Small-molecule antagonists of apoptosis suppressor XIAP exhibit broad antitumor activity

Aaron D. Schimmer,[1,5] Kate Welsh,[1,5] Clemencia Pinilla,[2,5,*] Zhiliang Wang,[1] Maryla Krajewska,[1] Marie-Josee Bonneau,[1] Irene M. Pedersen,[1] Shinichi Kitada,[1] Fiona L. Scott,[1] Beatrice Bailly-Maitre,[1] Gennadi Glinsky,[3] Dominick Scudiero,[4] Edward Sausville,[4] Guy Salvesen,[1] Adel Nefzi,[2] John M. Ostresh,[2] Richard A. Houghten,[2] and John C. Reed[1,*]

[1]The Burnham Institute, La Jolla, California 92037
[2]The Torrey Pines Institute for Molecular Studies, San Diego, California 92121
[3]The Sidney Kimmel Cancer Center, La Jolla, California 92037
[4]National Cancer Institute, Bethesda, Maryland 20817
[5]These authors contributed equally to the work.
*Correspondence: jreed@burnham.org (J.C.R.), cpinilla@tpims.org (C.P.)

Summary

Apoptosis resistance commonly occurs in cancers, preventing activation of Caspase family cell death proteases. XIAP is an endogenous inhibitor of Caspases overexpressed in many cancers. We developed an enzyme derepression assay, based on overcoming XIAP-mediated suppression of Caspase-3, and screened mixture-based combinatorial chemical libraries for compounds that reversed XIAP-mediated inhibition of Caspase-3, identifying a class of polyphenylureas with XIAP-inhibitory activity. These compounds, but not inactive structural analogs, stimulated increases in Caspase activity, directly induced apoptosis of many types of tumor cell lines in culture, and sensitized cancer cells to chemotherapeutic drugs. Active compounds also suppressed growth of established tumors in xenograft models in mice, while displaying little toxicity to normal tissues. These findings validate IAPs as targets for cancer drug discovery.

Introduction

At a fundamental level, cancer occurs or progresses because malignant cells fail to undergo apoptosis either spontaneously or in response to chemotherapy. Failure to activate Caspases may account for resistance to apoptosis. Caspases are a family of intracellular cysteine proteases that trigger the apoptotic program (Cryns and Yuan, 1999; Thornberry and Lazebnik, 1998). Caspases lie in a latent (zymogen) state in cells but become activated in response to a wide variety of cell death stimuli.

The Inhibitor of Apoptosis Proteins (IAPs) represent a family of endogenous Caspase inhibitors that share a conserved structure known as the BIR domain (Reed, 2001). Eight IAP-encoding genes are found in the human genome, and some of these are overexpressed in cancers (Ferreira et al., 2001; Hofmann et al., 2002; Tamm et al., 2000). Various proof-of-concept studies using antisense oligonucleotides or peptide inhibitors of IAP have suggested that these proteins are important for maintaining tumor cell survival or for affording resistance to apoptosis induction by anticancer drugs (Bilim et al., 2003; Fulda et al., 2002; Gordon et al., 2002; Sasaki et al., 2000; Yang et al., 2003b). Moreover, some tumor cells constitutively activate Caspases, apparently offsetting Caspases with overexpression of IAP family proteins (Yang et al., 2003a). Thus, small-molecule drugs that inhibit IAPs could be therapeutically useful in the treatment of malignancy.

XIAP is the best characterized of the IAP family members in terms of its Caspase inhibitory mechanism. The XIAP protein contains three BIR domains. BIR2 and flanking regions are responsible for binding and potently inhibiting active Caspases-3 and -7, while BIR3 and flanking regions suppress Caspase-9 (Deveraux et al., 1999; Takahashi et al., 1998). XIAP levels are pathologically elevated in many acute and chronic leukemias, prostate cancers, lung cancers, and other types of tumors (Byrd et al., 2002; Ferreira et al., 2001; Hofmann et al., 2002; Krajewska et al., 2003; Schimmer et al., 2003; Tamm et al., 2000). Here, we describe the generation and characterization of prototype small-molecule antagonists of XIAP targeting BIR2 that induce apoptosis of tumor cells in culture and that display antitumor activity in vivo. The findings validate certain IAP family proteins as suitable targets for cancer drug discovery.

SIGNIFICANCE

Inhibitor of Apoptosis Proteins (IAPs) are a family of apoptosis-suppressing proteins commonly overexpressed in human cancers. Some IAPs directly bind and suppress Caspases, the proteases responsible for apoptosis. We generated chemical compounds that target the BIR2 domain of XIAP. These compounds triggered apoptosis of most types of tumor cell lines and primary leukemias in culture. Active compounds also inhibited tumor growth in mice, while producing little toxicity to normal tissues. The findings suggest that cancer cells have an intrinsic drive to apoptosis that is held in check by IAPs. Negating IAPs allows the apoptosis program to proceed. Normal cells appear to be less dependent on IAPs, thus providing the basis for a therapeutic index.

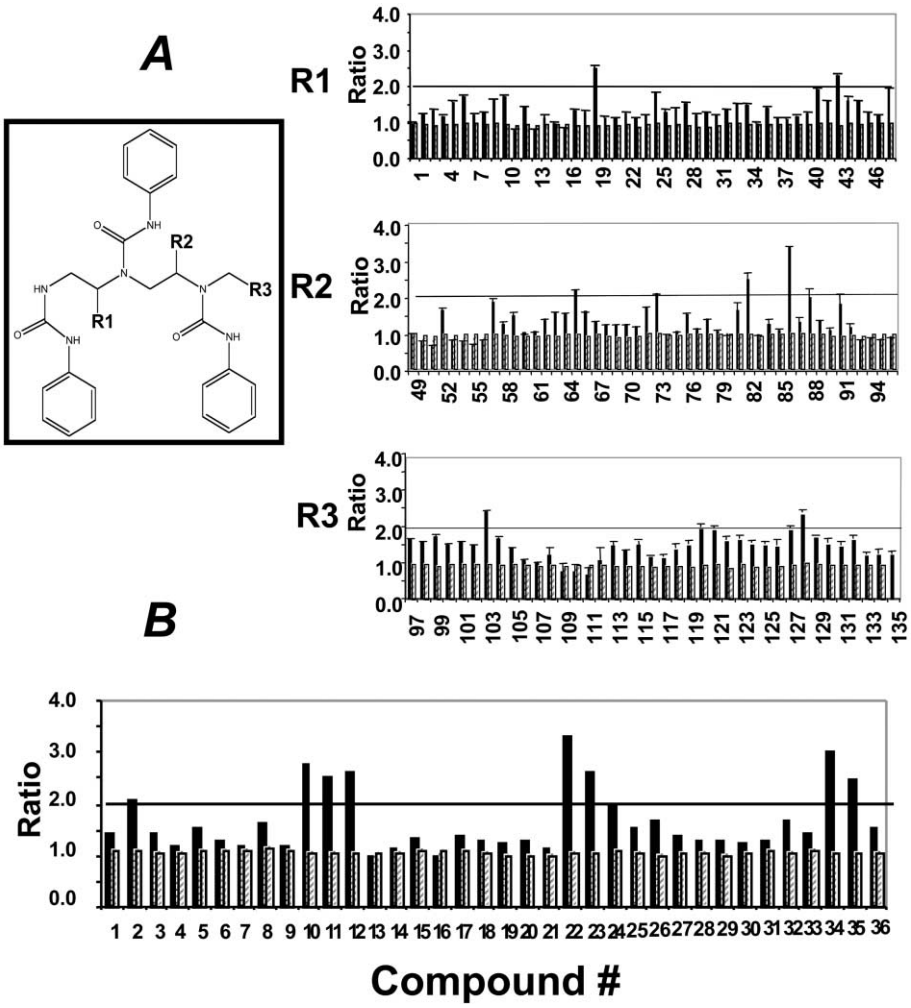

Figure 1. Identification of small-molecule XIAP inhibitors by screening combinatorial mixture-based libraries

A: Mixture-based combinatorial library of polyphenylureas was constructed in positional scanning format by fixing the functionality at the R_1, R_2, or R_3 position and combining into the mixture the variations of the other diversity groups. These mixture-based libraries were screened using the Caspase derepression assay for chemicals that overcome XIAP-mediated repression of Caspase-3. Aliquots from the mixtures were added to microtiter plates containing XIAP and Caspase-3 (black bars) or, as a control, Caspase-3 alone (gray bars). Caspase-3 activity was measured by monitoring cleavage of the fluorogenic substrate Ac-DEVD-AFC. Hits were empirically defined as compounds that increased Caspase-3 activity ≥2-fold in XIAP-inhibited reactions without affecting Caspase-3 alone. A representative screen of the positional scanning combinatorial library (final concentration 25 μg/ml) is shown. Caspase activity is presented as the fold increase in enzyme velocity after the addition of the compound.

B: Individual compounds were synthesized based on deconvolution of the polyphenylurea library. Thirty-six individual compounds were tested at 25 μg/mL using the Caspase derepression assay for their ability to increase Caspase-3 activity in the presence (black bars) or absence (gray bars) of XIAP, using a 2-fold elevation in the enzyme velocity as the cut-off for positivity.

Results

Identification and characterization of small-molecule antagonists of XIAP

Based on the knowledge that XIAP directly inhibits active Caspase-3, we devised an enzyme derepression assay in which libraries of chemical compounds were screened for molecules that relieve protease inhibition, allowing Caspase-3 to cleave a fluorogenic peptide substrate. Eleven mixture-based small-molecule combinatorial libraries (constituting approximately one million compounds) were screened, including a library of polyphenylureas (Nefzi et al., 2000) synthesized in the positional scanning format (Pinilla et al., 1992). All compounds in this particular chemical library shared a common polyphenylurea scaffold with three diversity positions (R_1, R_2, and R_3), where 48 different chemical moieties were linked at the R_1 and R_2 positions and 39 at the R_3 position, thus constituting a library of 135 mixtures and a total of 89,856 compounds (Figure 1A). Thus, three sublibraries based on the location of the defined position (R_1, R_2, or R_3) and composed of mixtures having a fixed chemical moiety at one of the positions were used to screen for XIAP inhibitors. Each mixture was screened for its effects on Caspase-3 activity in the presence and absence of XIAP, scoring as a "hit" those compound mixtures that increased Caspase-3 activity ≥2-fold when applied to XIAP/Caspase-3 assays without affecting Caspase-3 alone. The positive compound mixtures were then deconvoluted by standard methods (Pinilla et al., 1992, 2003), yielding 36 individual compounds, which were screened in the same enzyme derepression assay, resulting in the identification of eight compounds that increased Caspase-3 activity in XIAP-inhibited reactions by ≥2-fold (Figure 1B) (structures are provided in Table S1 of the Supplemental Data [http://www.cancercell.org/cgi/content/full/5/1/25/DC1]). In the process of library deconvolution, we also identified compounds such as 1396-28 that differed from active molecules at R_1, R_2, or R_3 and which did not inhibit XIAP ($EC_{50} > 100$ uM), serving as controls for subsequent experiments.

To determine the specificity of the active compounds, we compared their effects on inhibition of Caspase-3, -7, and -9 by XIAP. Figure 2 shows representative data for four active compounds (1396-11, 12, 22, and 34) and negative control (1396-28). Whereas all active compounds restored Caspase-3 and Caspase -7 activity toward normal, none of the compounds reversed XIAP-mediated suppression of Caspase-9 (Figures 2A and 2B and data not shown). Since it is known that the BIR2 domain and an upstream flanking segment of XIAP are sufficient to inhibit Caspases-3 and -7, we tested whether the active compounds could relieve Caspase suppression mediated by

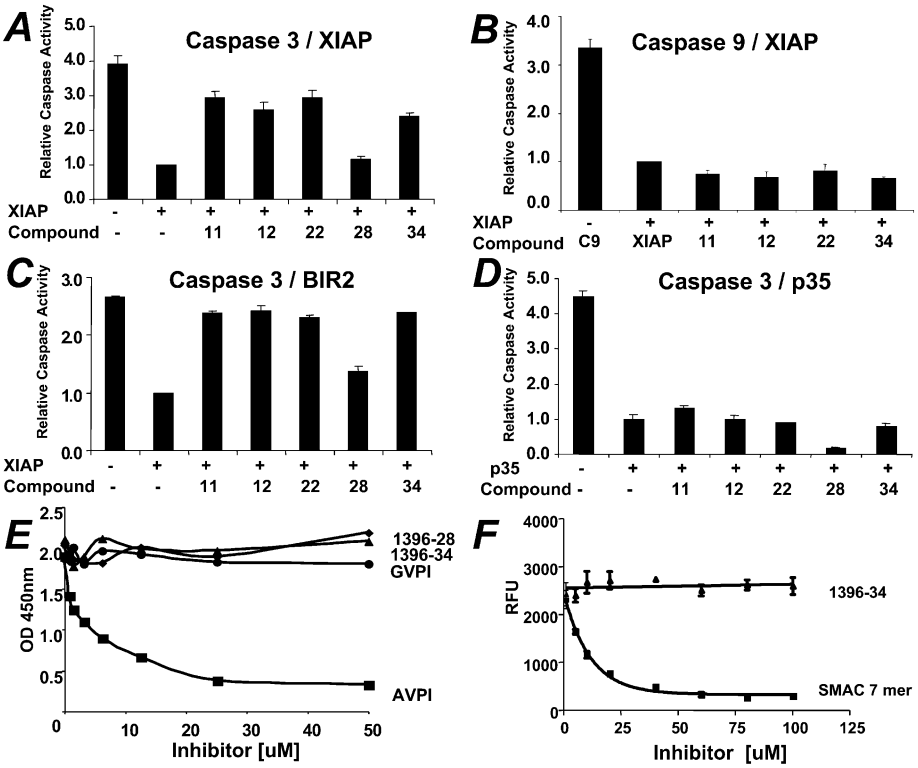

Figure 2. Characterization of biochemical mechanism of polyphenylurea XIAP antagonists

Four active XIAP antagonists (1396-11, 1396-12, 1396-22, 1396-34) or inactive control (1396-28) were added in excess (75 μM) to enzyme derepression assays containing (**A**) 46 nM full-length recombinant XIAP and 0.36 nM active Caspase-3; (**B**) 46 nM full-length XIAP and 2.2 nM active Caspase-9 (lacking its N-terminal CARD domain); (**C**) 1.2 nM GST-BIR2 fragment of XIAP and 0.36 nM active Caspase-3. (**D**) 3.4 nM recombinant baculovirus p35 and 0.36 nM active Caspase-3. Caspase activity was measured either by hydrolysis of Ac-DEVD-AFC (Caspase-3 substrate [**A, C, and D**]) or Ac-LEHD-AFC (Caspase-9 substrate [**B**]). Data represent enzyme velocity relative to XIAP-inhibited reactions in the absence of compound (= 1.0), where ratio of the enzyme rates with compound versus without compound are provided (mean ± SE [n = 3]). (**E**) Biotinylated SMAC (7 mer) was adsorbed to Neutravidin-coated plates, then GST-XIAP was added with or without compound or tetrapeptides, detecting bound GST-XIAP with anti-GST antibody. (**F**) GST-XIAP was adsorbed to plates and then incubated with biotinylated-SMAC (7 mer) with or without chemical compounds or SMAC heptapeptide, detecting bound biotinylated-SMAC peptide by a streptavidin-europium-based fluorescence method (mean ± SE; n = 3).

a GST-BIR2 fusion protein (Takahashi et al., 1998). All active compounds restored Caspase-3 activity toward normal in BIR2-suppressed reactions (Figure 2C). NMR-based binding assays also showed that active (but not inactive) analogs of the compounds directly bind purified recombinant BIR2 of XIAP but not control proteins (our unpublished data). Dose-response studies performed for these four XIAP antagonists revealed EC_{50} values of 32–58 μM against full-length XIAP versus 5–22 μM against BIR2 (EC_{50} = concentration required to restore Caspase activity to half-maximal rate). In contrast, the compounds did not overcome Caspase-3 suppression by p35 (Figure 2D), a viral inhibitor of Caspases with a mechanism of inhibition unrelated to IAPs (Xu et al., 2001), thus confirming their specificity. These compounds also did not show activity in other types of biochemical assays (e.g., luciferase activity; Bcl-X_L binding to BH3 peptides), again confirming a specific effect.

To further explore the mechanism of these small-molecule antagonists of XIAP, we performed binding assays, testing whether these compounds compete for interactions with the same sites on BIR domains that are targeted by certain endogenous inhibitors of XIAP, such as SMAC and HtrA2 (Liu et al., 2000; Suzuki et al., 2001). Accordingly, streptavidin was adsorbed to plates, biotin-SMAC peptide was bound, and then GST-XIAP was incubated in the presence or absence of candidate inhibitors, followed by detection with anti-GST antibody. As shown in Figure 2E, SMAC 4-mer peptide AVPI effectively competed with biotin-SMAC for binding to XIAP, while mutant peptide GVPI did not. In contrast, none of our XIAP-inhibitory polyphenylureas competed with biotin-SMAC (Figure 2E and data not shown). Similar results were obtained using an alternative binding assay where GST-XIAP was adsorbed to 96-well plastic plates, then biotinylated SMAC 7-mer peptide (AVPIAQK) was incubated with adsorbed XIAP in the presence or absence of candidate inhibitors, detecting plate-bound biotin-SMAC using streptavidin-europium in conjunction with a fluorescence-based method (Figure 2F). Thus, these polyphenylurea antagonists of XIAP act through a mechanism distinct from endogenous XIAP antagonist SMAC.

XIAP inhibitors induce Caspase activation and apoptosis of tumor cells

To evaluate the effects of our small-molecule XIAP inhibitors in cells, we incubated cultured tumor cell lines with active and structurally similar inactive polyphenylureas and measured cell viability at various times thereafter. Multiple tumor cell lines were thus empirically determined to be sensitive to these XIAP antagonists, undergoing apoptosis when incubated with active but not inactive polyphenylurea-based compounds. Figure 3A shows representative data using Jurkat leukemia cells. For these experiments, Jurkat cells were treated for 20 hr with increasing concentrations of five of the most potent XIAP inhibitors (1396-10, 11, 12, 22, 34) and compared with five inactive structural analogs (1396-13, 14, 16, 28, 31) (for structures, see Table S1 of the Supplemental Data [http://www.cancercell.org/cgi/content/full/5/1/25/DC1]). Cells were also treated with the SMAC tetrapeptide (AVPI) for comparison. After incubation, dead cells were identified by Annexin-V staining. All of the compounds that displayed XIAP-inhibitory activity in the in vitro enzyme derepression assay induced apoptosis of Jurkat leukemia cells in a dose-dependent manner, with the concentration required for killing half the cells (lethal dose-50%; LD_{50}) of 6–17 μM. In contrast, none of the inactive structural analogs killed Jurkat cells, at concentrations up to 50 μM. Similar results were obtained for several other cancer cell lines (see below). In

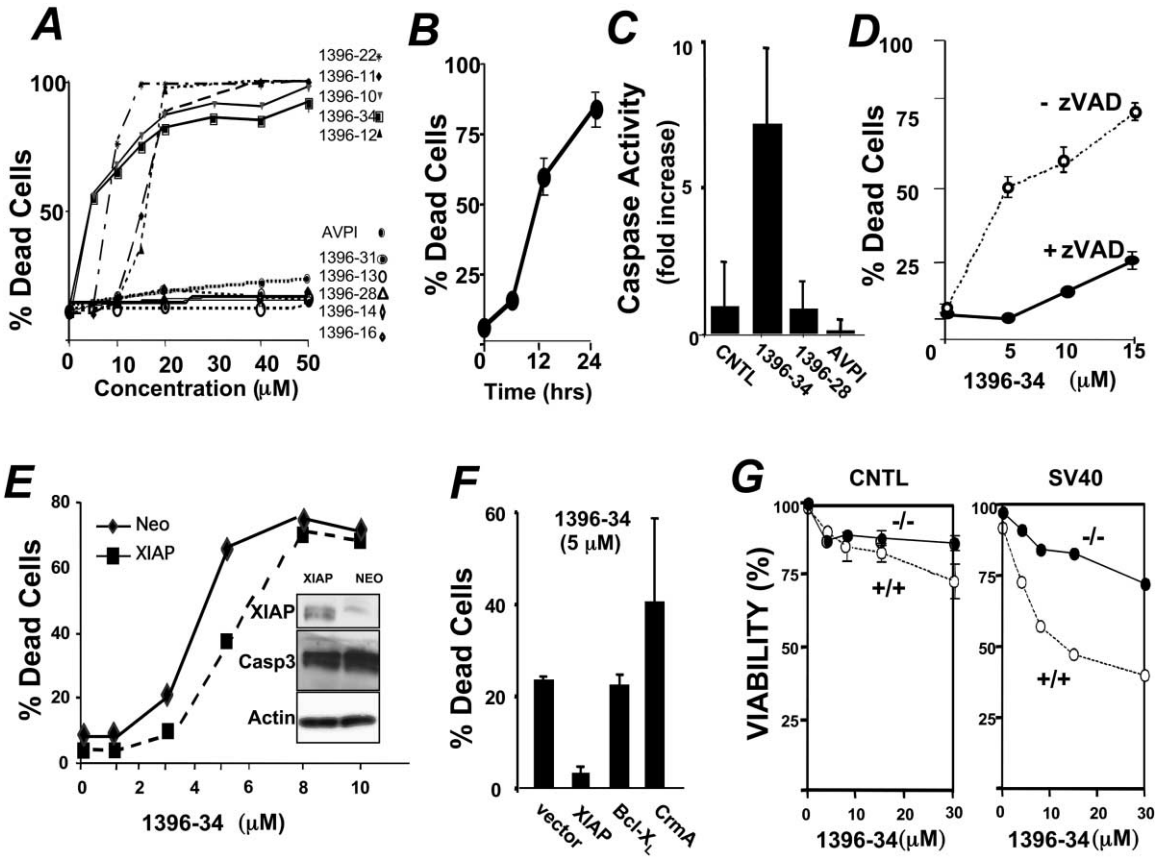

Figure 3. Characterization of cellular activity of polyphenylurea XIAP antagonists

A: Jurkat leukemia cells were cultured for 20 hr with various concentrations of either active (top) (1396-10, 11, 12, 22, 34) or inactive (bottom) (1396-13, 14, 16, 28, 31) polyphenylureas. SMAC tetrapeptide (AVPI) was also included in the study. The percentage of dead cells was determined by annexin-V staining.
B: Jurkat cells were cultured with 10 μM 1396-34 for various times before measuring percentage cell death by annexin-V staining (mean ± SE; n = 3).
C: Jurkat cells were cultured with the XIAP antagonist 1396-34, the structurally related control compound 1396-28, or SMAC 4-mer AVPI peptide at final concentrations of 8 μM for 20 hr. After incubation, Caspase-3/7 activity was measured in whole cells using a cell-permeable substrate. Data represent fold increase relative to untreated cells (mean ± SD; n = 3).
D: Jurkat cells were cultured with various concentrations of XIAP antagonists 1396-34 with (solid line) or without (dashed line) 100 μM zVAD-fmk. Percentage cell death was measured 20 hr later by annexin-V staining (mean ± SD; n = 3).
E: U937 cells stably overexpressing XIAP (squares) or neomycin control transfectants (diamonds) were cultured with various concentrations of the XIAP inhibitor 1396-34 for 20 hr. Percentage cell death was measured by annexin-V staining. Inset shows immunoblot analysis of lysates prepared from the U937 cells. Samples were normalized for total protein content and analyzed by SDS-PAGE/immunoblotting using antibodies specific for XIAP, Pro-Caspase-3, and β-Actin. Note that differences in antibody affinities and blot exposure times preclude quantitative comparisons of the ratio of XIAP:Pro-Caspase-3.
F: HeLa cells were transfected with plasmids encoding XIAP, Bcl-X_L Crm A, or empty vector. At 2 days after transfection, cells were treated with the XIAP antagonist 1396-34 (5 μM) for 20 hr, then percentage dead cells was measured by annexin-V staining (mean ± SD; n = 3).
G: Cultures of mouse embryo fibroblasts (MEFs) were established and either tested directly at low passage (left panels) or after transformation by infection with a retrovirus encoding SV40 large T antigen (right panels). Cells were cultured for 1 day with various concentrations of active compound 1396-34. Cell viability was measured by MTT assay, expressing data as a percentage relative to control, untreated cells. Data represent mean ± SD of triplicate determinations. Death of *xiap*$^{-/-}$ MEFs remained ≤10% above background for up to 3 days in cultures containing 5 μM 1396-34 (data not shown).

contrast to BIR2-targeting phenylureas, SMAC 4-mer also failed to kill Jurkat cells, possibly due to poor cell permeability. We therefore tested the effects of a SMAC peptide containing a polyarginine (membrane-penetrating) sequence (SMAC-Arg8), but it also failed to induce apoptosis of Jurkat leukemia cells at concentrations up to 50 μM (data not shown).

Among 18 compounds tested based on the polyphenylurea pharmacophore, none were identified that induced apoptosis of cancer cells that were inactive in the in vitro Caspase derepression assay. Conversely, none were identified that were active in the Caspase derepression assay but inactive in the cell-based assay. Differences in the shape of the cytotoxicity dose-response curve among XIAP-suppressing compounds may reflect subtle differences in their stability, cell permeability, serum protein binding, metabolism, or activity against other IAP family members besides XIAP.

The kinetics of apoptosis induction of Jurkat cells by XIAP antagonists such as 1396-34 was rapid, with half-maximal killing achieved at approximately 12 hr and maximum killing at ~24 hr (Figure 3B). As expected, XIAP antagonists such as 1396-34 induced Caspase activation, while inactive compounds such as 1396-28 (which differs from 1396-34 only at R2) did not (Figure 3C). The apoptosis induced by these compounds was suppressible by coculturing the cells with a broad spectrum

Caspase inhibitor, zVAD-fmk (Figure 3D), consistent with their intended mechanism.

Consistent with IAPs representing the primary target of the polyphenylureas described here, transient or stable overexpression of XIAP rendered tumor cell lines more resistant to apoptosis induction by active compounds, shifting the dose-response curve to the right, so that higher concentrations of compound were required (Figure 3E). In contrast, overexpressing antiapoptotic proteins Bcl-X_L or CrmA did not alter sensitivity of tumor cell lines to the XIAP antagonists (Figure 3F), demonstrating a specific effect. Bcl-X_L overexpression however did afford resistance to traditional anticancer drugs such as etoposide, and CrmA (a Caspase-8 inhibitor) (Zhou et al., 1997) protected cells from apoptosis induced by TRAIL (data not shown), confirming that these antiapoptotic proteins were functional in these experiments.

To further address the mechanism of these compounds, we tested their activity against normal and transformed mouse embryo fibroblasts (MEFs) derived from wild-type (*xiap*$^{+/+}$) versus knockout (*xiap*$^{-/-}$) mice (gift of C. Duckett) (Harlin et al., 2001). XIAP antagonists such as 1396-34 displayed greater cytotoxic activity against transformed *xiap*$^{+/+}$ than *xiap*$^{-/-}$ cells, consistent with mechanism-based activity (Figure 3G). If these compounds killed cells through a nonspecific mechanism, we would expect to observe more apoptosis rather than less apoptosis in XIAP-deficient cells. Interestingly, transformed MEFs displayed greater sensitivity to XIAP antagonists than normal cells (Figure 3G). In contrast to XIAP antagonists, the conventional anticancer drug daunorubicin was equally cytotoxic against normal and transformed MEFs and against *xiap*$^{+/+}$ and *xiap*$^{-/-}$ cells (Figure S1 [http://www.cancercell.org/cgi/content/full/5/1/25/DC1]). Unlike conventional cytotoxic anticancer drugs, polyphenylurea XIAP antagonists also displayed little cytotoxic activity against normal cultured rat hepatocytes, human epithelial cells, or bone marrow cells at concentrations $\leq$10 μM (Figure S2 and data not shown).

Broad activity of XIAP antagonists against tumor cell lines and leukemia cells

To assess the spectrum of apoptotic activity of IAP antagonists, we tested selected polyphenylureas on the NCI panel of 60 human tumor cell lines (Figure 4A and Figure S3 [http://www.cancercell.org/cgi/content/full/5/1/25/DC1]). All of these tumor cell lines were previously shown to express endogenous XIAP protein (Tamm et al., 2000). Cells were cultured with these compounds for 48 hr, followed by measurement of the relative number of viable cells (Alley et al., 1988). The XIAP inhibitors 1396-11, 1396-12, 1396-22, and 1396-34 induced reductions in viable cell numbers, with an average LD_{50} for the 60 cell lines of 10 ± 2.8 μM (median = 17 μM), 7.6 ± 12 μM (median = 6 μM), 11 ± 2.6 μM (median = 22 μM), and 22 ± 5 μM (median = 23 μM), respectively. Moreover, the LD_{50} was <10 μM for over one-third of the tumor cells treated with the active compounds. In contrast, LD_{50} was not reached for any of the 60 tumor cell lines after treatment with up to 70 μM of the structurally related control compound 1396-28 (Figure 4A). By comparison, when using this same assay, the mean LD_{50} for the anticancer drug etoposide in the NCI 60 cell panel is 200 ± 2.5 μM, with none of cells having LD_{50} <10 μM, and the LD_{50} for doxorubicin is 12 μM (http://dtp.nci.nih.gov) (Table S2 [http://www.cancercell.org/cgi/content/full/5/1/25/DC1]). Thus, the potency of these XIAP-inhibitory compounds compares favorably with drugs currently used in the treatment of cancer.

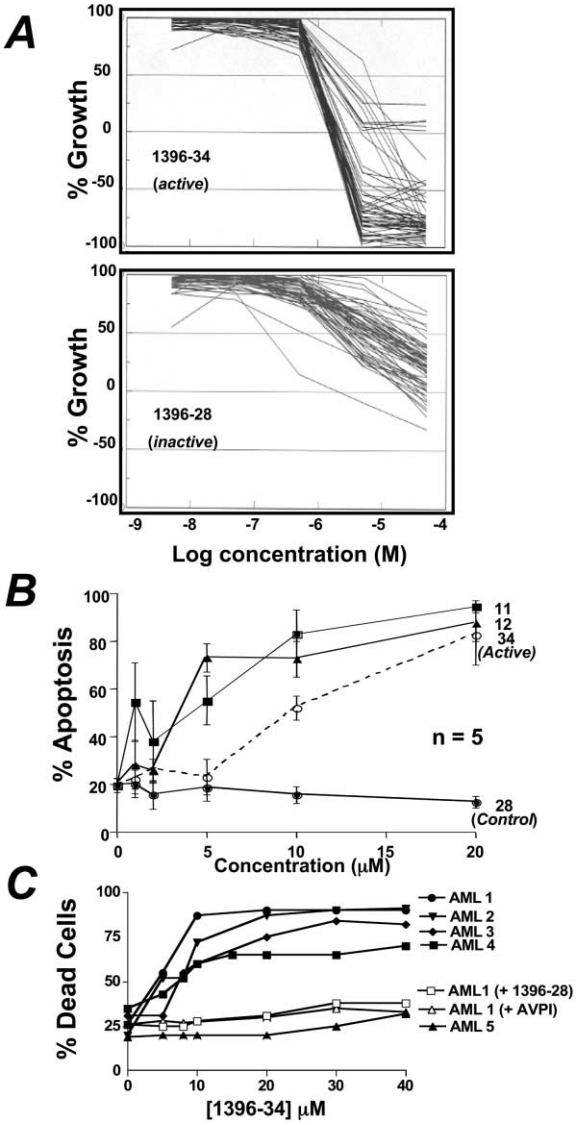

Figure 4. Broad antitumor activity of XIAP antagonists

A: Sixty human tumor cell lines were cultured with various concentrations of active XIAP antagonist 1396-34 or inactive control 1396-28 for 2 days, then relative cell numbers were determined by a protein-based colorimetric assay, expressing data as percentage growth relative to cells treated with solvent control alone. Each line represents a tumor line.

B: CLL B cells from five patients were cultured with various concentrations of active polyphenylureas 1396-11, 12, or 34 or with inactive polyphenylurea 1396-28 (negative control). After 1 day, the percentage of apoptotic cells was determined by annexin-V/propidium iodide staining, with FACS analysis. Data represent mean ± SE for n = 5.

C: AML cells were isolated from peripheral blood of five patients and cultured with various concentrations of polyphenylurea IAP antagonist 1396-34. Percentage cell death was measured 1 day later by annexin-V staining. All samples were treated with both active (1396-34) and inactive (1396-28) polyphenylurea as well as AVPI peptide, but the complete data set is shown only for AML-1. Comparable results were obtained with control compound for the other leukemia specimens.

Since many tumor and leukemia cell lines proliferate faster than normal cells, we explored whether XIAP antagonists could

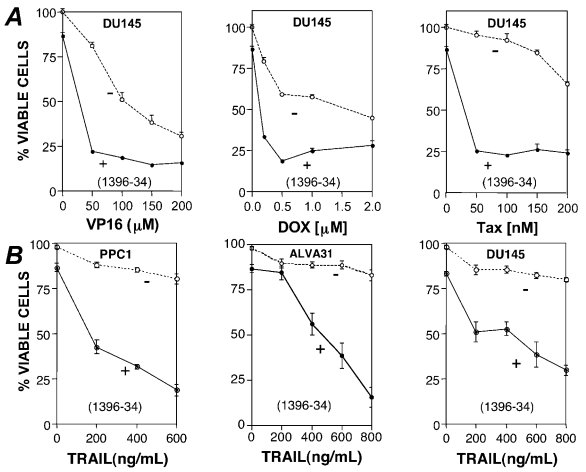

Figure 5. IAP antagonists sensitize cancer cells to chemotherapy and TRAIL

A: Du145 prostate cancer cells were cultured for 48 hr with various concentrations of Etoposide (VP16), Doxrubicin (DOX), or Paclitaxel (TAXOL), with (+) or without (−) 10 μM XIAP antagonist 1396-34. The percentage of viable cells relative to control was determined by MTT assay (mean + SE; n = 3). **B:** Alternatively, cancer cell lines were treated with various concentrations of TRAIL alone (dashed line) or in combination with (solid line) IAP antagonist 1396-34 at 1 μM. Control compounds did not affect sensitivity to chemotherapy or TRAIL (data not shown).

induce apoptosis of nonreplicating malignant cells. Accordingly, freshly isolated chronic lymphocytic leukemia (CLL) B cells from five patients and freshly isolated leukemic blasts from five patients with acute myelogenous leukemia (AML) were treated for 20–24 hr in vitro with the XIAP inhibitors 1396-11, 1396-12, or 1396-34 versus 1396-28 control compound or AVPI peptide, and the percentage of cell death was measured by annexin-V staining (Figures 4B and 4C). These leukemic cell samples contained only small percentages of cycling cells and did not replicate under standard culture conditions. XIAP antagonists induced dose-dependent cell death of primary-cultured leukemia cells in 5 of 5 CLL (Figure 4B) and 4 of 5 AML (Figure 4C) specimens examined, with LD_{50} achieved at doses of approximately 5 μM after correction for spontaneous apoptosis in culture. In contrast, the inactive control compound 1396-28 and AVPI peptide did not induce apoptosis of these leukemia cells. The membrane-penetrating SMAC-Arg8 peptide also failed to induce apoptosis of primary CLL cells (Figure S4 [http://www.cancercell.org/cgi/content/full/5/1/25/DC1]). Thus, cell replication is not required for sensitivity to BIR2-targeting polyphenylurea XIAP antagonists.

XIAP antagonists were also active against transformed hematopoietic cells from mice, inducing death of mouse 70Z/3 lymphoma and immortalized 32D myeloid cells with EC_{50} values of 8–12 μM (data not shown).

XIAP antagonists sensitize tumor cells to anticancer drugs and TRAIL

We explored whether XIAP antagonists could collaborate with conventional anticancer drugs to induce killing of tumor cells. Figure 5A presents representative data for Du145 prostate cancer cells, showing that XIAP antagonist 1396-34 significantly increased dose-dependent cytotoxicity of Etoposide (VP16), Doxorubicin (DOX), and Paclitaxel (TAXOL). Similar results were obtained for PC3 and PPC1 prostate cancer cells treated with VP16, DOX, or TAXOL and for H460 lung cancer cells treated with VP16 or DOX (Figure S5 [http://www.cancercell.org/cgi/content/full/5/1/25/DC1]). Inactive polyphenylureas failed to sensitize tumor cells to anticancer drugs (data not shown).

We similarly tested the effects of XIAP antagonists on apoptosis induction by the biological agent TRAIL, an apoptosis-inducing member of the Tumor Necrosis Factor (TNF) family (Ashkenazi and Dixit, 1998). Prototype XIAP antagonist 1396-34 sensitized PPC1, ALVA31, Du145, and HeLa cells to TRAIL-induced apoptosis (Figure 5B and data not shown). Inactive control compounds did not display this activity (data not shown).

Clonogenic survival of cancer cells is reduced by XIAP antagonists

In addition to short-term cytotoxicity assays, selected XIAP antagonists were tested for effects on clonogenic survival of cancer cells in colony formation assays, often considered a more stringent test of anticancer activity. Figure 6A shows data from two prostate cancer cell lines arbitrarily chosen for testing. Prototype XIAP antagonist 1396-34 diminished clonogenic survival of these cancer lines in a concentration-dependent manner, with an average EC_{50} dose of 3 μM ± 0.5 μM (mean ± SE). At a dose of 10 μM, colony formation was reduced to <5% of control, in contrast to inactive control compounds which had relatively little effect (Figure 6).

XIAP inhibitors are effective in tumor xenograft models

Selected IAP antagonists were tested for antitumor activity in vivo, using human tumor xenografts grown in immunocompromised mice. First, we explored what doses of IAP-inhibitory compounds were tolerated by mice, finding that 200–400 mg/kg delivered i.p. as a single or in divided doses resulted in no gross toxicity. Then, for tumor xenograft studies, PPC1 prostate or HCT116 colon cancer cells were injected subcutaneously into the flanks of Balb/c *nu/nu* mice, measuring tumor volume externally with calipers and sacrificing mice 19–24 days after tumor inoculation to weigh excised tumors. Tumors were allowed to grow for 6–7 days (when tumor were ~125 mm^3), then mice were dosed with 30 mg/kg i.p. of either solvent diluent (control) or XIAP antagonists 1396-34 (Figures 6B and 6C) or 1396-22 (data not shown), repeating the dosing the next day (2 day treatment) or for two additional days (3 day treatment). Dosing mice for just 2 or 3 days with XIAP antagonist 1396-34 or 1396-22 significantly slowed the rate of growth of both PPC1 and HCT116 tumors, thus demonstrating in vivo antitumor activity of these chemical compounds.

Analysis of tumor tissue from mice treated with XIAP antagonists showed evidence of proteolytic processing of Caspases and Caspase substrates (Figure 6D), as well as histological evidence of tumor regression by apoptosis (Figures 6D and 6E). XIAP antagonists did not appear to alter tumor cell proliferation, as judged by PCNA immunostaining. In contrast to tumor tissues, normal tissues such as liver, kidney, and spleen remained histologically normal. Also, these compounds did not induce significant abnormalities in blood cell counts or serum chemistries at doses that were sufficient for antitumor activity (Figure S6 [http://www.cancercell.org/cgi/content/full/5/1/25/DC1]).

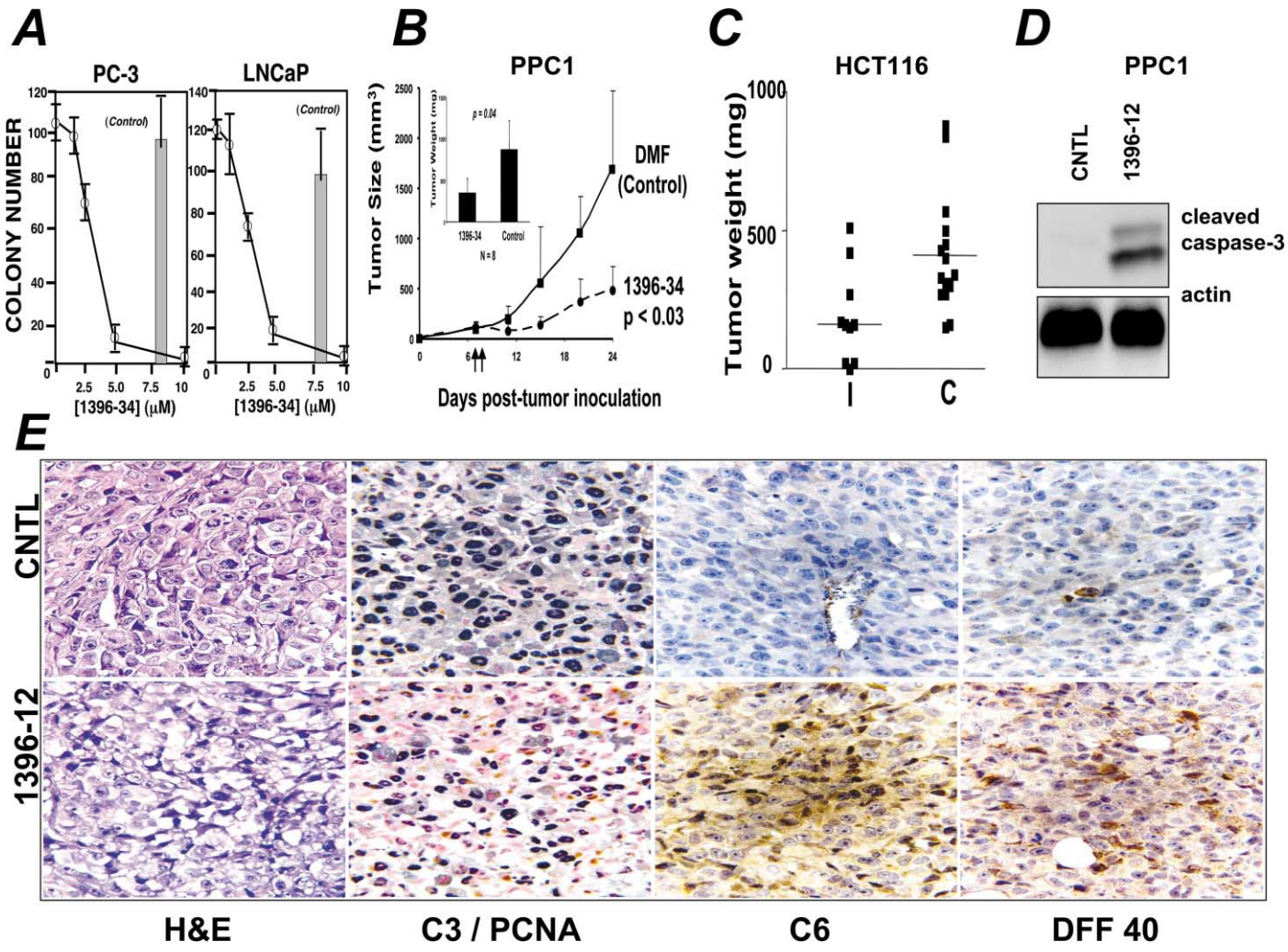

Figure 6. IAP antagonists demonstrate antitumor activity in clonogenic survival assays and tumor xenograft studies

A: IAP antagonist 1396-34 inhibits clonogenic survival of cancer cells. Two prostate cancer lines were cultured with IAP antagonist 1396-34 for 3 days, then culture medium was changed and colonies were counted 1 week later. Control compound is represented by the bars, showing only the 10 μM dose results.

B: Male Balb/C *nu*⁻/*nu*⁻ mice were injected s.c with 2.5 million PPC1 prostate cancer cells (n = 8). Half the animals received ip injections of 1396-34 in DMF at 30 mg/kg at day 7 and day 8, while the other half received DMF diluent alone. Tumor growth was monitored at least twice weekly by external calipers (mean ± SE). (Inset) At 24 days after compound injections, mice were sacrificed and tumors were excised and weighed (mean + SE).

C: HCT-116 colon cancer cells (2.5×10^6) were injected subcutaneously in the flanks of female Balb/C *nu*⁻/*nu*⁻ mice. On days 6, 7, and 8, when tumors were ~125 mm^3, mice were treated with 30 mg/kg of XIAP inhibitor 1396-34 (I) (n = 10) or solvent control (C) (n = 19) by IP injection. On day 19, the mice were sacrificed, the tumors excised and weighed. Differences in tumor size and weight on day 19 are significant by the rank sum test ($p < 0.001$). Bars represent the median tumor size or weight.

D and E: In vivo activation of Caspases in tumors by IAP antagonists. Tumor-bearing Balb/c mice (8 weeks age) were either injected i.p. for three successive days with 30 mg/kg of polyphenylurea compound 1396-12 or with an equal volume of diluent alone (CNTL). Mice were sacrificed at 24 hr after the last dose, and tumor tissue was analyzed either by (**D**) immunoblot analysis using an antibody specific for cleaved caspase-3 or actin or by (**E**) immunohistochemistry, using antibodies recognizing PCNA (blue stain [nuclei]) or the cleaved forms of Caspase-3, Caspase-6, and DFF40 (brown staining). The first panels (left) represent H&E stained sections. A double-staining method was used to assess PCNA and cleaved Caspase 3 simultaneously.

Discussion

The regulation of apoptosis is complex and multifaceted, with multiple checkpoints for either enhancing or suppressing the cell death machinery. Caspases are the ultimate effectors of apoptosis (Cryns and Yuan, 1999; Thornberry and Lazebnik, 1998). The activity of these intracellular proteases is countered by certain IAP family proteins. Thus, IAPs define a distal checkpoint for cell death control, downstream of p53, Bcl-2, and many other proteins of relevance to tumor biology. Many strategies for restoring apoptosis sensitivity in refractory cancers have been envisioned, and some are undergoing clinical testing in humans. A potential advantage of IAPs as drug targets is that they operate at a distal point in apoptosis pathways, potentially bypassing many upstream defects in apoptosis-regulatory mechanisms in tumors. However, this strategy is only valid if tumor and normal cells demonstrate differential sensitivities to IAP suppression.

In this report, we describe nonpeptidic, small-molecule inhibitors of the IAP family protein, XIAP, resulting from a screen of nearly one million synthetic chemicals. At least eight active compounds were identified within a library of polyphenylureas, the most active of which induce apoptosis of a broad range of tumor and leukemia cells in vitro as single agents, while having comparatively little toxicity on normal cells. Moreover, a perfect correlation was observed between the inhibitory activity of these compounds on XIAP in a Caspase-3 enzyme derepression assay and their ability to induce apoptosis of malignant cells in culture. Cell death induction by XIAP antagonists was blocked by Caspase inhibitor zVAD-fmk and reduced by overexpressing XIAP but unaffected by overexpression of upstream apoptosis suppressors Bcl-X_L and CrmA, which are characteristics consistent with mechanism-based cytotoxicity. These compounds were also considerably less toxic against transformed *xiap*$^{-/-}$ cells lacking the intended drug target, further validating their mechanism. While this manuscript was in preparation, Wu et al. described a structurally distinct class of XIAP antagonists (Wu et al., 2003). However, the activity of those compounds against endogenous XIAP in tumor cells in vitro or in vivo and their toxicity to normal cells were not determined.

The results obtained with our XIAP antagonists suggest that cancer cells have an intrinsic drive to activate Caspases, such that functional removal of IAPs permits apoptosis to occur. This hypothesis is consistent with recent data showing evidence of processed Caspase-3 in tumor cell lines and tumor tissues, offset by overexpression of XIAP or other IAP family members (Yang et al., 2003a). In this regard, many causes of Caspase activation in tumor cells can be envisioned, including protooncogene activation, disobeyance of cell cycle checkpoints associated with defective DNA replication and chromosome segregation, and loss of cell attachment (anchorage-independent growth), all of which are known to drive apoptosis unless countered by antiapoptotic proteins (Evan and Vousden, 2001). In contrast, normal cells would be expected to have fewer drives to Caspase activation, thus rendering them less dependent on IAPs—a notion supported by gene ablation studies in mice, which have revealed little or no adverse phenotypes in animals lacking XIAP, cIAP1, cIAP2, or NAIP (Harlin et al., 2001; Holcik et al., 2000) (T. Mak, personal communication).

XIAP is the best-studied of the human IAP family proteins from the standpoint of biochemical mechanism (Stennicke et al., 2002), and its overexpression in several types of human cancers has been documented (Ferreira et al., 2001; Hofmann et al., 2002; Krajewska et al., 2003; Tamm et al., 2000). XIAP suppresses the downstream effector proteases Caspase-3 and -7 via its BIR2 region, while suppressing upstream initiator protease Caspase-9 via the BIR3 region (Deveraux et al., 1997, 1999; Takahashi et al., 1998). Our objective was to search for agents that overcome the inhibitory effect of XIAP on the effector proteases Caspase-3 and -7, favoring this strategy over screens targeting the Caspase-9-suppressing activity of XIAP. The polyphenylurea-based compounds described here overcome the inhibitory effects of XIAP on Caspases-3 and -7 but not Caspase-9 in vitro. Also, they are active against a fragment of XIAP which is known to be sufficient for binding and inhibiting Caspase-3, comprised only of the BIR2 region with an accompanying upstream flanking segment (Riedl et al., 2001; Sun et al., 1999; Takahashi et al., 1998). The mechanism of these small-molecule compounds remains to be elucidated, but they differ fundamentally from the endogenous XIAP antagonist SMAC, which is known to target BIR3 and overcome Caspase-9 suppression (Liu et al., 2000; Srinivasula et al., 2001; Wu et al., 2000). Furthermore, the polyphenylurea-based XIAP antagonists do not compete with SMAC peptides for binding to XIAP, adding further weight to the evidence that they suppress XIAP through a non-SMAC mechanism. It remains to be determined whether the polyphenylurea-based compounds described here mimic the effects of other endogenous antagonists of XIAP, such as XAF1 (Liston et al., 2001), versus suppressing XIAP through a novel mechanism. It also remains formally possible that our compounds bind an adjacent site in the SMAC tetrapeptide binding pocket of BIR domains, thus acting through an analogous mechanism as SMAC but favoring BIR2 rather than BIR3 of XIAP. Differences in the mechanism of XIAP inhibition by our polyphenylurea-based compounds (which target the Caspase-3-suppressing activity of XIAP) compared to previously described SMAC peptides (which target the Caspase-9-suppressing activity of XIAP) presumably explain why the small-molecule antagonists described here induce apoptosis directly, while SMAC peptides generally sensitize tumor cells to apoptosis-inducing agents but often fail to induce apoptosis as single agents (Fulda et al., 2002; Yang et al., 2003b). The difficulties of delivering peptides into cells could account for some of the bioactivity differences compared to our compounds, but even membrane-penetrating SMAC-Arg8 failed to induce apoptosis of leukemia cells at concentrations 5–10 times higher than our BIR2-targeting XIAP antagonists.

The human genome encodes eight IAP family proteins. While all of these proteins contain BIR domains (by definition), the mechanisms by which they suppress apoptosis vary. For example, while XIAP, cIAP1, and cIAP2 have been demonstrated to directly bind and suppress Caspase-3 in vitro (Deveraux et al., 1997; Roy et al., 1997), inhibition of this Caspase by the IAP family member Survivin is controversial (Reed, 2001). Also, ML-IAP may operate as a SMAC antagonist rather than as a Caspase inhibitor (Vucic et al., 2002). The inhibitory mechanisms for keeping IAP family protein in check in vivo also vary, given that SMAC binds XIAP but not Survivin (Marusawa et al., 2003). Thus, it is unlikely that the polyphenylurea-based XIAP antagonists described here are active against all IAP family proteins. Additional structure-activity relationship studies will define the repertoire of IAP family proteins that are affected by these compounds, thus guiding future decisions about which types of cancer might be most sensitive to this structural class of antagonists. The potential for these compounds to inhibit other IAP family members besides XIAP may account for the lack of correlation of XIAP protein levels with EC_{50} in the NCI panel of 60 tumor cell lines (Tamm et al., 2000) (http://dtp.nci.nih.gov), though other explanations also are possible, such as differences among tumor cell lines in the ratio of XIAP to endogenous XIAP antagonists (e.g., XAF1, SMAC, HtrA2) and regulation of XIAP by posttranslational modifications.

XIAP blocks apoptosis at a distal step in apoptosis pathways, at the convergence of cell death pathways activated by mitochondria-dependent death stimuli (e.g., DNA-damaging drugs; x-irradiation; growth factor deprivation) and by mitochondria-independent TNF/Fas family death ligands (e.g., TNF, FasL, TRAIL). Consistent with this mechanism, we observed that small-molecule antagonists of XIAP sensitized tumor cells to cell death induced by anticancer drugs as well as TNF family

member TRAIL. The ability of XIAP antagonists to collaborate with a broad range of anticancer drugs in vitro raises the intriguing possibility of exploiting such compounds as chemosensitizers for cancer therapy but requires further exploration with respect to effects on sensitivity of normal cells to chemotherapy. The ability of polyphenylurea-based XIAP antagonists to suppress tumor growth in vivo in tumor xenograft models as single agents delivered at modest doses suggests that these chemical compounds could serve as prototypes for drug development, providing suitable toxicological and pharmacokinetic profiles can be achieved. Regardless, the results provide validation of IAPs as targets for cancer drug discovery based on a small-molecule chemical strategy that seeks to overcome the ability of these proteins to suppress downstream effector Caspases.

Experimental Procedures

Caspase derepression assay

Recombinant proteins were produced in bacteria and purified as described (Deveraux et al., 1997; Stennicke and Salvesen, 1997; Takahashi et al., 1998). GST-XIAP (46 nM) or GST-BIR2 (1.2 nM) was added to active Caspase-3-His6 (0.36 nM) (Stennicke and Salvesen, 1997) in 100 μL of 50 mM HEPES (pH 7.4), 10% sucrose, 1 mM EDTA, 0.1% CHAPS, 100 mM NaCl, and 10 mM DTT to achieve ~75% inhibition of protease activity. Recombinant GST-XIAP (50 nM) was added to active (ΔCard)Caspase 9 E306A (2.2 nM) (Stennicke et al., 1999). Activity of Caspase-3 and -9 was measured by monitoring cleavage of the fluorogenic tetrapeptide substrates acetyl-Asp-Glu-Val-Asp-AFC and acetyl-Leu-Glu-His-Asp-AFC (BIOMOL, Plymouth, PA) at 100 μM, respectively. Generation of fluorogenic AFC (7-amino-4-trifluoromethyl coumarin) product was measured with a spectrofluorometric plate reader in kinetic mode for 30 min at 37°C using excitation and emission wavelengths of 405 nm and 510 nm, respectively. Chemical compounds were screened at 6.25, 12.5, and 25.0 μg/ml to identify compounds that increased Caspase-3-induced cleavage of DEVD-AFC, setting a 2-fold rise in protease activity as the threshold for positivity. Control reactions lacked XIAP, and all assays were conducted in the linear range of substrate hydrolysis to avoid substrate depletion artifacts.

Chemical libraries

Eleven mixtured-based small-molecule combinatorial libraries constituting approximately one million compounds were screened. The library from which the described XIAP inhibitors were identified was a polyphenylurea library composed of a total of 89,856 compound (Nefzi et al., 2000) synthesized in the positional scanning format (Pinilla et al., 1992) with 48 functionalities at the R_1 and R_2 positions and 39 functionalities at the R_3 position using the libraries from libraries approach (Ostresh et al., 1994) ($48 \times 48 \times 39 = 89{,}856$). Three separate sublibraries were synthesized representing the same diversity and differing solely by the location of the defined position, allowing for library deconvolution essentially as described (Pinilla et al., 1992, 2003). Individual compounds were synthesized by solid-phase methods, purified by 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 preparations of purified individual compounds.

SMAC peptide competition assay

Two types of assays were developed. For the first assay, GST-XIAP or control protein GST-Bid at 1 μM in PBS (pH 7.4) was adsorbed overnight at 4°C to 96-well flat-bottom black plates (Corning Inc, Corning, NY) using 100 μL per well. Plates were washed extensively with H_20, then incubated for 1 hr with 0.01% gelatin in phosphate-buffered saline (pH 7.4) (PBS). SMAC 7-mer (AVPIAQK), conjugated at the C terminus with biotin, was added to the plates in 100 μL of PBS containing 0.01% gelatin with or without XIAP-inhibitory compounds in ≤1% (v:v) DMSO and incubated for 2 hr at room temperature. Plates were then washed five times with H_2O, and 0.1 mL/well was added of a solution containing DELFIA assay buffer and Europium (EU)-conjugated Streptavidin (Perkin-Elmer). After 1 hr incubation, plates were washed in H_2O and 0.1 mL/well of Delphia Enhancement solution was added and the plates were incubated for 15 min. EU-based fluorescence was measured with a plate reader (LJL analyst #1) using 360 nm excitation and 620 nm emission wavelengths with a dichroic mirror at 400 nm. For the second assay, biotinylated SMAC 7-mer peptide (50 ng) was bound to 96-well plates coated with NeutrAvidin (Pierce, Rockford, IL) at 1 μg/mL in 100 μL per well of 50 mM HEPES (pH 7.4), 100 mM NaCL, 1 mM EDTA, 10% sucrose, 0.1% CHAPS, 10 mM DTT. Then, GST-XIAP was added at 0.1 μg/mL in 100 μL with or without chemical compounds in DMSO. After incubation for 1 hr at room temperature, plates were washed with PBS with 0.05% Tween 20, and bound GST-XIAP was detected by addition of mouse anti-GST monoclonal antibody (1:2000 dilution), followed by anti-mouse horseradish peroxidase (HRP)-conjugated IgG and 3,3′5,5′-Tetramethylbenzidine Base (TMB) substrate, using a plate reader at 450 nm.

Cell culture

Tumor cell lines and primary cells were cultured in RPMI 1640, DMEM (high-glucose), DMEM-H21, or IMDM medium, with 5%–10% fetal calf serum (FCS), 1 mM L-glutamine, and penicillin/streptomycin. Primary epithelial cells were obtained from Cambrex (Walkersville, MD). Normal PBLs, bone marrow mononuclear cells (BMMCs), and primary leukemia cells were isolated by density centrifugation of samples from whole blood or bone marrow aspirates obtained from either healthy donors or patients, with informed consent under IRB approval. AML blasts were verified by immunofluorescence flow cytometry to be composed of >80% CD-34-positive cells. PBLs and CLLs were verified to be comprised of >80% T cells and >95% B cells, respectively, by flow cytometry. Chemical compounds were added to cultures in DMSO. Apoptosis-inducing agents included TRAIL (BioMol), Paciltaxel (Sigma, St. Louis, MO), Etoposide (Sigma), and Doxorubicin (Sigma).

Cell death assays

Various methods were employed to assess effects of XIAP antagonists on cell viability, including Annexin-V staining, MTT dye reduction, and Sulforhodamine B staining, essentially as described (Alley et al., 1988; Dimitroulakos et al., 2001; Schimmer et al., 2003), in either 12-, 24-, or 96-well formats. For some experiments, Annexin-V staining was assessed using a 96-well compatable flow cytometer (Guava Technologies, Inc.).

Caspase activity

To measure cellular Caspase activity, 10^4 cells per well were seeded into 96-well flat-bottom black plates (Corning Inc, Corning, NY). Cells were then cultured 1 day later with the XIAP inhibitors or control compounds for 20 hr. Caspase-3 activity was detected by the addition of the cell-permeable "CellProbe HT Caspase 3/7 substrate" according to the manufacturer's instructions (Beckman Coulter, Palo Alto, CA). Assays were performed at 1 hr after incubation with the Caspase-3 substrate, measuring release of fluorescent rhodamine dye using a fMax spectrofluorimeter plate reader (excitation at 485 nm and emission at 538 nm) (Sunnyvale, CA).

Clonogenic survival assays

Tumor cells (n = 200) in RPMI with 10% FCS were seeded into 12-well plates and cultured with polyphenylurea XIAP inhibitors or inactive control compounds for 3 days. The medium was then replaced, and cells were cultured for 1 week before fixing with 4% paraformaldehyde and staining with methylene blue and counting colonies. All assays were performed in triplicate (mean ± SD).

Cell transfections

For transient transfections, HeLa cells were transfected with pcDNA3 (control) or pcDNA3-based plasmids encoding human XIAP, Bcl-X_L, or CrmA, using Lipofectamine Plus (Gibco-BRL, Carlsbad, CA), essentially as described (Deveraux et al., 1997, 1998). Cells were treated with the XIAP inhibitors or control compounds in <1% DMSO at 2 days after transfection. Stably transfected U937 cells were generously provided by D. Kufe (Dana Farber; Boston). Expression was confirmed by immunoblotting.

Tumor xenografts

PPC1 or HCT116 (2.5×10^6) cells in 100 μL of RPMI were injected subcutaneously into the flanks of male Balb-c *nu*$^-$/*nu*$^-$ mice (Simonsen, Gilroy, CA) age 4–6 weeks. At various times thereafter when tumors were ~125 mm^3 (day 6 or 7), mice were injected i.p. with 30 mg/kg of chemical compounds or an equivalent volume of solvent diluent control (DMSO or DMF). The dose

was repeated the next day or for 2 additional days. Tumor size was measured at least twice weekly with calipers for approximately 3 weeks. Mice were sacrificed by CO_2 asphixiation at 19–24 days after tumor inoculation, tumor excised, and weighed. In some experiments, mice were sacrificed 24 hr after the last dose and tumor tissue was excised and either homogenized for immunoblot analysis or fixed and sectioned for histological analysis. Tissue sections were subjected either to hematoxylin-eosin staining or were stained with antibodies specific for cleaved Caspase-3, Caspase-6, or DFF40, alone or in combination with anti-PCNA antibody. All animal experiments were conducted in accordance with the NIH guidelines under institutional approval.

Acknowledgments

We thank D. Kufe and C. Duckett for reagents; M.D. Minden, M. Andreeff, and T. Kipps (CLL Research Consortium) for patient samples; C. Stehlik and S. Kitada for control assays; and R. Cornell and J. Valois for secretarial assistance. We acknowledge the generous support of the NIH (NCI-CA78040, NIA-AG15402, and NCI-CA89827), CaP-CURE, Canadian Institutes of Health Research, Foundation for Medical Research (FRM), Philippe Foundation, Leukemia Lymphoma Society, and the American Society of Hematology.

Received: September 23, 2003
Revised: November 12, 2003
Accepted: November 13, 2003
Published: January 19, 2004

References

Alley, M.C., Scudiero, D.A., Monks, A., Hursey, M.L., Czerwinski, M.J., Fine, D.L., Abbott, B.J., Mayo, J.G., Shoemaker, R.H., and Boyd, M.R. (1988). Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res. *48*, 589–601.

Ashkenazi, A., and Dixit, V.M. (1998). Death receptors: signaling and modulation. Science *281*, 1305–1308.

Bilim, V., Kasahara, T., Hara, N., Takahashi, K., and Tomita, Y. (2003). Role of XIAP in the malignant phenotype of transitional cell cancer (TCC) and therapeutic activity of XIAP antisense oligonucleotides against multidrug-resistance TCC in vitro. Int. J. Cancer *103*, 29–37.

Byrd, J.C., Kitada, S., Flinn, I., Aron, J.L., Pearson, M., Lucas, D., and Reed, J.C. (2002). The mechanism of tumor cell clearance by Rituximab in vivo in patients with B-cell chronic lymphocytic leukemia: evidence of caspase activation and apoptosis induction. Blood *99*, 1038–1043.

Cryns, V., and Yuan, Y. (1999). Proteases to die for. Genes Dev. *12*, 1551–1570.

Deveraux, Q.L., Takahashi, R., Salvesen, G.S., and Reed, J.C. (1997). X-linked IAP is a direct inhibitor of cell death proteases. Nature *388*, 300–304.

Deveraux, Q.L., Roy, N., Stennicke, H.R., Van Arsdale, T., Zhou, Q., Srinivasula, M., Alnemri, E.S., Salvesen, G.S., and Reed, J.C. (1998). IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J. *17*, 2215–2223.

Deveraux, Q.L., Leo, E., Stennicke, H.R., Welsh, K., Salvesen, G.S., and Reed, J.C. (1999). Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. EMBO J. *18*, 5242–5251.

Dimitroulakos, J., Ye, L.Y., Benzaquen, M., Moore, M.J., Kamel-Reid, S., Freedman, M.H., Yeger, H., and Penn, L.Z. (2001). Differential sensitivity of various pediatric cancers and squamous cell carcinomas to lovastatin-induced apoptosis: therapeutic implications. Clin. Cancer Res. *7*, 158–167.

Evan, G.I., and Vousden, K.H. (2001). Proliferation, cell cycle and apoptosis in cancer. Nature *411*, 342–348.

Ferreira, C.G., van der Valk, P., Span, S.W., Jonker, J.M., Postmus, P.E., Kruyt, F.A., and Giaccone, G. (2001). Assessment of IAP (inhibitor of apoptosis) proteins as predictors of response to chemotherapy in advanced non-small-cell lung cancer patients. Ann. Oncol. *12*, 799–805.

Fulda, S., Wick, W., Weller, M., and Debatin, K.M. (2002). Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma in vivo. Nat. Med. *8*, 808–815.

Gordon, G.J., Appasani, K., Parcells, J.P., Mukhopadhyay, N.K., Jaklitsch, M.T., Richards, W.G., Sugarbaker, D.J., and Bueno, R. (2002). Inhibitor of apoptosis protein-1 promotes tumor cell survival in mesothelioma. Carcinogenesis *23*, 1017–1024.

Harlin, H., Reffey, S.B., Duckett, C.S., Lindsten, T., and Thompson, C.B. (2001). Characterization of XIAP-deficient mice. Mol. Cell. Biol. *21*, 3604–3608.

Hofmann, H.S., Simm, A., Hammer, A., Silber, R.E., and Bartling, B. (2002). Expression of inhibitors of apoptosis (IAP) proteins in non-small cell human lung cancer. J. Cancer Res. Clin. Oncol. *128*, 554–560.

Holcik, M., Thompson, C.S., Yaraghi, Z., Lefebvre, C.A., MacKenzie, A.E., and Korneluk, R.G. (2000). The hippocampal neurons of neuronal apoptosis inhibitory protein 1 (NAIP1)-deleted mice display increased vulnerability to kainic acid-induced injury. Proc. Natl. Acad. Sci. USA *97*, 2286–2290.

Krajewska, M., Krajewski, S., Turner, B., Bubendorf, L., Kallioniemi, O.-P., Shabaik, A., Vitiello, A., Goddison, S., and Reed, J.C. (2003). Elevated expression of inhibitor of apoptosis proteins (IAPs) in prostate cancer. Clin. Cancer Res. *9*, 4914–4925.

Liston, P., Fong, W.G., Kelly, N.L., Toji, S., Miyazaki, T., Conte, D., Tamai, K., Craig, C.G., McBurney, M.W., and Korneluk, R.G. (2001). Indentificaiton of XAF1 as an antagonist of XIAP anti-caspase activity. Nat. Cell Biol. *3*, 128–133.

Liu, Z., Sun, C., Olejniczak, E.T., Meadows, R.P., Betz, S.F., Oost, T., Herrmann, J., Wu, J.C., and Fesik, S.W. (2000). Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. Nature *408*, 1004–1008.

Marusawa, H., Matsuzawa, S.-I., Welsh, K., Zou, H., Armstrong, R., Tamm, I., and Reed, J.C. (2003). HBXIP functions as a cofactor of survivin in apoptosis suppression. EMBO J. *22*, 2729–2740.

Nefzi, A., Ong, N., and Houghten, R.A. (2000). An efficient two-step synthesis of mono-, di- and triureas from resin-bound amides. Tetrahedron Lett. *41*, 5441–5446.

Ostresh, J.M., Husar, G.M., Blondelle, S.E., Dörner, B., Weber, P.A., and Houghten, R.A. (1994). Libraries from libraries: Chemical transformation of combinatorial libraries to extend the range and repertoire of chemical diversity. Proc. Natl. Acad. Sci. USA *91*, 11138–11142.

Pinilla, C., Appel, J.R., Blanc, P., and Houghten, R.A. (1992). Rapid identification of high affinity peptide ligands using positional scanning synthetic peptide combinatorial libraries. Biotechniques *13*, 901–905.

Pinilla, C., Appel, J.R., Borras, E., and Houghten, R.A. (2003). Advances in the use of synthetic combinatorial chemistry: mixture-based libraries. Nat. Med. *9*, 118–122.

Reed, J.C. (2001). The survivin saga goes in vivo. J. Clin. Invest. *108*, 965–969.

Riedl, S.J., Renatus, M., Schwarzenbacher, R., Zhou, Q., Sun, C., Fesik, S.W., Liddington, R.C., and Salvesen, G.S. (2001). Structural basis for the inhibition of caspase-3 by XIAP. Cell *104*, 791–800.

Roy, N., Deveraux, Q.L., Takashashi, R., Salvesen, G.S., and Reed, J.C. (1997). The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J. *16*, 6914–6925.

Sasaki, H., Sheng, Y., Kotsuji, F., and Tsang, B.K. (2000). Down-regulation of X-linked inhibitor of apoptosis protein induces apoptosis in chemoresistant human ovarian cancer cells. Cancer Res. *60*, 5659–5666.

Schimmer, A.D., Munk-Pedersen, I., Kitada, S., Demiralp, E., Minden, M.D., Pinto, R., Mah, K., Andreeff, M., Kipps, T., and Reed, J.C. (2003). Functional blocks in Caspase activation pathways are common in leukemia and predict patient-response to induction chemotherapy. Cancer Res. *63*, 1242–1248.

Srinivasula, S.M., Hegde, R., Saleh, A., Datta, P., Shiozaki, E., Chai, J., Lee, R.-A., Robbins, P.D., Fernandes-Alnemri, T., Shi, Y., and Alnermi, E.S. (2001).

A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. Nature *410*, 112–116.

Stennicke, H.R., and Salvesen, G.S. (1997). Biochemical characteristics of caspase-3, -6, -7, and -8. J. Biol. Chem. *272*, 25719–25723.

Stennicke, H.R., Deveraux, Q.L., Humke, E.W., Reed, J.C., Dixit, V.M., and Salvesen, G.S. (1999). Caspase-9 can be activated without proteolytic processing. J. Biol. Chem. *274*, 8359–8362.

Stennicke, H.R., Ryan, C.A., and Salvesen, G.S. (2002). Reprieval from execution: the molecular basis of caspase inhibition. Trends Biochem. Sci. *27*, 94–101.

Sun, C., Cal, M., Gunasekera, A., Meadows, R., Wang, H., Chen, J., Zhang, H., Wu, W., Xu, M., Ng, S., and Fesik, S. (1999). NMR structure and mutagenesis of the inhibitor-of-apoptosis protein XIAP. Nature *401*, 818–821.

Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., and Takahashi, R. (2001). A serine protease HtrA2/Omi, is released from the mitochondria and interacts with XIAP, induces cell death. Mol. Cell *8*, 613–621.

Takahashi, R., Deveraux, Q.L., Tamm, I., Welsh, K., Assa-Munt, N., Salvesen, G.S., and Reed, J.C. (1998). A single BIR domain of XIAP sufficient for inhibiting caspases. J. Biol. Chem. *273*, 7787–7790.

Tamm, I., Kornblau, S.M., Segall, H., Krajewski, S., Welsh, K., Kitada, S., Scudiero, D.A., Tudor, G., Qui, Y.H., Monks, A., et al. (2000). Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias. Clin. Cancer Res. *6*, 1796–1803.

Thornberry, N.A., and Lazebnik, Y. (1998). Caspases: enemies within. Science *281*, 1312–1316.

Vucic, D., Deshayes, K., Ackerly, H., Pisabarro, M.T., Kadkhodayan, S., Fairbrother, W.J., and Dixit, V.M. (2002). SMAC negatively regulates the anti-apoptotic activity of melanoma inhibitor of apoptosis (ML-IAP). J. Biol. Chem. *277*, 12275–12279.

Wu, G., Chai, J., Suber, T.L., Wu, J.-W., Du, C., Wang, X., and Shi, Y. (2000). Structural basis of IAP recognition by Smac/DIABLO. Nature *408*, 1008–1012.

Wu, T.Y., Wagner, K.W., Bursulaya, B., Schultz, P.G., and Deveraux, Q.L. (2003). Development and characterization of nonpeptidic small molecule inhibitors of the XIAP/caspase-3 interaction. Chem. Biol. *10*, 759–767.

Xu, G., Cirilli, M., Huang, Y., Rich, R.L., Myszka, D.G., and Wu, H. (2001). Covalent inhibition revaled by the crystal structure of the caspase-8/p35 complex. Nature *410*, 494–497.

Yang, L., Cao, Z., Yan, H., and Wood, W.C. (2003a). Coexistence of high levels of apoptotic signaling and inhibitor of apoptosis proteins in human tumor cells: implication for cancer specific therapy. Cancer Res. *63*, 6815–6824.

Yang, L., Mashima, T., Sato, S., Mochizuki, M., Sakamoto, H., Yamori, T., Oh-Hara, T., and Tsuruo, T. (2003b). Predominant suppression of apoptosome by inhibitor of apoptosis protein in non-small cell lung cancer H460 cells: therapeutic effect of a novel polyarginine-conjugated Smac peptide. Cancer Res. *63*, 831–837.

Zhou, Q., Snipas, S., Orth, K., Muzio, M., Dixit, V.M., and Salvesen, G.S. (1997). Target protease specificity of the viral serpin CrmA: analysis of five caspases. J. Biol. Chem. *272*, 7797–7800.