonists block interaction of downstream effector caspases with XIAP, thus inducing apoptosis of tumor cell lines through a caspase-dependent, Bcl-2/Bax-independent mechanism.

Defects in apoptosis play important roles in cancer pathogenesis and progression and have been linked to chemoresistance, radioresistance, and hormonal therapy failures (1, 2). A need exists therefore to reduce the roadblocks to apoptosis in advanced cancers, placing tumor cells into a more vulnerable state and making them easier to eradicate.

Caspases are the principal mediators of apoptosis (3, 4). These intracellular proteases become activated in response to various cell death stimuli, triggering a characteristic set of biochemical reactions responsible for cellular demise in the context of normal cell turnover and immune-based elimination of virus-infected and malignant cells. Caspases cleave and activate each other in proteolytic networks, characterized by upstream initiator proteases (e.g. caspases 2, 8, 9, and 10) and downstream effector proteases (e.g. caspases 3, 6, and 7) (5). At least four major pathways for apoptosis have been delineated (6–11), including pathways activated by: (a) TNF/Fas family cytokine receptors, which recruit certain caspases to ligand-activated receptor complexes; (b) granzyme B, a protease injected into target cells by cytolytic T-cells and NK cells, which directly cleaves and activates caspases; (c) mitochondria, which release caspase-activating proteins into the cytosol in response to various stimuli; and (d) endoplasmic reticulum, where organelar stress links to caspase activation through various mechanisms (12, 13). These pathways for apoptosis are kept in check by endogenous antagonists that operate at specific points in caspase signaling cascades to interrupt the cell death program. Unfortunately, tumor cells commonly over-express apoptosis-suppressing proteins, thus avoiding elimination by either natural immune defenses or iatrogenic means (hormonal therapy, chemotherapy).

Certain caspases are inhibited by IAP family proteins (14). Several members of the IAP family have been shown to bind and directly suppress the activity of selected caspases, thereby suppressing apoptosis (1, 15–28). Over-expression of IAPs has been documented in several types of human cancers (1, 20, 27, 29–38), and proof-of-concept data using antisense and peptide
inhibitors suggest an important role for these proteins in maintaining tumor survival and promoting resistance to apoptosis induction by anticancer drugs (39–43).

We recently described small molecule chemical inhibitors of the IAP family member XIAP, showing that these compounds induce apoptosis of cancer cell lines and primary leukemia specimens in culture and display anti-tumor activity in mouse xenograft models (44). Here, we have extended these studies, exploring the mechanisms of action of these chemical inhibitors and addressing questions about the apoptosis pathways that they activate in tumor cells and the roadblocks to apoptosis that these compounds bypass or overcome. Altogether, the results show that these IAP-binding compounds activate a downstream point in apoptosis pathways, releasing effector caspases to induce apoptosis and thus functioning distal to the actions of anti-apoptotic proteins such as Bcl-2 and Bcl-XL, which are commonly over-expressed in chemoresistant cancers.

MATERIALS AND METHODS

Chemical Compounds—XIAP antagonist polyphenylurea compounds were identified by screening mixture-based combinatorial chemical libraries for compounds that reversed XIAP-mediated repression of caspase-3 in a high-throughput enzyme derepression assay in vitro (44). Individual compounds were synthesized by solid-phase methods, purified by high pressure liquid chromatography (HPLC), and analyzed by mass spectrometry to confirm identity and >90% purity. Data for prototype active XIAP antagonists are based on more than one independent preparation of purified compound. All compounds were dissolved in dimethyl sulfoxide (Me2SO), and stored at 20 °C. Compounds were subsequently diluted in RPMI 1640 medium so that the final concentration of Me2SO was in all cases 0.05% (v/v).

Cell Culture—Jurkat, MDA-MB-468, and HeLa cells were maintained in either RPMI 1640 or Dulbecco’s modified Eagle’s medium with 10% serum, 1 mM l-glutamine, and antibiotics. 697-neo and 697-Bcl-2 stable transfectants have been described (45). Mouse fibroblasts established from bax+/−bak−+/− or xiap+/− embryos by transformation with SV40 large T antigen have been described (46, 47). HeLa Tet-on cells that stably express reverse tetracycline transactivator (rtTA) regulator protein or HeLa Tet-off cells that stably express tetracycline-controlled transactivator (tTA) were obtained from Clontech, Inc.

Plasmid Construction and Cell Transfections—The tetracycline-inducible or -repressible plasmids, pTRE2-hyg expression vectors, were obtained from Clontech, Inc. cDNAs encoding full-length CrmA, Bcl-XL, or BI-1 or encoding a fragment of XIAP encompassing the BIR2 and BIR3 domains (residues 124–356) were subcloned into pTRE2-hyg vectors. Plasmids were transfected into HeLa-tet-on or HeLa-tet-off cells using LipofectAMINE-Plus, and stable clones derived from individual colonies were selected in Dulbecco’s modified Eagle’s medium supple-
mented with 10% of Tet-system-approved fetal bovine serum (Clontech) in the presence of 400 μg/ml hygromycin. To test for the induced expression of these genes, stable clones were left untreated or treated for 24 h with 1 μg/ml doxycycline, harvested, and analyzed for expression of the appropriate protein by immunoblot as described. For each case, several clones displaying conditional expression were identified.

Apoptosis and Cell Death Assays—For annexin V assays, 10⁶ cells were double-stained with fluorescein isothiocyanate-conjugated annexin V and propidium iodide using a kit according to the manufacturer’s instructions (Bвисion, Moutain View, CA). The percentage of apoptotic (annexin V positive) cells was determined by flow cytometric analysis (BD Biociences). Alternatively, cell viability was assessed by a dye exclusion method using a 96-well compatible flow cytometer and the ViaCount assay reagent (Guava Technologies, Inc.). Mitochondrial membrane potential (ΔΨm) was monitored by flow cytometry using 3,3′-dioxyloxyacarbocyanine iodide (DiO) (48).

Caspase Activity Assays—Effector caspase activity in cell lysates was examined using substrates acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) according to a standard protocol (Bio-Rad). Activity was measured using Max spectrofluorometer plate reader (excitation at 405 nm and emission at 510 nm) (Molecular Devices, Sunnyvale, CA) to measure relative fluorescent product, normalized relative to total protein content.

Protein Interaction Assays—GST pull-down experiments were performed using the ProFound pull-down GST protein-protein interaction kit (Pierce). Purified GST-XIAP protein (1 μg) and caspase-3 or caspase-9 (0.5 μg) were incubated with 20 μl of glutathione beads for 1 h in 400 μl of phosphate-buffered saline (PBS) (pH 7.4) solution followed by three washes with 1 ml of PBS. Various amounts of chemical or peptide XIAP antagonists were then added to the beads, and the mixture was incubated for another 2 h. After five washes, the bound protein was eluted using 100 μl free reduced glutathione in PBS. Each sample was separated by one-dimensional SDS-PAGE in an acrylamide gel and transferred to nitrocellulose, and the resulting blots were incubated with various antibodies as described below.

Immunoblotting—Immunoblot analysis was performed essentially as described previously (49). In brief, for each sample, 25 μg of protein/lane was separated by 10% SDS-PAGE and transferred to nitrocellulose (Schleicher & Schuell, Keene, NH). Blots were incubated in PBS-Tween 20 (0.05%) supplemented with 5% nonfat dry milk for 1 h at room temperature and then incubated for 2 h in fresh blocking solution with an appropriate dilution of primary antibodies as follows: polyclonal rabbit-antihuman caspase-3, 1:1000 (50); polyclonal rabbit-antihuman Bcl-X₅, 1:2000; polyclonal rabbit-antihuman XIAP, 1:1000 (50); CRM197, 1:1000 (Pharminogen, San Diego, CA); anti-hemagglutinin, 1:2000 (Santa Cruz Biotechnology); or monoclonal mouse anti-β-actin, 1:3000 (Sigma Inc.). Blots were washed three times for 5 min in PBS-Tween 20 and then incubated with a 1:2000 (v/v) dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Bio-Rad) for 1 h. Blots were again washed three times for 5 min in PBS-Tween 20 and then developed by enhanced chemiluminescence (Amersham Biosciences).

RESULTS

We exploited the ability of IAPs to suppress active caspases in vitro, developing a high throughput screening assay for identification of compounds that block the caspase-suppressing activity of these anti-apoptotic proteins (44). This assay utilized purified protein components in an enzyme derepression assay employing human XIAP and caspase-3. Compounds were identified that inhibited XIAP by releasing caspase-3 to cleave the fluorogenic peptide substrate Ac-DEVD-AFC. We reported established observations.

Previous studies showed that the BIR2 domain and an upstream flanking segment of XIAP are sufficient to bind and inhibit caspase-3 (17, 51), whereas the BIR3 domain binds and inhibits caspase-9 (52, 53). We therefore compared the ability of our active phenylurea series compounds to displace effector protease caspase-3 and an initiator protease caspase-9 from BIR2 and BIR3 of XIAP, respectively, making comparisons with a synthetic peptide corresponding to the N terminus of SMAC, an endogenous antagonist of IAPs (54). Active compounds such as 1396-11 displaced caspase-3 from GST-BIR2, as assessed in GST pull-down assays (Fig. 1A), and although structurally related, inactive control compounds such 1396-28 did not. NMR-based binding assays also showed that active (but not inactive) analogs of the compounds directly bind purified recombinant BIR2 of XIAP but not caspase-3.² In contrast to caspase-3, active phenylurea-based XIAP antagonists did not displace caspase-9 from GST-BIR3. By comparison, SMAC

² Z. Wang, K. Welsh, C. Pinilla, M. Pellechia, and J. C. Reed, unpublished observations.
peptide displaced both caspase-3 and caspase-9 as predicted (54–56).

Consistent with these protein binding data, active phenylurea series compounds such as 1396-11 restored caspase-3 but not caspase-9 activity in XIAP-inhibited reactions in a dose-dependent manner (Fig. 1B), consistent with our recently reported data (44). To determine whether the compounds could activate pro-caspase-3 in vitro, purified pro-caspase-3 was incubated with various concentrations of active XIAP inhibitors. As expected, none of our compounds induces activation of pro-caspase-3 in vitro (data not shown). XIAP antagonists such as 1396-11 induced rapid, concentration-dependent apoptosis of a variety of tumor cell lines in culture, including HeLa cervical cancer, MDA-MB-468 breast cancer, and Jurkat leukemia cells (Fig. 2). In contrast, inactive phenylureas such as 1396-28 did not induce apoptosis. The concentration of active phenylurea-based compounds required to induce apoptosis of 50% of tumor cells within 24 h ranged from 5 to 10 μM. Apoptosis was detected as early as 4–8 h after addition of active compounds, indicating a rapid onset of action.

The apoptosis induced by these active phenylurea-based compounds was associated with activation of caspases, as measured in cell lysates with a fluorogenic substrate peptide, Ac-DEVD-AFC (Fig. 3A). The activation of caspases was suppressible by co-culturing the cells with a broad-spectrum caspase inhibitor, Z-VAD-fmk, prior to producing cell lysates (Fig. 3A). Active XIAP antagonists such as 1396-11 also induced proteolytic cleavage of effector protease caspase-3, as demonstrated by immunoblotting methods (Fig. 3C).

**XIAP Antagonists Induce Cellular Apoptosis through a Caspase-dependent Pathway**—We characterized the effects of XIAP antagonists at the cellular level. Active XIAP antagonists such as 1396-11 induced rapid, concentration-dependent apoptosis of a variety of tumor cell lines in culture, including HeLa cervical cancer, MDA-MB-468 breast cancer, and Jurkat leukemia cells (Fig. 2). In contrast, inactive phenylureas such as 1396-28 did not induce apoptosis. The concentration of active phenylurea-based compounds required to induce apoptosis of 50% of tumor cells within 24 h ranged from 5 to 10 μM. Apoptosis was detected as early as 4–8 h after addition of active compounds, indicating a rapid onset of action.

The apoptosis induced by these active phenylurea-based compounds was associated with activation of caspases, as measured in cell lysates with a fluorogenic substrate peptide, Ac-DEVD-AFC (Fig. 3A). The activation of caspases was suppressible by co-culturing the cells with a broad-spectrum caspase inhibitor, Z-VAD-fmk, prior to producing cell lysates (Fig. 3A). Active XIAP antagonists such as 1396-11 also induced proteolytic cleavage of effector protease caspase-3, as demonstrated by immunoblotting methods (Fig. 3C).
To further characterize the mechanism of our XIAP antagonists, we compared the ability of various peptidyl caspase inhibitors to block apoptosis induced by active compounds such as 1396-11. Selective caspase-3 and -7 inhibitor (Ac-DEVD-fmk) was compared with partially selective inhibitors of upstream initiator proteases caspase-8 and -10 (Ac-IETD-fmk), caspase-9 (Ac-LEHD-fmk), and downstream effector caspase-6 (Ac-VEID-fmk). Although DEVD effectively blocked apoptosis induction by active phenylurea-based IAP antagonists, the other selective inhibitors, IETD, LEHD, and VEID, did not (Fig. 3D). In contrast, when we used the kinase inhibitor staurosporine (STS), a known activator of the intrinsic mitochondrial pathway for apoptosis (11), the caspase-9-selective inhibitor Ac-LEHD-fmk partially inhibited apoptosis. Also, when TRAIL protein (representing an activator of the extrinsic death-receptor pathway for apoptosis) was used, all of the caspase inhibitors displayed anti-apoptotic activity (Fig. 3D).

These data derived from various peptide inhibitors of caspases clearly demonstrate a different inhibitor pattern for XIAP antagonists compared with agents that are known to trigger either the intrinsic or extrinsic apoptosis pathways.

XIAP Antagonists Induce Apoptosis prior to Mitochondrial Membrane Depolarization—Loss of mitochondrial membrane potential ($\Delta \Psi m$) represents a common concomitant of apoptosis, which can either occur as an early initiating event prior to caspase activation or as a late, downstream consequence of caspase activation (reviewed in Refs. 11 and 57). We therefore compared the effects on mitochondrial depolarization of XIAP
antagonist 1396-11 with an agent known to trigger activation of the mitochondrial pathway for apoptosis, the anti-cancer drug paclitaxel (Taxol™), by flow cytometry using the dye 3,3'-dihexyloxacarbocyanine iodide. Apoptosis was monitored in parallel by annexin V staining, using fluorescein isothiocyanate-conjugated annexin, and by assessment of changes in the light-scattering properties of cells.

Fig. 4 shows representative data contrasting the effects of 1396-11 and paclitaxel on 697 leukemia cells. At 24 h after drug exposure, the ΔΨm remained normal in cultures of leukemia cells treated with XIAP antagonists 1396-11 but not in paclitaxel-treated cultures (top). Despite the normal ΔΨm measurements, extensive apoptosis was induced in 1396-11-treated cultures within 24 h, as determined by annexin V staining (middle) and by changes in light-scattering (bottom). Days later, the ΔΨm began to decline in 1396-11-treated cultures (not shown), suggesting that loss of mitochondrial membrane polarization is a late event in cells induced to undergo apoptosis by XIAP antagonists.

Bcl-2-independent Induction of Apoptosis by XIAP Antagonists—Bcl-2 and related anti-apoptotic proteins associate with mitochondrial membranes and prevent release of mitochondrial proteins that activate caspases in the cytosol (reviewed in Ref. 58). Most anti-cancer drugs trigger apoptosis through the mitochondrial pathway, and chemoresistant cancers commonly develop defects in this cell death pathway as a result of over-expression of Bcl-2 (59). We therefore compared the effects of XIAP antagonist 1396-11 with the anti-cancer drug paclitaxel with respect to apoptosis induction in Bcl-2 over-expressing cells. A well characterized pair of genetically engineered cell lines was employed for these experiments, 697-neo and 697-Bcl-2, which stably contain control (empty) and Bcl-2-encoding expression vectors (45). Over-expression of Bcl-2 reduced apoptosis induction by paclitaxel but had no effect on apoptosis induced by the XIAP antagonist 1396-11 (Fig. 5). These data thus corroborate our prior findings based on transient transfection studies (44), indicating that phenylurea-based XIAP antagonists induce apoptosis through a Bcl-2-independent mechanism. These findings are also consistent with the observation that mitochondrial membrane depolarization is a very late, post-apoptotic event in tumor cell lines treated with these XIAP antagonists (Fig. 4).

Genetic Analysis of XIAP Antagonist Mechanisms Shows Specificity for Caspase-dependent Pathways—To further explore the mechanism by which phenylurea-based XIAP antagonists induce apoptosis of tumor cell lines, we used genetically engineered cells. Complementary approaches of gene ablation and gene over-expression were used.

First, we generated a series of tetracycline-inducible or -repressible HeLa cell lines conditionally over-expressing (a) XIAP, (b) Bcl-XL, (c) CrmA, or (d) BI-1. These anti-apoptotic proteins represent prototypical antagonists of the (a) downstream convergence, (b) mitochondrial (intrinsc), (c) death receptor (extrinsic), and (d) endoplasmic reticulum stress pathways for apoptosis, respectively (15, 60–62). Immunoblot analysis demonstrated the conditional expression of the intended proteins in these stably transfected cell lines (Fig. 6). Note that for XIAP, we used a fragment of the protein containing the BIR2 and BIR3 domain because of protein instability caused by the E2-binding RING domain found within the full-length XIAP protein (63). This fragment of XIAP contains the domains required for inhibiting both effector (caspase-3 and -7) and initiator (caspase-9) proteases. The induced proteins were verified to suppress apoptosis induced by prototypical apoptotic stimuli known to engage these various cell death pathways, such as doxorubicin (mitochondrial/intrinsic), TNF (death recep-

![Fig. 6. Conditional expression of anti-apoptotic proteins in HeLa cells.](image1)

![Fig. 7. Effect of XIAP, Bcl-XL, CrmA, and BI-1 on XIAP antagonist 1396-11-induced killing.](image2)
Next, we tested the effects of phenylurea XIAP antagonists, including 1396-11 (shown). As expected, inducible expression of XIAP shifted the dose-response curve to the right for 1396-11, consistent with the presence of more drug target in the cells; thus, more compound was required to achieve a similar effect on cell viability (Fig. 7). CrmA, a selective inhibitor of caspase-1 and -8 (60), also slightly shifted the dose-response curve to the right, consistent with reports showing that activated caspase-3 can cleave and activate caspase-8 in epithelial cancer cell lines (64). In contrast, over-expression of either Bel-X\(_\text{L}\) or BI-1 failed to alter the dose response. These data thus provide additional evidence that these phenylurea-based XIAP antagonists induce apoptosis through the intended mechanism. Importantly, these compounds reveal a Bcl-2/Bcl-X\(_\text{L}\)-independent route to induction of tumor cells, unlike conventional cytotoxic anticancer drugs.

To complement these protein over-expression studies, we compared the effects of phenylurea-based XIAP antagonists on SV40 large T-transformed mouse embryo fibroblasts (MEFs) from xiap\(^{-/-}\) mice (generous gift of C. Duckett (47)) with bax\(^{-/-}\)-bak\(^{-/-}\) double knock-out cells (generous gift of S. Korsmeyer (46)). Phenylurea-based XIAP antagonists such as 1396-11 induced concentration-dependent apoptosis of bax\(^{-/-}\)+/+ control and bax\(^{-/-}\)-bak\(^{-/-}\) double knock-out cells with equal efficiency (Fig. 8A), providing further evidence that the cytotoxic mechanism of the XIAP-inhibitory compounds is independent of the Bax/Bcl-2 mitochondrial pathway for apoptosis. By comparison, bax\(^{-/-}\)-bak\(^{-/-}\) cells were highly resistant to STS and serum deprivation, stimuli known to induce cell death through a Bax/Bak-dependent mechanism (46). Active XIAP antagonists such as 1396-11 also induced activation of effector protease caspases in bax\(^{-/-}\)-bak\(^{-/-}\) cells, as measured in cell lysates with a fluorogenic substrate peptide, Ac-DEVD-\text{AFC} (Fig. 9). In contrast to bax\(^{-/-}\)-bak\(^{-/-}\) double knock-out cells, transformed xiap\(^{-/-}\) cells were less sensitive to our phenylurea-based XIAP antagonists compared with normal xiap\(^{+/+}\) cells (Fig. 8B). This finding suggests that cells lacking the primary target of our compounds (XIAP) are less dependent on XIAP and therefore less sensitive to the compounds. However, because active compounds kill xiap\(^{-/-}\) cells when applied at higher concentrations, these data indicate that other secondary targets of the compounds remain in xiap\(^{-/-}\) cells. The most likely candidates for these secondary targets are other members of the IAP family, such as cIAP1 and cIAP2, which have a great structural and mechanistic similarity to XIAP (15, 16).

**DISCUSSION**

IAP family proteins play critical roles in apoptosis regulation. A pathological over-expression of some of these anti-apoptotic proteins has been documented in cancer, suggesting the possibility of developing targeted therapies directed against IAPs. To this end, we recently generated small molecule antagonists of XIAP using an enzyme derepression assay to obtain chemical tools for studying the function of IAPs in cellular contexts, particularly tumor cells (44). Here, we have further characterized the mechanism of action of these chemical XIAP antagonists.

IAPs vary in their mechanisms, with at least some of them functioning as caspase inhibitors. However, the specific caspases that these anti-apoptotic proteins inhibit can differ.
For example, XIAP uses different domains to suppress both the downstream effector caspases that operate at points of convergence of apoptosis pathways (caspase-3 and -7) and the apical protease in the mitochondrial pathway for apoptosis (caspase-9) (15, 17, 18). In contrast, ML-IAP and survivin are suppressors only of caspase-9. Moreover, ML-IAP and survivin suppress this caspase-9 through different mechanisms (27, 66). Consequently, developing chemical inhibitors of various IAP family members may require individualized strategies.

The phenylurea-based compounds described here dissociate caspase-3 but not caspase-9 from XIAP and restore caspase-3 but not caspase-9 activity in XIAP-inhibited reactions. These findings are consistent with antagonism of the BIR2 domain functionality of XIAP, since caspase-3 suppression can be achieved by a region of XIAP that comprises the BIR2 domain and an upstream flanking region (17, 19, 51). This inhibitory mechanism of the phenylurea-based XIAP antagonists differs from SMAC peptides, which have been shown to bind tightly to the BIR3 domain and with lower affinity to BIR2 of XIAP, such that BIR3 is the more relevant target of these inhibitory peptides in cell-based assays. Previously, we showed that these phenylurea-based compounds do not compete with SMAC 7'-mer peptide for binding to XIAP (44). The effects of our phenylurea-based XIAP compounds thus can be compared with SMAC-based peptidyl inhibitors. BIR3-targeting SMAC peptides induce apoptosis only of certain human tumor cell lines as single agents, but they broadly sensitize tumor cell lines to other types of apoptosis inducers such as conventional anti-cancer drugs or biologicals such as TNF family death ligands (67, 68). In contrast, our BIR2-targeting phenylurea-based antagonists were shown to induce apoptosis as single agents of most tumor cell lines (44), presumably by acting at a more downstream point in apoptosis pathways (Fig. 10). Thus, chemical reagents targeting the ability of XIAP to target downstream effector caspases offer unique tools relative to SMAC peptides for addressing questions about the endogenous roles of these anti-apoptotic proteins. More precisely than gene ablation strategies, these compounds can address questions about the roles of specific domains or functional sites on IAPs.

Evidence of over-expression of IAPs in cancer has been obtained suggesting a role for these apoptosis suppressors in malignancy (35, 36, 37). Various IAP family proteins are overexpressed in specific types of cancer. However, some tumors over-express more than one member of the family simultaneously. For example, in prostate cancers, evidence has been obtained that protein levels of XIAP, cIAP1, cIAP2, and survivin are often simultaneously increased (69), suggesting redundancy in expression of these anti-apoptotic proteins. With regards to redundancy, induction of apoptosis of \textit{xiap} \textit{−/−} transformed cells with phenylurea-based compounds required higher concentrations compared with \textit{xiap} \textit{+/+} cells but was achieved nevertheless. These data are consistent with XIAP representing a significant target, but not the only target, of these phenylurea-based compounds. Other likely candidates are IAP family proteins such as cIAP1 and cIAP2, which exhibit similar mechanisms and also bind effector caspases (16). In this regard, SMAC peptides have been demonstrated to target minimally XIAP, cIAP1, and ML-IAP (53, 67, 70, 71). Thus, the phenylurea-based XIAP antagonists characterized here may also inhibit other IAP family members, accounting for differences in apoptosis-inducing activity compared with XIAP antisense or small interfering RNA (65).

The point of action of the IAPs can be contrasted with other known apoptosis suppressors of relevance to cancer, such as Bcl-2 family proteins and FLIP, which act upstream of the IAPs. Consistent with a downstream point of action of phenylurea-based XIAP antagonists, we observed that over-expression of Bel-2, Bel-\textit{X}\textsubscript{L}, and BI-1 failed to alter cellular sensitivity. In contrast, sensitivity to conventional anti-cancer drugs such as doxorubicin and paclitaxel was reduced by Bel-2 and Bel-\textit{X}\textsubscript{L} in side-by-side comparisons, and sensitivity to the endoplasmic reticulum stress agent tunicamycin was reduced by BI-1. Also, XIAP antagonists induced apoptosis through a caspase-dependent mechanism where mitochondrial membrane depolarization occurs as a late rather than early event. Altogether, these findings provide further evidence that phenylurea-based XIAP antagonists induce apoptosis through the intended mechanism. They also demonstrate potential advantages to the use of IAP antagonists for cancer cell eradication, provided an acceptable therapeutic index can be achieved by agents that hit at a very downstream step in apoptosis pathways.

**Acknowledgments**—We thank J. Valois and M. Lamie for manuscript preparation, C. Duckett and S. Korsmeyer for cells, and G. Salvesen for pro-caspase-3 and caspase-9.

**References**

1. Deveraux, Q. L., and Reed, J. C. (1999) *Genes Dev.* 13, 239–252
2. Lowe, S. W., and Lin, A. W. (2000) *Carcinogenesis* 21, 485–495
3. Thornberry, N. A., and Lazebnik, Y. (1998) *Science* 281, 1312–1316
4. Cryns, V., and Yuan, Y. (1999) *Genes Dev.* 12, 1551–1570
5. Salvesen, G. S., and Duckett, C. S. (1997) *Cell* 91, 443–446
6. Talanian, R., Yang, X., Turbov, J., Seth, P., Ghayur, T., Casiano, C., Orth, K., and Froelich, C. (1997) *J. Exp. Med.* 186, 1323–1331
7. Quan, L. T., Towari, M., O’Bourke, K., Dixit, V., Shipas, S. J., Poirier, G. G., Ray, C., Pickup, D. J., and Salvesen, G. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 1972–1976
8. Lochely, R. M., Killeen, N., and Lenardo, M. J. (2001) *Cell* 104, 487–501
9. Wallach, D., Varfolomeev, E. E., Malinin, N. L., Goltsvev, Y. V., Kovalenko, A. V., and Boldin, M. P. (1999) *Annu. Rev. Immunol.* 17, 331–367
10. Yuan, J. (1997) *Curr. Opin. Cell Biol.* 9, 247–251
11. Green, D. R., and Reed, J. C. (1998) *Science* 281, 1309–1312
12. Zong, W. X., Li, C., Hatzivassiliou, G., Lindsten, T., Yu, Q. C., Yuan, J., and Thompson, C. B. (2003) *J. Cell Biol.* 162, 59–69
13. Szagredi, E., Fitzgerald, U., and Samali, A. (2003) *Ann. N. Y. Acad. Sci.* 1010, 186–194
14. Salvesen, G. S., and Duckett, C. S. (2002) *Nat. Rev. Mol. Cell Biol.* 3, 401–410
15. Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997) *EMBO J.* 16, 6914–6925
16. Roy, N., Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997) *EMBO J.* 16, 6914–6925
17. Takahashi, R., Deveraux, Q. L., Tam, I., Walsh, K., Assa-Munt, N., Salvesen,
Cellular, Biochemical, and Genetic Analysis of Mechanism of Small Molecule IAP Inhibitors
Zhiliang Wang, Michael Cuddy, Temesgen Samuel, Kate Welsh, Aaron Schimmer, Farid Hanaii, Richard Houghten, Clemencia Pinilla and John C. Reed

J. Biol. Chem. 2004, 279:48168-48176.
doi: 10.1074/jbc.M405022200 originally published online August 27, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M405022200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 71 references, 33 of which can be accessed free at http://www.jbc.org/content/279/46/48168.full.html#ref-list-1