Published OnlineFirst March 5, 2012; DOI:10.1158/1078-0432.CCR-11-1430

Clinical Cancer Research

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

Natalia Edison, Tali-Haviv Reingewertz, Yossi Gottfried, et al.

Clin Cancer Res 2012;18:2569-2578. Published OnlineFirst March 5, 2012.

Updated Version	Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-11-1430
Supplementary Material	Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2012/03/08/1078-0432.CCR-11-1430.DC1.html

Cited Articles	This article cites 49 articles, 11 of which you can access for free at: http://clincancerres.aacrjournals.org/content/18/9/2569.full.html#ref-list-1

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

Downloaded from clincancerres.aacrjournals.org on May 2, 2012
Copyright © 2012 American Association for Cancer Research

Published OnlineFirst March 5, 2012; DOI:10.1158/1078-0432.CCR-11-1430

Clinical Cancer Research

Cancer Therapy: Preclinical

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

Natalia Edison[1,2], Tali-Haviv Reingewertz[3], Yossi Gottfried[1,4], Tali Lev[1], Dotan Zuri[1], Inbal Maniv[1], Marie-Jeanne Carp[1], Gil Shalev[4], Assaf Friedler[3], and Sarit Larisch[1]

Abstract

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. *Clin Cancer Res; 18(9); 2569–78. ©2012 AACR.*

Introduction

Apoptosis, or programmed cell death, plays a critical role in development and homeostasis (1). Deregulation of the apoptotic process leads to various diseases and the inhibition of apoptosis is the hallmark of most, if not all, cancer cell types (2). The main executioners of apoptosis are caspases, a family of cysteine proteases that preferentially cleave substrates after aspartate (3). Caspases can be activated either through binding of ligands to cell-surface death receptors or through promoting the release of proapoptotic proteins from mitochondria (4).

The apoptotic process is tightly controlled through the action of both activators and inhibitors of caspases (5). Inhibitors of apoptosis (IAP) are a family of proteins that can directly bind and inhibit caspases (6). All IAP proteins are structurally related and contain at least one baculovirus IAP repeat (BIR) domain, which is required for binding to caspases (7). Some of the IAP proteins contain a RING domain that bestows an E3-ubiquitin ligase activity (8).

X-linked IAP (XIAP) is the best characterized IAP and probably the most potent inhibitor of caspases (9, 10). XIAP contains a RING domain with an E3-ligase activity, ubiquitin associated domain, and 3 BIR domains that can directly inhibit caspase-3, caspase-7, and caspase-9 (11, 12).

In dying cells, the inhibition of caspases by XIAP is overcome to enable the initiation of apoptosis. This is achieved by the function of natural IAP antagonist proteins. Several mammalian XIAP antagonists have been identified, including Smac/DIABLO (hereforth referred to as Smac; refs. 13, 14), Omi/HtrA2 (15, 16), and ARTS (17, 18). Most known mammalian IAP antagonists including Smac and Omi/HtrA2 contain a short, conserved IAP-binding motif

Authors' Affiliations: [1]Cell Death Research Laboratory, Department of Biology, Faculty of Natural Sciences, University of Haifa, Mount Carmel; [2]The B. Rappaport Faculty of Medicine, Technion–Israel Institute of Technology, Haifa; [3]Institute of Chemistry, Hebrew University of Jerusalem, Givat Ram, Jerusalem; and [4]Simetra-Pharm LTD, Kiryat Shmona, Israel

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Sarit Larisch, Cell Death Research Laboratory, Department of Biology, Faculty of Natural Sciences, Multi-purpose Building, University of Haifa, Mount Carmel, Haifa, 31905, Israel. Phone: 972-544-314671; Fax: 972-4-8288763; E-mail: saritlarisch@gmail.com

doi: 10.1158/1078-0432.CCR-11-1430

©2012 American Association for Cancer Research.

Downloaded from clincancerres.aacrjournals.org on May 2, 2012
Copyright © 2012 American Association for Cancer Research

Published OnlineFirst March 5, 2012; DOI:10.1158/1078-0432.CCR-11-1430

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, Ascenta, 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. 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.

(IBM) and are released to the cytosol upon apoptotic induction (reviewed in ref. 8). This IBM was originally described in *Drosophila* IAP antagonists Reaper, Hid and Grim (19, 20). *Drosophila* IAP antagonists as well as mammalian IAP antagonists use their N-terminal IBM for IAP-binding and inhibition. This conserved 4-residue motif (AVPI/F) binds to the surface groove on the BIR domains of the IAP proteins in which caspases also bind and allows the release and activation of caspases (21).

Another natural IAP antagonist is ARTS (Sept4_i2; ref. 17). ARTS is localized at the mitochondrial outer membrane (MOM; ref. 22). Upon induction of apoptosis, ARTS translocates from the mitochondria to the cytosol and antagonizes XIAP, causing activation of caspases and cell death (18, 23).

Translocation of ARTS from MOM precedes the pore opening during MOM permeabilization (MOMP), which allows the release of Smac and Cytochrome c residing at the intermembrane space (22). Moreover, ARTS seems to be required for MOMP and the release of Cytochrome c and Smac, as HeLa cells in which ARTS expression is knocked down, exhibit a significant inhibition in MOMP and release of these proteins (22). Although ARTS lacks any recognizable IBM, ARTS can bind directly to BIR3 domain of XIAP and promote apoptosis (17, 18, 24). ARTS is derived from the human septin gene *Sept4* (17). Septins have been traditionally studied for their role in cytokinesis and filament forming abilities, but subsequently have been implicated in diverse functions, including determination of cell polarity, cytoskeletal reorganization, membrane dynamics, vesicle trafficking, and exocytosis (25). ARTS is exceptional both in terms of its mitochondrial localization and its proapoptotic function, not shared by any other known septin family member (26, 27). ARTS expression is frequently lost in acute lymphoblastic leukemia (ALL), lymphoma, and hepatocellular carcinoma patients, suggesting it functions as a tumor suppressor protein (ref. 28 and H. Steller, personal communication). Moreover, *Sept4*/ARTS-deficient mice reveal increased numbers of hematopoietic stem and progenitor cells and elevated XIAP protein levels, increased resistance to cell death, and augmented tumor incidence (29). Importantly, the apoptosis, stem cell, and tumor phenotypes of *Sept4*/ARTS null mice are all suppressed by inactivation of XIAP. These findings confirm that XIAP is a major target for ARTS-induced caspase activation and tumor suppression (29).

Because IAPs are overexpressed in a variety of tumors, these proteins have become attractive targets for designing new anticancer therapeutics (30, 31). *In vivo* and *in vitro* studies have shown that downregulation of XIAP potentiates apoptosis in different tumors (32, 33) and antisense oligonucleotides or RNA interference (RNAi)-based technologies can be used for this purpose (34, 35).

Another strategy to antagonize IAP proteins is by using IBM peptidomimetics that mimic one (monovalent) or 2 IBM sequences in tandem (bivalent; refs. 36, 37). These compounds sensitize cancer cells to apoptosis because of elimination of XIAP-mediated caspase inhibition as well as proteasomal degradation of the cIAP and activation of NF-κB pathway (38, 39).

In this study we show that ARTS can preferentially kill cancer cells. The binding of ARTS to XIAP involves sequences that are distinct from all other known IAP antagonists. ARTS does not contain the canonical IBM and binds to XIAP via a unique sequence that we term ARTS-IBM (AIBM). This AIBM sequence is sufficient to bind XIAP and promote apoptosis. Moreover, AIBM-based peptides can bind to BIR3/XIAP and reduce XIAP levels inducing apoptosis in a mechanism similar to function of full-length ARTS protein. These peptides penetrate cancer cells, induce caspase activation, and apoptosis. These results provide proof-of-concept for developing ARTS-based cancer therapeutics.

Materials and Methods

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 (D64E10, #5625, Cell Signalling), and actin (c4, #691001, MP) were used.

Downloaded from clincancerres.aacrjournals.org on May 2, 2012
Copyright © 2012 American Association for Cancer Research

Published OnlineFirst March 5, 2012; DOI:10.1158/1078-0432.CCR-11-1430

Constructs

The pSC2-6myc-ARTS construct with 6myc tag, which was attached to the N-terminus of ARTS, was generated using PCR with the following primers:

F *Bam*HI-5′- EcoRI 5′-CGAATTCCATGATCAAGCGTTTC-3′ and the reverse primer: pEG-ARTS-R *Xho*I 5′-TACCGCTCGAGCTAGTGGCAGCCCTGCCC-3′. For the GST pull-down assays, we cloned full-length ARTS into the pGEX4T (Pharmacia Biotech) construct; GST–ARTS fusion protein was generated using PCR method with the following primers:

*Bam*HI-5′-TCGAGGATCCATCAAGCGTTTCCTGGAGGACACCACGG-3′ and

*Eco*RI-5′-CTAGTGGCAGCCCTGCCCCTGGTGC-3′, and cloned into *Bam*HI and *Eco*RI sites in pGEX4T. The pEBG expression constructs encoding N-terminus GST fusion proteins, together with XIAP-BIR3, were a kind gift from Colin Duckett (Departments of Pathology and Internal Medicine, University of Michigan, Ann Arbor, MI).

Peptide synthesis labeling and purification

Pep1–YGPSLRLLA, **Pep2**–PPGAVKGTG, **Pep3**–QEHQGQGCH.

The peptides were synthesized, labeled, purified, and analyzed as described in Reingewertz and colleagues (40).

Mammalian cell culture

COS-7 and HeLa cells were grown in Dulbecco's modified Eagle medium with 4.5g/L D-glucose. K562 and CCRF-CEM cells were grown in RPMI medium. Media were supplemented with 10% fetal calf serum, penicillin 100 U/mL, streptomycin 100 mg/mL, and glutamine 2 mmol/L (Biological Industries). All other cell lines were purchased from American Type Culture Collection (ATCC) and growth media was used according to ATCC instructions.

Transient transfection of cells

For HeLa cells transfection jetPEI (Polyplus Transfection) was used according to the manufacturer's protocol.

Nucleofection of normal lymphocytes

Blood samples taken from healthy donors were immediately transferred into heparin containing tubes. Following dilution with 2 volumes of PBS-0.5% bovine serum albumin (BSA), the entire volume was laid over Ficoll-Paque-Plus test tubes (#71-7167-00 AD of Amersham Biosciences) and centrifuged at 750 g for 20 minutes at 4°C (swinging bucket rotor, without brake). Interface peripheral blood mononuclear cells were transferred into new tubes, washed 3 times with PBS-0.5% BSA and spun at 350 g for 10 minutes at 4°C. The cell pellet obtained was exploited for nucleofection using the U-16 program of Nucleofactor (Amaxa). For that purpose, 20 μg DNAs were taken for aliquots of 5×10^6 to 7×10^6 cells. Subsequently, cells were incubated in a final volume of 2 mL of RPMI media (#01-100-1 of Biological Industries). This incubation was taking place in a 12-well plate for 16 hours before cell harvesting.

Western blot analyses

Western blot analyses were carried out as described in Lotan and colleagues (23). Visualization was done using LAS4000 luminesent 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 (pH8), 1% NP-40, 0.5% deoxycholate acid containing protease inhibitors (mini Complete; Roche)]. The samples were left rotating for 4 hours at 4°C with the GST fusion proteins coupled to the 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/XIAP. 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.

In vivo *coimmunoprecipitation of ARTS-derived peptides.* HeLa or COS-7 cells were transfected with pEBG-XIAP-BIR3 or pEBG-cIAPI or pEBG empty vector. Cell lysates were incubated with the indicated peptides for 3 hours, followed by GST pull-down using glutathione beads for additional 2 hours. After 3 washes, fluorescence emission was measured using a fluorimeter reader (Enspire 2300 multilabel reader; Perkin Elmer).

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 +

Downloaded from clincancerres.aacrjournals.org on May 2, 2012
Copyright © 2012 American Association for Cancer Research

Published OnlineFirst March 5, 2012; DOI:10.1158/1078-0432.CCR-11-1430

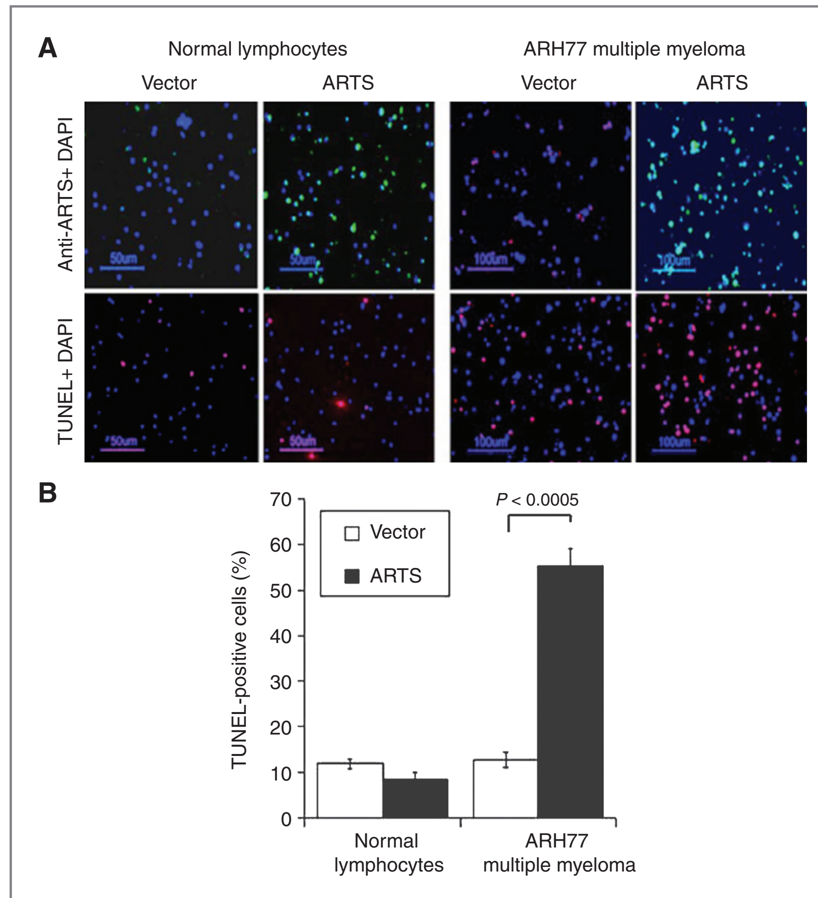

Figure 1. Exogenous ARTS induces apoptosis in ARH77—multiple myeloma cells but not in normal lymphocytes. A, ARH77 human multiple myeloma cells (right) or human normal lymphocytes isolated from healthy donors (left) were transfected with ARTS or empty vector (top, green) using AMAXA nucleofector system. *In situ* cell death detection assay—TUNEL (bottom, red) was carried out to measure apoptosis rate. The results were visualized using fluorescent microscope Nikon 50i, Japan. B, apoptosis rate was calculated as percent of TUNEL-positive cells out of counted transfected cells (mean ± SEM, $n = 4$). Despite high expression levels of ARTS, normal lymphocytes exhibited low apoptosis rates (8.47% ± 1.5). In contrast, high levels of apoptosis (55.3% ± 3.2) were found in the ARTS-transfected multiple myeloma cells.

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. Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL) 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. TUNEL-positive cells represent 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 1×10^6 to 2×10^6 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

Downloaded from clincancerres.aacrjournals.org on May 2, 2012
Copyright © 2012 American Association for Cancer Research

Published OnlineFirst March 5, 2012; DOI:10.1158/1078-0432.CCR-11-1430

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 Carboxyfluorescein FLICA (FAM-DEVD-FMK) 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 and B). 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 and B). 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. 2AI 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 other 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

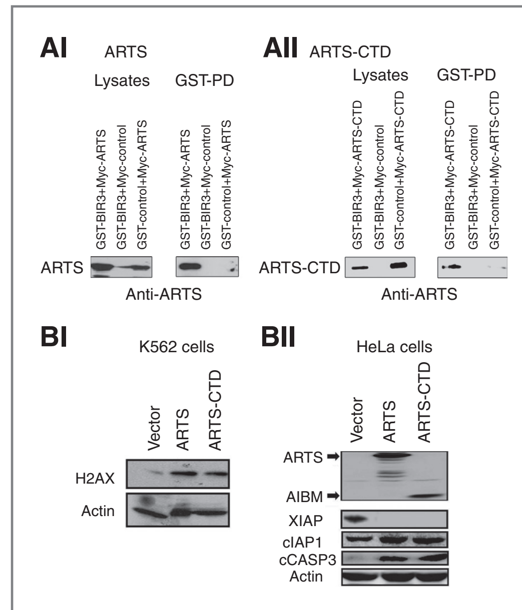

Figure 2. The unique ARTS-CTD, defined as AIBM, is sufficient for binding to BIR3/XIAP and promotes apoptosis. AI, ARTS can bind to BIR3/XIAP. COS-7 cells were cotransfected with pSC-6myc-ARTS or pSC-6myc empty vector constructs together with pEBG-XIAP-BIR3 or pEBG empty vector. GST-pull down assays 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) show that ARTS binds to BIR3/XIAP. AII, 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 (BI) and HeLa–cervical cancer 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.

apoptosis (28). Therefore, we reasoned that this unique ARTS-CTD may be important for its proapoptotic function and IAP binding. To test this hypothesis, we investigated whether the 27 mer motif alone is sufficient to bind BIR3/XIAP and to induce apoptosis in different cell lines. Indeed, expression of the myc-tagged C-terminal 27 residues of ARTS was sufficient for binding to BIR3/XIAP (Fig. 2AII). This construct was able to promote apoptosis in leukemia K562 and cervical carcinoma HeLa cells as efficiently as the full-length ARTS (Fig. 2BI and BII). In addition, ARTS-CTD

Downloaded from clincancerres.aacrjournals.org on May 2, 2012
Copyright © 2012 American Association for Cancer Research

Published OnlineFirst March 5, 2012; DOI:10.1158/1078-0432.CCR-11-1430

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 hereforth 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. 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 cIAPI, 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 EC_{50} 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).

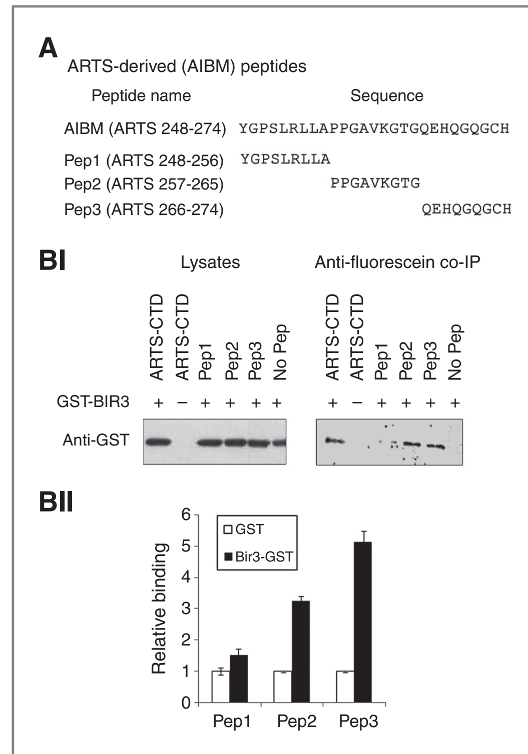

Figure 3. AIBM-derived peptides are sufficient for binding to BIR3/XIAP. A, a scheme describing the sequence of the unique 27mer AIBM and its division into 3 nonoverlapping 9 mer peptides (ARTS 248–256, ARTS 257–265, and ARTS 266–274), termed Pep1, Pep2, and Pep3 respectively. B, Pep2 and Pep3, AIBM-derived 9 mer peptides, show binding to GST-BIR3. BI, peptides spanning the 27 residues of AIBM and 3 consecutive 9 mer peptides: Pep1, Pep2, and Pep3 were tested for their binding to BIR3/XIAP. *In vitro* binding assay was carried out using purified GST-BIR3/XIAP and fluorescein-labeled peptides. Coimmunoprecipitation (co-IP) assay was carried out using anti-fluorescein antibodies conjugated to protein A/G sepharose beads, followed by Western blot analysis with anti-GST antibody. AIBM peptide as well as Pep2 and Pep3 derivatives show binding to BIR3/XIAP. BII, HeLa cells were transfected with pEBG-XIAP-BIR3 or pEBG empty vector. Cell lysates were incubated with the indicated peptides for 3 hours, followed by GST-pull down using glutathione beads for additional 2 hours. After 3 washes, fluorescence emission was measured using a fluorimeter reader (Enspire 2300 multilabel reader; Perkin Elmer). Relative fluorescence units representing relative binding are shown for each peptide. The fluorescence intensity of each BIR3–GST peptide complex was normalized to the fluorescence of GST-beads incubated with each peptide (mean ± SEM, $n = 4$).

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 (H248A Pep3) with impaired binding to BIR3/XIAP (40) had compromised cell killing activity (Supplementary Fig. S2). In all

Downloaded from clincancerres.aacrjournals.org on May 2, 2012
Copyright © 2012 American Association for Cancer Research

Published OnlineFirst March 5, 2012; DOI:10.1158/1078-0432.CCR-11-1430

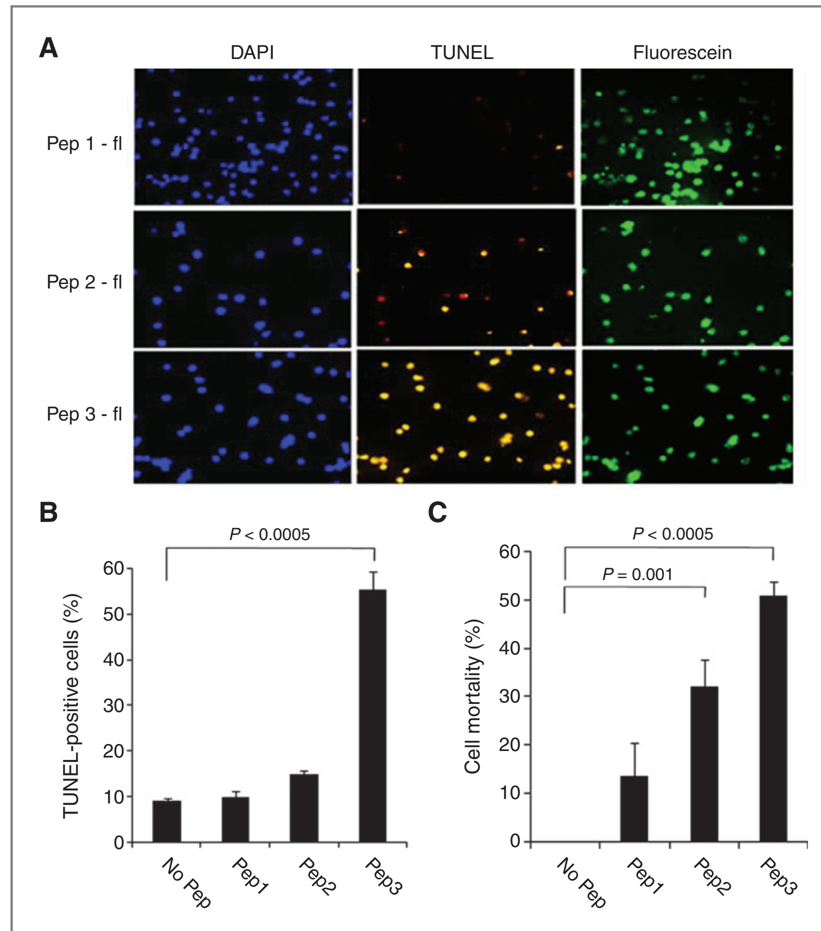

Figure 4. AIBM-derived 9-mer peptides can penetrate cancer cells and induce cell death. A, K562 leukemia cells were incubated with the AIBM-derived fluorescein-labeled 9 mer peptides (Pep1-fl, Pep2-fl, and Pep3-fl) for 8 hours. TUNEL assay was carried out to measure apoptosis rate. Though all 3 peptides were efficiently taken up by the leukemia cells (green), Pep3 exhibited the highest apoptosis rate, Pep2 showing mild apoptotic effect (middle), whereas Pep1 had no apoptotic effect. This suggests that these peptides exhibit a specific differential effect on these cancer cells rather than nonspecific cytotoxicity. B, apoptosis rate was calculated as percent of TUNEL-positive cells out of peptides containing cells (mean ± SEM, $n = 6$). Quantification analyses of TUNEL assays revealed that Pep2 administration resulted in 14.8% ± 0.62 TUNEL-positive cells, whereas Pep3 resulted in 55.37% ± 3.88. C, mortality rates were determined by cell morphology following staining with DAPI. Cell mortality numbers are composed of average cell counts of 8 different fields for each sample using fluorescent microscope and presented as mean ± SEM, $n = 7$. Pep2 and Pep3 showed potent killing of K562 leukemia cells.

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

Downloaded from clincancerres.aacrjournals.org on May 2, 2012
Copyright © 2012 American Association for Cancer Research

Published OnlineFirst March 5, 2012; DOI:10.1158/1078-0432.CCR-11-1430

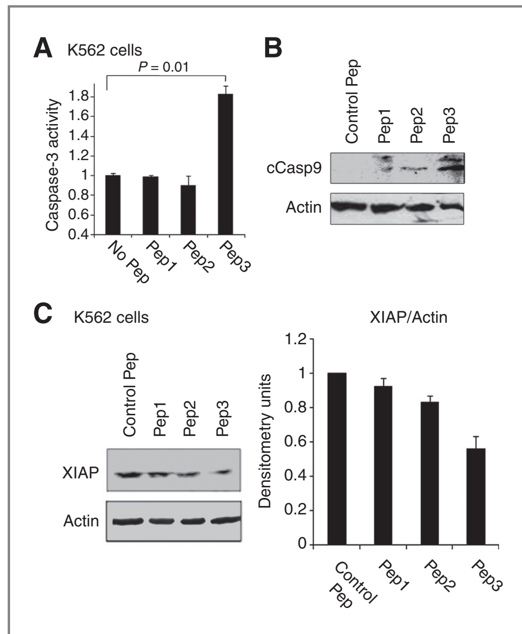

Figure 5. AIBM-derived peptides kill cancer cells through activation of caspases and reduction of XIAP levels. A, Pep3 promotes caspase-3 activation. K562 cells were incubated with AIBM-derived peptides (Pep1, Pep2, and Pep3) for 24 hours. The activation of caspase-3 was determined using carboxyfluorescein 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. Scrambled peptide was used as a control. Pep2 and Pep3 induced a significant increase in activation of caspase-9 in K562 cancer cells. C, Pep2 and Pep3 promote a specific reduction of XIAP levels in K562 cancer cells. K562 cells were incubated for 24 hours with either Pep1, Pep2, or Pep3 or with scrambled peptide which had no effect on the cells. XIAP levels were determined by 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, $n = 4$).

malignancies (28) and *Sept4*/ARTS-deficient mice develop spontaneous hematopoietic malignancies (29). Moreover, these *Sept4*/ARTS null mice exhibit elevated XIAP protein levels and increased resistance to cell death (29). Importantly, the tumor and apoptosis phenotypes of *Sept4*/ARTS-deficient mice are all suppressed by inactivation of XIAP. These findings confirm that XIAP is a major target for ARTS-induced caspase activation and tumor suppression (29).

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 better, 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

Downloaded from clincancerres.aacrjournals.org on May 2, 2012
Copyright © 2012 American Association for Cancer Research

Published OnlineFirst March 5, 2012; DOI:10.1158/1078-0432.CCR-11-1430

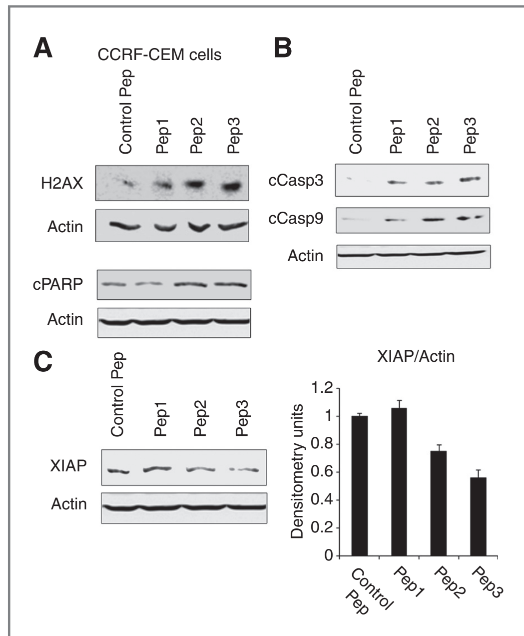

Figure 6. AIBM-derived 9 mers, Pep2, and Pep3 induce apoptosis in CCRF-CEM T-cell leukemia cells. A, CCRF-CEM T-cell leukemia cells were incubated with AIBM-derived 9 mer peptides (Pep1, Pep2, and Pep3) for 24 hours, followed by Western blot analyses. Scrambled peptide was used as a control. Apoptosis was detected by determining the levels of phosphorylated H2AX and cleaved PARP (cPARP). Pep2 and Pep3 induced a potent apoptotic response in CCRF-CEM cells, as evident by a strong increase in H2AX and cPARP levels. B, Pep2 and Pep3 induce caspase-9 and caspase-3 cleavage and activation. CCRF-CEM cells were incubated with AIBM-derived peptides for 24 hours. Scrambled peptide was used as a control. The levels of cleaved caspase-9 (cCasp9) and cleaved caspase-3 (cCasp3) were determined using Western blot analysis. Pep2 and Pep3 induced a significant increase in activation of caspase-3 and caspase-9 in CCRF-CEM cancer cells. C, Pep2 and Pep3 cause reduction of XIAP levels in CCRF-CEM cancer cells. CCRF-CEM cells were incubated with either Pep1, Pep2, or Pep3 or with scrambled control peptide that has no effect for 24 hours. XIAP levels were determined by 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, $n = 4$).

intrinsically disordered and lack significant secondary structure (27, 40). Pep3 was found to be the most potent activator of caspase-9 and caspase-3 (Figs. 5–6). Pep2 showed variable potency in killing cancer cells (Figs. 4–6). We speculate that these variable results may reflect the different sensitivities of the different experimental methods used. Alternatively, this may support our hypothesis that residues outside of Pep3 are also important for binding and induction of apoptosis.

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 cIAPI (22). Similarly, whereas Pep3 shows highest binding to BIR3/XIAP, it also binds to cIAPI, 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 therapeutics. 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.

Acknowledgments

The authors thank Colin Duckett for generously providing us with GST-BIR3/XIAP construct used in this article; Juliana Kagan and Bavat Bornstein for technical assistance; and Hermann Steller for critical reading of the manuscript.

Grant Support

This work was supported by funds from BSF (US Israel Binational Science Foundation) grant #2003085 (to S. Larisch), ISF (Israel Science Foundation), grant #1264/06 (to S. Larisch), grant from Israel Cancer Association (ICA; to S. Larisch), and by generous contributions from the Charles Wolfson Charitable Trust, England, and by Ms. Helen Steyer and Mr. Tommy Steyer, USA.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 3, 2011; revised February 8, 2012; accepted February 17, 2012; published OnlineFirst March 5, 2012.

References

1. Meier P, Finch A, Evan G. Apoptosis in development. Nature 2000;407:796–801.
2. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science 1995;267:1456–62.
3. Thornberry NA, Lazebnik Y. Caspases: enemies within. Science 1998;281:1312–6.
4. Degterev A, Boyce M, Yuan J. A decade of caspases. Oncogene 2003;22:8543–67.

Downloaded from clincancerres.aacrjournals.org on May 2, 2012
Copyright © 2012 American Association for Cancer Research

Published OnlineFirst March 5, 2012; DOI:10.1158/1078-0432.CCR-11-1430

5. Shi Y. Mechanisms of caspase activation and inhibition during apoptosis. Mol Cell 2002;9:459–70.
6. Deveraux QL, Reed JC. IAP family proteins-suppressors of apoptosis. Genes Dev 1999;13:239–52.
7. Srinivasula SM, Ashwell JD. IAPs: what's in a name? Mol Cell 2008;30:123–35.
8. Galban S, Duckett CS. XIAP as a ubiquitin ligase in cellular signaling. Cell Death Differ 2010;17:54–60.
9. Schile AJ, Garcia-Fernandez M, Steller H. Regulation of apoptosis by XIAP ubiquitin-ligase activity. Genes Dev 2008;22:2256–66.
10. Eckelman BP, Salvesen GS, Scott FL. Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. EMBO Rep 2006;7:988–94.
11. Deveraux QL, Takahashi R, Salvesen GS, Reed JC. X-linked IAP is a direct inhibitor of cell-death proteases. Nature 1997;388:300–4.
12. Gyrd-Hansen M, Darding M, Miasari M, Santoro MM, Zender L, Xue W, et al. IAPs contain an evolutionarily conserved ubiquitin-binding domain that regulates NF-kappaB as well as cell survival and oncogenesis. Nat Cell Biol 2008;10:1309–17.
13. Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 2000;102:33–42.
14. Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, et al. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 2000;102:43–53.
15. Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. Mol Cell 2001;8:613–21.
16. van Loo G, van Gurp M, Depuydt B, Srinivasula SM, Rodriguez I, Alnemri ES, et al. The serine protease Omi/HtrA2 is released from mitochondria during apoptosis. Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity. Cell Death Differ 2002;9:20–6.
17. Larisch S, Yi Y, Lotan R, Kerner H, Eimerl S, Tony Parks W, et al. A novel mitochondrial septin-like protein, ARTS, mediates apoptosis dependent on its P-loop motif. Nat Cell Biol 2000;2:915–21.
18. Gottfried Y, Rotem A, Lotan R, Steller H, Larisch S. The mitochondrial ARTS protein promotes apoptosis through targeting XIAP. EMBO J 2004;23:1627–35.
19. Goyal L, McCall K, Agapite J, Hartwieg E, Steller H. Induction of apoptosis by *Drosophila* reaper, hid and grim through inhibition of IAP function. EMBO J 2000;19:589–97.
20. Kornbluth S, White K. Apoptosis in *Drosophila*: neither fish nor fowl (nor man, nor worm). J Cell Sci 2005;118:1779–87.
21. Liu Z, Sun C, Olejniczak ET, Meadows RP, Betz SF, Oost T, et al. Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. Nature 2000;408:1004–8.
22. Edison N, Zuri D, Maniv I, Bornstein B, Lev T, Gottfried Y, et al. The IAP-antagonist ARTS initiates caspase activation upstream of cytochrome C and SMAC/Diablo. Cell Death Differ 2012;19:356–68.
23. Lotan R, Rotem A, Gonen H, Finberg JP, Kemeny S, Steller H, et al. Regulation of the proapoptotic ARTS protein by ubiquitin-mediated degradation. J Biol Chem 2005;280:25802–10.
24. Bornstein B, Gottfried Y, Edison N, Shekhtman A, Lev T, Glaser F, et al. ARTS binds to a distinct domain in XIAP-BIR3 and promotes apoptosis by a mechanism that is different from other IAP-antagonists. Apoptosis 2011;16:869–81.
25. Longtine MS, DeMarini DJ, Valencik ML, Al-Awar OS, Fares H, De Virgilio C, et al. The septins: roles in cytokinesis and other processes. Curr Opin Cell Biol 1996;8:106–19.
26. Carp M-J, Larisch S. Septins and apoptosis. In:Hall PA, Russell SEH, Pringle JR, editors. The Septins. London: Wiley Blackwell; 2008. p. 281–95.
27. Mandel-Gutfreund Y, Kosti I, Larisch S. ARTS, the unusual septin: structural and functional aspects. Biol Chem 2011;392:783–90.
28. Elhasid R, Sahar D, Merling A, Zivony Y, Rotem A, Ben-Arush M, et al. Mitochondrial pro-apoptotic ARTS protein is lost in the majority of acute lymphoblastic leukemia patients. Oncogene 2004;23:5468–75.
29. Garcia-Fernandez M, Kissel H, Brown S, Gorenc T, Schile AJ, Rafii S, et al. Sept4/ARTS is required for stem cell apoptosis and tumor suppression. Genes Dev 2010;24:2282–93.
30. Tamm I, Kornblau SM, Segall H, Krajewski S, Welsh K, Kitada S, et al. Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias. Clin Cancer Res 2000;6:1796–803.
31. Hunter AM, LaCasse EC, Korneluk RG. The inhibitors of apoptosis (IAPs) as cancer targets. Apoptosis 2007;12:1543–68.
32. Sasaki H, Sheng Y, Kotsuji F, Tsang BK. Down-regulation of X-linked inhibitor of apoptosis protein induces apoptosis in chemoresistant human ovarian cancer cells. Cancer Res 2000;60:5659–66.
33. Carter BZ, Mak DH, Schober WD, Koller E, Pinilla C, Vassilev LT, et al. Simultaneous activation of p53 and inhibition of XIAP enhance the activation of apoptosis signaling pathways in AML. Blood 2009;115:306–14.
34. Tamm I. AEG-35156, an antisense oligonucleotide against X-linked inhibitor of apoptosis for the potential treatment of cancer. Curr Opin Investig Drugs 2008;9:638–46.
35. McManus DC, Lefebvre CA, Cherton-Horvat G, St-Jean M, Kandimalla ER, Agrawal S, et al. Loss of XIAP protein expression by RNAi and antisense approaches sensitizes cancer cells to functionally diverse chemotherapeutics. Oncogene 2004;23:8105–17.
36. LaCasse EC, Mahoney DJ, Cheung HH, Plenchette S, Baird S, Korneluk RG. IAP-targeted therapies for cancer. Oncogene 2008;27: 6252–75.
37. Vucic D, Fairbrother WJ. The inhibitor of apoptosis proteins as therapeutic targets in cancer. Clin Cancer Res 2007;13:5995–6000.
38. Fulda S, Wick W, Weller M, Debatin KM. Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma *in vivo*. Nat Med 2002;8:808–15.
39. Varfolomeev E, Blankenship JW, Wayson SM, Fedorova AV, Kayagaki N, Garg P, et al. IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. Cell 2007;131:669–81.
40. Reingewertz TH, Shalev DE, Sukenik S, Blatt O, Rotem-Bamberger S, Lebendiker M, et al. Mechanism of the interaction between the intrinsically disordered C-terminus of the pro-apoptotic ARTS protein and the Bir3 domain of XIAP. PLoS One 2011;6:e24655.
41. Martins LM. The serine protease Omi/HtrA2: a second mammalian protein with a Reaper-like function. Cell Death Differ 2002;9:699–701.
42. Zieger B, Tran H, Hainmann I, Wunderle D, Zgaga-Griesz A, Blaser S, et al. Characterization and expression analysis of two human septin genes, PNUTL1 and PNUTL2. Gene 2000;261:197–203.
43. Jost PJ, Grabow S, Gray D, McKenzie MD, Nachbur U, Huang DC, et al. XIAP discriminates between type I and type II FAS-induced apoptosis. Nature 2009;460:1035–9.
44. Dean EJ, Ranson M, Blackhall F, Dive C. X-linked inhibitor of apoptosis protein as a therapeutic target. Expert Opin Ther Targets 2007;11:1459–71.
45. Li L, Thomas RM, Suzuki H, De Brabander JK, Wang X, Harran PG. A small molecule Smac mimic potentiates TRAIL- and TNFalpha-mediated cell death. Science 2004;305:1471–4.
46. Sun H, Nikolovska-Coleska Z, Lu J, Meagher JL, Yang CY, Qiu S, et al. Design, synthesis, and characterization of a potent, nonpeptide, cell-permeable, bivalent Smac mimetic that concurrently targets both the BIR2 and BIR3 domains in XIAP. J Am Chem Soc 2007;129:15279–94.
47. Vince JE, Wong WW, Khan N, Feltham R, Chau D, Ahmed AU, et al. IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell 2007;131:682–93.
48. Wu JW, Cocina AE, Chai J, Hay BA, Shi Y. Structural analysis of a functional DIAP1 fragment bound to grim and hid peptides. Mol Cell 2001;8:95–104.
49. Garrison JB, Correa RG, Gerlic M, Yip KW, Krieg A, Tamble CM, et al. ARTS and Siah collaborate in a pathway for XIAP degradation. Mol Cell 2010;41:107–16.
50. Petersen SL, Wang L, Yalcin-Chin A, Li L, Peyton M, Minna J, et al. Autocrine TNFalpha signaling renders human cancer cells susceptible to Smac-mimetic-induced apoptosis. Cancer Cell 2007;12:445–56.

Downloaded from clincancerres.aacrjournals.org on May 2, 2012
Copyright © 2012 American Association for Cancer Research