Peptides Mimicking the Unique ARTS-XIAP Binding Site Promote Apoptotic Cell Death in Cultured Cancer Cells

Marie-Jeanne Carp

Purpose: XIAP [X-linked inhibitor of apoptosis (IAP) protein] is the best characterized mammalian caspase inhibitor. XIAP is frequently overexpressed in a variety of human tumors, and genetic inactivation of XIAP in mice protects against lymphoma. Therefore, XIAP is an attractive target for anticancer therapy. IAP antagonists based on a conserved IAP-binding motif (IBM), often referred to as "Smac-mimetics," are currently being evaluated for cancer therapy in the clinic. ARTS (Sept4_i2) is a mitochondrial proapoptotic protein which promotes apoptosis by directly binding and inhibiting XIAP via a mechanism that is distinct from all other known IAP antagonists. Here, we investigated the ability of peptides derived from ARTS to antagonize XIAP and promote apoptosis in cancer cell lines.

Experimental Design: The ability of synthetic peptides, derived from the C-terminus of ARTS, to bind to XIAP, stimulate XIAP degradation and induce apoptosis was examined. We compared the response of several cancer cell lines to different ARTS-derived peptides. Pull-down assays were used to examine binding to XIAP, and apoptosis was evaluated using terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling, caspase activation, and Western blot analyses of caspase substrates.

Results: The C-terminus of ARTS contains a unique sequence, termed ARTS-IBM (AIBM), which is important for binding to XIAP and cell killing. AIBM peptides can bind to XIAP-BIR3, penetrate cancer cells, reduce XIAP levels, and promote apoptosis.

Conclusions: Short synthetic peptides derived from the C-terminus of ARTS are sufficient for binding to XIAP and can induce apoptosis in cancer cells. These results provide proof-of-concept for the feasibility of developing ARTS-based anticancer therapeutics.
Translational Relevance

Because inhibitors of apoptosis (IAP) are overexpressed in a variety of tumors, they have become promising targets for developing novel anticancer therapeutics, and several companies (including Genentech, Novartis, Ascenda, Tetralogics, and others) are currently carrying out clinical trials with compounds targeting IAPs. Importantly, all these companies use IAP antagonists based on the IAP-binding motif (IBM). ARTS does not contain an IBM. Instead ARTS uses a different, unique IBM not found in any other protein, which we termed ARTS-IBM (AIBM). Moreover, recently it has been shown that these IBM-based IAP antagonists initially designed to target XIAP, which is considered the most potent caspase inhibitor, were found to preferentially induce degradation of cIAPs, but not XIAP (Schimmer, 2004; Dean and colleagues, 2007; Gaither and colleagues, 2007; Petersen and colleagues, 2007; Varfolomeev and colleagues, 2007; Vinc and colleagues, 2007; Ashwell, 2008). Our work shows that ARTS-based peptides can penetrate and kill cancer cells by targeting XIAP. These AIBM-based peptides provide proof-of-concept for the feasibility of developing new ARTS-based anticancer therapeutics.

Experimental Procedures

Antibodies

Antibodies to the various proteins were purchased from the indicated companies and used as instructed. Importantly, unless noted otherwise, in all our assays we used the monoclonal anti-ARTS antibody (Sigma), which is the only currently commercially available antibody directed against the unique C-terminus of ARTS. Antibodies against GST (glutathione S-transferase, B-14, sc-138, Santa Cruz), H2AX (DR1016; Calbiochem), XIAP (#610716; BD), cIAP (AF818; R&D systems), caspase-9 (C7729, Sigma; #9502, Cell Signalling), caspase-3 (5A1E, Cell Signaling; #9661, Cell Signaling), cleaved PARP (97, 98)}
Western blot analyses

Western blot analyses were carried out as described in Lotan and colleagues (23). Visualization was done using LAS4000 luminescent image analyzer (Fujifilm). For densitometry analyses, intensity of signals was compared with actin using TotalLab TL100 graphic software (Nonlinear Dynamics Ltd).

Immunofluorescence assay

Immunofluorescence assay was carried out as described in Lotan and colleagues (23).

Image analysis was carried out using confocal laser microscopy (Zeiss LSM 510) or fluorescent microscopy (Nikon 50i).

Binding assays

In vivo GST pull-down binding studies. COS-7 cells were cotransfected with pEBG empty vector or pEBG-XIAP-BIR3 together with pCS2-6myc-ARTS or pCS2-6myc vector. The cells were lysed in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8), 1% NP-40, 0.5% deoxycholate acid containing protease inhibitors (mini Complete; Roche)]. The samples were left rotating for 4 hours at 4°C, followed by GST pull-down using glutathione beads. Samples were centrifuged at 4,000 rpm at 4°C for 4 minutes and washed 5 times in lysis buffer. Proteins were eluted from beads following 5 minutes of boiling in sample buffer. Proteins were separated on 12.5% SDS–PAGE gel, followed by Western blot analysis.

In vitro coimmunoprecipitation of ARTS-derived peptides. The fluorescein-labeled 27 mer ARTS-CTD and the fluorescein-labeled 9 mer peptides (100 nmol/L) were incubated with purified GST-BIR3. The complexes were incubated with anti-fluorescein antibodies conjugated to protein A and G sepharose beads (Amersham Biosciences). Samples were centrifuged at 4,000 rpm at 4°C for 5 minutes and washed 3 times in PBS. Proteins were eluted from beads following 5 minutes of boiling in sample buffer. Proteins were separate on 12.5% SDS–PAGE gel, followed by Western blot analysis.

Mortality assay

K562 cells were incubated in 24-well plate at 0.2 × 10^6 cells per well in hypotonic media (0.5 mL RPMI medium +...
0.5 mL DDW) containing fluorescein-labeled peptides. After 4 hours the media was replaced with complete RPMI medium containing the peptides, and incubation was continued for additional 4 hours. Cells were harvested and washed once with PBSx1. Cells were suspended in equal volumes of PBSx1 and seeded on slides precoated with poly-L-lysine (Sigma). Paraformaldehyde (4%) was used to fix the cells for 30 minutes, followed by 2 repeated washes with PBSx1. Permeabilization was done using 0.1% TritonX-100 and 0.1% sodium citrate in DDW for 5 minutes. Staining with DAPI (4′, 6-diamidino-2-phenylindole; Vectoshield, Vector, H-1200) and scoring cells was done using fluorescent microscope Nikon 50i. Counts for each sample represent the average number of viable cells in 8 different fields.

In situ cell death detection assay
K562 cells were incubated with 1 μmol/L fluorescein-labeled 9 mer peptides (Pep1, Pep2, and Pep3 or without peptide additions) as is described in previous section. TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling) assay (In Situ Cell Death Detection Kit; Roche, #12-156-792-910) was done on the slides followed by image analysis using fluorescent microscopy (Nikon 50i). DAPI stain (Vectoshield, Vector, H-1200) was used to assess total cell number. Penetration of peptides to cells was measured by ratio of cells containing fluorescein-labeled peptides to total DAPI-stained cells. Ratio of TUNEL-positive cells out of total cells represented the number of apoptotic cells. The percentage of TUNEL-positive cells was calculated as the number of TUNEL-positive cells divided by the number of cells containing fluorescein-labeled peptides.

Caspase-3 activity assay
To measure caspase-3 activity K562 cells were seeded by $1 \times 10^6$ to $2 \times 10^6$ cells per well. Following overnight incubation, 100 nmol/L of peptides were administrated in the absence of serum for 2 hours; serum was added to a final concentration of 10% afterwards. Following serum administration, cells were further incubated for about 20 hours before cell harvesting. Cells were spun at 1,000 rpm for 10 minutes, washed once at room temperature in 1xPBS.
solution, and treated with 80 μL of lysis buffer (kit #BF1100; R&D) for 30 minutes on ice. Protein concentration of cell lysates was determined by the BCA Protein Assay Kit (Pierce) according to manufacturer’s instructions. Hundred microgram total protein lysate was used to analyze caspase-3 activity using the Carboxyfluorescin FLICA (FAM-DEVDFMK) Apoptosis Detection Kit (Immunochemistry Technologies, LLC). FL600 Microplate Fluorescence Reader (Bio-Tek, USA) was used to examine the fluorescent readout of the samples.

Results

ARTS can preferentially kill cancer cells

Overexpression of ARTS alone is sufficient for induction of apoptosis in several tumor cell lines (18, 23, 28). Expression of ARTS is frequently lost in ALL and lymphoma patients (28). Moreover, Sept4/ARTS-deficient mice exhibit increased incidence of spontaneous tumors and accelerated tumor development in an Eμ-Myc background (29). Together these data suggested that loss of ARTS can provide a survival advantage for tumor cells and that treating cancer cells with ARTS mimetics might restore the ability of cancer cells to undergo apoptosis. Therefore, we first decided to investigate whether exogenous ARTS can selectively affect cancer but not normal cells. For this purpose, we used electroporation to introduce exogenous ARTS into ARH77 multiple myeloma cells and into normal lymphocytes isolated from healthy donors. TUNEL assay was carried out in these cells to determine apoptosis. Despite high expression levels of exogenous ARTS in normal lymphocytes, no increase in apoptosis was seen in these cells when compared with transfection with control vector (Fig. 1A left panel, Fig. 1B). In contrast, despite equivalent expression levels of exogenous ARTS detected in ARH77 multiple myeloma cells, a prominent increase in apoptosis was shown in these cancer cells (Fig. 1A right panel, Fig. 1B). These results suggested that exogenous ARTS can preferentially kill cancer cells without causing significant toxicity to normal cells.

The unique C-terminal domain of ARTS is sufficient to bind to XIAP-BIR3 and induce apoptosis

The main mechanism by which ARTS promotes apoptosis is through binding and antagonizing XIAP (18). In addition, we have recently discovered that ARTS binds specifically to the BIR3 domain in XIAP (Fig. 2A1 and ref. 24). IAP antagonists in Drosophila–Reaper, Hid and Grim, use a conserved N-terminal IBM for binding and induction of apoptosis (19, 20). This IBM is necessary and sufficient for IAP inhibition and it is conserved among all known IAP antagonists, including mammalian Smac and Omi/HtrA2 (13, 14, 21, 41). ARTS lacks any recognizable IBM. Instead, ARTS contains a stretch of 27 residues at its C-terminus, which is not found in any other known protein (17, 18). Sept4_i1 (H5/PNUTL2) is the other splice variant of Sept4 gene (26, 42). Although Sept4_i1 shares 83% amino acid identity with ARTS, it does not contain the 27 mer C-terminal domain of ARTS (ARTS-CTD) and cannot induce apoptosis (28). Therefore, we reasoned that this unique ARTS-CTD, defined as AIBM, is sufficient for binding to BIR3/XIAP and promotes apoptosis. A1, ARTS can bind to BIR3/XIAP. COS-7 cells were cotransfected with pSC-6myc-ARTS or pSC-6myc-empty vector constructs together with pEBG-XIAP-BIR3 or pEBG empty vector. GST-pull down assays were carried out using glutathione beads, followed by Western blot analyses with monoclonal anti-ARTS antibodies (Sigma). Whole lysates (the input–left) show that ARTS is expressed in considerable amounts in these transfected cells. Results of a GST-pull down (GST-PD) assay (right) shows that ARTS binds to BIR3/XIAP. All, the unique C-terminal domain of ARTS (ARTS-CTD) alone can bind to BIR3/XIAP. COS-7 cells were cotransfected with pSC-6myc-ARTS-CTD or pSC-6myc-empty vector constructs together with pEBG-XIAP-BIR3 or pEBG empty vector. GST-pull down assays were carried out using glutathione beads, followed by Western blot analyses with anti-ARTS antibodies. Whole lysates (the input–left) show that ARTS-CTD is expressed in considerable levels in these transfected cells. GST-pull down (GST-PD) assay (right) shows that the ARTS-CTD alone can bind efficiently to BIR3/XIAP. B, AIBM sequence alone induces apoptosis as efficiently as the full-length ARTS protein. K562 leukemia cells (B1) and HeLa-cervical carcinoma cells (BII) were transfected with pSC-6myc-ARTS or pSC-6myc-ARTS-CTD (AIBM) constructs containing the 27 mer AIBM sequence. SDS-PAGE followed by Western blot analysis using various antibodies show that the AIBM sequence alone could induce a significant increase in apoptosis which is comparable with the full-length ARTS. Apoptosis is determined by an increase in the phosphorylated H2AX apoptotic marker, the appearance of the cleaved form of caspase-3 (cCASP3), and reduction in XIAP levels.
construct specifically reduced the levels of XIAP, but not cIAP1, in these cells (Fig. 2BII). Similar results exhibiting specific reduction of XIAP, but not cIAP1, were seen following transfection of full-length ARTS in HeLa cells (22). This suggested that similar to the full-length ARTS, the ARTS-CTD construct can promote activation of caspase-3 and an increase in levels of the apoptotic marker H2AX through degradation of XIAP. Because these 27 mers bear no similarity with other IAP-binding proteins and lack any detectable IBM consensus sequences, it seems that ARTS contains a novel IAP-binding motif, which we will hereafter refer to as ARTS-IBM (AIBM).

**AIBM 9aa peptide derivatives can bind to XIAP-BIR3**

We have found that ARTS specifically binds to the BIR3 domain in XIAP (24). In addition, nuclear magnetic resonance (NMR) analysis of ARTS-CTD bound to BIR3/XIAP revealed that the last third of ARTS-CTD is particularly important for that binding (40). To investigate which of the residues in AIBM could substitute the function of the full-length ARTS protein, we subdivided the 27 mer AIBM into 3 nonoverlapping 9 mer peptides (ARTS 248–256, ARTS 257–265, and ARTS 266–274), termed Pep1, Pep2, and Pep3, respectively (Fig. 3A). These peptides were labeled with fluorescein as well as the 27 mer AIBM (ARTS 248–274). Because the 27 mer AIBM construct could bind to BIR3/XIAP, it served as a positive control in this assay (Fig. 2A). The ability of the peptides to bind to BIR3/XIAP was examined. Incubation of the fluorescein-labeled 9 mer peptides with COS-7 cells expressing BIR3/XIAP-GST resulted in efficient uptake of these peptides without any further treatment (Fig. 4A). Moreover, as shown in Fig. 3B, coimmunoprecipitation with anti-fluorescein antibodies revealed that the AIBM peptide as well as its derivatives, Pep2 and Pep3, can bind to BIR3/XIAP. Pep3 showed highest binding to BIR3/XIAP (Fig. 3BII). In addition, we found that Pep3 can also bind to cIAP1, although to a lesser extent, than to BIR3/XIAP (Supplementary Fig. S3).

**AIBM-based peptides reduce XIAP levels and induce apoptosis in cancer cells through activation of caspase-9 and caspase-3**

To explore whether the ARTS-derived AIBM peptides can penetrate and kill cancer cells, K562 leukemia cells were incubated with the fluorescein-labeled peptides and apoptosis was determined using TUNEL assay. All 3 fluorescein-labeled 9 mer peptides were able to penetrate K562 cells with high efficiency (Fig. 4A). However, only Pep2 and Pep3, which could bind to BIR3/XIAP in immunoprecipitation assays, were also able to induce cell death as visualized by increased numbers of TUNEL-positive cells (Fig. 4A). Pep3 exhibited the strongest ability to potentiate apoptosis in K562 leukemia cells (Fig. 4B), with an EC₅₀ of approximately 200 nmol/L (Supplementary Fig. S2). Similar results were obtained using cell mortality assays (Fig. 4C, Supplementary Fig. S2). Pep3 was also most potent in promoting caspase-3 activity (Fig. 5A), caspase-9 activity (Fig. 5B), and in reducing XIAP levels (Fig. 5C). Similar results were obtained when using CCRF-CEM T-cell leukemia cell line (Fig. 6).

In summary, AIBM derivatives Pep2 and Pep3, which can bind to XIAP also showed efficient cell killing of K562 and CCRF-CEM cancer cell lines (Figs. 4–6). The ability of AIBM derivatives to bind XIAP seems to be linked with their proapoptotic activity, as a mutated Pep3 (H1248A Pep3) with impaired binding to BIR3/XIAP (40) had compromised cell killing activity (Supplementary Fig. S2). In all
these assays, Pep3 was the most potent inducer of cell death (Figs. 5–6), with highest ability to reduce XIAP levels in both CCRF-CEM and K562 cells (Fig. 5E, 6B). Importantly, the AIBM-derived Pep2- and Pep3-induced apoptosis involved activation of caspase-9 and caspase-3 and reduction of XIAP levels (Fig. 5B and C, 6B).

To test whether AIBM-derived peptides have general cytotoxicity, we tested Pep3 on several other cancer cell lines. We found that Pep3 could also promote apoptosis in HeLa cells, but 3 other cancer cell lines—T98G, a human glioblastoma line; DU145, a human prostate carcinoma cell line; and PANC1, a human pancreatic cancer cell line—were completely nonresponsive (data not shown). Therefore, Pep3 does not seem to cause general, unspecific cell death.

This suggests that, despite their small size, AIBM-based 9 mer peptides retain at least some of the proapoptotic activity of full-length ARTS and may therefore specifically kill cancer cells, by a similar, if not identical mechanism. Collectively, our experiments define the 27 mer ARTS-CTD as a novel IAP-binding motif, which we term AIBM. AIBM peptides are sufficient to bind XIAP, reduce XIAP levels, and induce apoptosis in a variety of cancer cell lines, although other members of the IAP family of proteins may also serve as possible targets for ARTS-derived peptides. These results provided a proof-of-concept to explore the use of AIBM derivatives for cancer therapy.

Discussion

In recent years, IAP proteins have emerged as promising targets for cancer therapy (31, 36, 37). XIAP is considered to be the most potent inhibitor of caspases in vitro and elevated levels of this protein are found in human tumors (10, 30, 31). On the other hand, because mice deficient for XIAP are viable, the physiologic function of XIAP in situ has remained unclear. However, it was shown that loss of XIAP function causes elevated caspase-3 enzyme levels and sensitizes certain primary cells toward apoptosis (9). In addition, XIAP mutant mice are protected against Eμ-Myc–driven lymphoma due to increased apoptosis of premalignant lymphocytes (9, 43). Conversely, loss of ARTS function has been implicated in hematopoietic

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Figure 5. AIBM-derived peptides kill cancer cells through activation of caspases and reduction of XIAP levels. A, Pep3 promotes caspase-3 activation. K562 cells were incubated with AIBM-derived peptides (Pep1, Pep2, and Pep3) for 24 hours. The activation of caspase-3 was determined using carboxyfluorescein FLICA kit. The results are normalized to control (No Pep), which is considered to be 1 and presented as mean ± SEM, n = 3. B, Pep3 induces caspase-9 cleavage and activation. K562 cells were incubated with AIBM-derived peptides for 24 hours. Apoptosis was detected by determining the levels of cleaved caspase-9 (cCasp9) using Western blot analysis. Densitometry analyses were carried out using TotalLab TL100 graphic software, measurements are presented relative to actin levels, which served as loading control (mean ± SEM, n = 4).

Several approaches for developing anticancer drugs have focused on specifically antagonizing XIAP (44). These approaches include antisense oligonucleotides or RNAi-based technologies selectively inhibiting expression of XIAP (34, 35). In addition, small molecules mimicking IBM domain were designed and tested in clinical trials (36, 37, 39, 45–47).

Overexpression of ARTS can promote apoptotic cell death in a variety of cancer cell lines (18, 23), but seems to have no effect on normal lymphocytes (Fig. 1). Moreover, certain cancer cell lines such as human glioblastoma (T98G), prostate carcinoma (DU145), and pancreatic cancer cell line (PANC1) were completely nonresponsive to Pep3 administration, suggesting that the effect of ARTS is selective and not due to general cytotoxicity.

We have recently shown that ARTS initiates caspase activation upstream of MOMP (22). ARTS is localized at the MOM (22). Following induction of apoptosis, ARTS rapidly translocates to the cytosol where it binds to XIAP. The translocation of ARTS from mitochondria occurs within minutes following apoptotic stimuli and precedes MOMP and the release of cytochrome C and SMAC seen hours after induction of apoptosis (22). Moreover, knockdown of ARTS strongly inhibits the release of SMAC and cytochrome C, suggesting that ARTS is required for the proper timing of MOMP and the release of these proteins (22). We therefore propose that ARTS-based mimetics could be useful in initiating apoptosis in cancer cells.

In an effort to better define the binding site of ARTS to XIAP and explore the feasibility of developing ARTS-mimetics, we initiated structure-function analyses. We found that the 27 residues covering the unique C-terminus of ARTS are sufficient for binding to XIAP and inducing apoptosis similar to full-length ARTS (Fig. 2). Because the 27 residues residing at the extreme C-terminus of ARTS show no detectable sequence similarity to any known motif and have a composition entirely distinct from the IBM, we conclude that it contains a novel IBM that we term ARTS-IBM (AIBM). Of notice, deletion of these 27 residues did not completely abrogate the ability of ARTS to bind to XIAP (data not shown). However, deleting 68 residues from the C-terminus of ARTS abolished the ability of ARTS to bind to XIAP (18). We therefore suggest that the unique 27 residues of ARTS are sufficient for binding of ARTS to XIAP, but there are probably additional residues that are needed for a more efficient binding. A somewhat similar situation is seen for the IBM. Although the proapoptotic function of Smac requires a conserved 4 residue IBM (AVPI), additional residues downstream make a second contact with the XIAP-BIR3 domain (13, 14, 21, 48). Similarly, the Drosophila IBM containing proteins, Reaper/Hid/Grim use sequences beyond the fifth amino acid to bind to Diap1 (48). However, the significance of additional residues for binding to XIAP beyond the 27 residues comprising the unique C-terminus of ARTS, awaits further structural analysis studies using X-ray crystallography.

Importantly, NMR analyses revealed that BIR3/XIAP interacts with C-terminal part of AIBM (40). These data support our results showing that Pep3 located at the very C-terminal part of AIBM is most potent in binding and promoting apoptosis (Figs. 3B, 4–6). In addition, 2 different studies have recently described the properties of the unique C-terminus of ARTS using various biochemical and structural methods, including CD and NMR (27, 40). On the basis of these studies, it seems that the C-terminus of ARTS is highly disordered. Moreover, these studies indicate that peptides derived from the C-terminal domain of ARTS are...
ARTS was shown to promote apoptosis through specifically inducing proteasome-mediated degradation of XIAP (18, 49). Similarly, we now show that Pep3 promotes downregulation of XIAP levels (Figs. 5C, 6C, Supplementary Fig. S1) in a caspase-independent manner (Supplementary Fig. S1). Therefore, degradation of XIAP following peptide administration is not a consequence of general apoptosis, but rather directly induced by these peptides. This reduction of XIAP levels is associated with caspase activation and induction of apoptosis (Figs. 4, 5A and B, 6A and B). ARTS was shown to bind to other IAP family members such as cIAP2 (22). Similarly, whereas Pep3 shows highest binding to BIR3/XIAP, it also binds to cIAP1, although to a lesser extent (Supplementary Fig. S3). Thus, we cannot rule out that the AIBM derivatives may have additional targets such as other members of IAP family and/or that they could activate other apoptosis pathways in addition to targeting XIAP in a way that full-length ARTS does.

Interestingly, IBM-based IAP antagonists initially designed to target XIAP were found to preferentially induce degradation of cIAPs but not XIAP (39, 47, 50). In contrast, our results suggest that ARTS-based agonists can act by preferentially targeting XIAP (Figs. 5C, 6B). These results provide a proof-of-concept for the development of ARTS-based small-molecule XIAP antagonists as cancer therapies. Such compounds are expected to be particularly effective against tumors exhibiting loss of ARTS, as well as for those overexpressing XIAP.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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