ARTS-based peptides promote apoptosis of cancer cells

Peptides mimicking the unique ARTS-XIAP binding site promote apoptotic cell death in cultured cancer cells

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Statement of translational relevance

Because IAPs are over-expressed in a variety of tumors, they have become promising targets for developing novel anti-cancer therapeutics, and several companies (including Genentech, Novartis, Ascenta, Tetralogics, etc.) are currently performing 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 IAP binding motif 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 et al., 2007; Gaither et al., 2007; Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 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 anti-cancer therapeutics.
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Abstract

Purpose: XIAP (X-linked IAP-Inhibitor of Apoptosis Protein) is the best characterized mammalian caspase inhibitor. XIAP is frequently over-expressed in a variety of human tumors, and genetic inactivation of XIAP in mice protects against lymphoma. Therefore, XIAP is an attractive target for anti-cancer 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 pro-apoptotic 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 TUNEL, 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 anti-cancer therapeutics.
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Introduction

Apoptosis, or programmed cell death plays a critical role in development and homeostasis (1). De-regulation 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 pro-apoptotic proteins from mitochondria (4).

The apoptotic process is tightly controlled through the action of both activators and inhibitors of caspases (5). Inhibitor of Apoptosis (IAP) are a family of proteins which 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 which 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 three BIR domains that can directly inhibit caspases-3, -7 and -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 (here forth referred to as Smac) (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 (IBM) and are released to the cytosol upon apoptotic induction (reviewed in (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 four-residue motif (AVPI/F) binds to the surface groove on the BIR domains of the IAP proteins where caspases also bind and allows the release and activation of caspases (21).

Another natural IAP antagonist is ARTS (Sept4_i2) (17). ARTS is localized at the mitochondrial outer membrane (MOM) (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 mitochondrial outer membrane permeabilization (MOMP) which allows the release of Smac and Cytochrome c residing at the intermembrane space (IMS) (22). Moreover, ARTS seems to be required for MOMP and the release 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
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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 pro-apoptotic 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 (HCC) patients, suggesting it functions as a tumor-suppressor protein ((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).

Since IAPs are over-expressed in a variety of tumors, these proteins have become attractive targets for designing new anti-cancer therapeutics (30, 31). In vivo and in vitro studies have demonstrated that down-regulation of XIAP potentiates apoptosis in different tumors (32, 33) and anti-sense oligonucleotides or RNAi-based technologies can be used for this purpose (34, 35).

Another strategy to antagonize IAP proteins is by using IBM peptido-mimetics, which mimic one (monovalent) or two IBM sequences in tandem (bivalent) (36, 37). These compounds sensitize cancer cells to apoptosis due to 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 which 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.
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**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, St. Louis) which is the only currently commercially available antibody directed against the unique C-terminus of ARTS. Antibodies against GST (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.

** 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 BamHI-5’- EcoRI 5’- CGAATTCCATGATCAAGCGTTTC-3’ and the reverse primer: pEG-ARTS-R XhoI 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:

BamHI-5’-TCGAGGATCCATCAAGCGTTTCCTGGAGGACACCACGG-3’ and
EcoRI-5’-CTAGTGCGACCCCTGCCCCTGGTGC-3’, and cloned into BamHI and EcoRI sites in pGEX4T. The pEBG expression constructs encoding N-terminus GST fusion proteins together with XIAP-BIR3 were a kind gift from Colin Duckett.

**Peptide synthesis labeling and purification**

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

The peptides were synthesized, labeled, purified and analyzed as described in (42).

**Mammalian cell culture**

COS-7 and HeLa cells were grown in Dulbecco’s modified Eagle medium (DMEM) with 4.5g/l D-glucose. K562 and CCRF-CEM cells were grown in RPMI medium. Media were supplemented with 10% fetal calf serum (FCS), penicillin 100U/ml, streptomycin 100mg/ml, and glutamine 2mM (Biological Industries, Israel). All other cell lines were purchased from ATCC and growth media was used according to ATCC instructions.
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**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% BSA, the entire volume was laid over Ficoll-Paque-Plus test tubes (#71-7167-00 AD of Amersham Biosciences) and centrifuged at 750g for 20 min at 4°C (swinging bucket rotor, without brake). Interface peripheral blood mononuclear cells (PMBC) were transferred into new tubes, washed 3 times with PBS-0.5% BSA and spun at 350g for 10 min at 4°C. The cell pellet obtained was exploited for nucleofection using the U-16 program of Nucleofactor (Amaxa). For that purpose, 20µg DNA were taken for aliquots of 5-7x10⁶ cells. Subsequently, cells were incubated in a final volume of 2ml of RPMI media (#01-100-1 of Biological Industries, Israel). This incubation was taking place in a 12-well plate for 16h before cell harvesting.

**Western blot analyses**
Western blot analyses were performed as described in (23). Visualization was performed using LAS4000 luminous image analyzer (Fujifilm). For densitometry analyses intensity of signals was compared to actin using TotalLab TL100 graphic software (Nonlinear Dynamics Ltd).

**Immunofluorescence assay**
Immunofluorescence assay was carried out as described in (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 co-transfected with pEBG-empty vector or pEBG-XIAP-BIR3 together with pCS2-6myc-ARTS or pCS2-6myc-vector. The cells were lysed in RIPA buffer (150mM NaCl, 50mM 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 4000 rpm at 4°C for 4 minutes and washed five times in lysis buffer. Proteins were eluted from beads following 5min of boiling in sample buffer. Proteins were separate on 12.5% SDS–PAGE gel, followed by Western blot analysis.
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**In vitro co-immunoprecipitation of ARTS-derived peptides.** The fluorescein-labeled 27mer ARTS-CTD and the fluorescein-labeled 9mer peptides (100nM) 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 4000 rpm at 4°C for 5min and washed three times in PBS. Proteins were eluted from beads following 5min of boiling in sample buffer. Proteins were separate on 12.5% SDS–PAGE gel, followed by Western blot analysis.

**In vivo co-immunoprecipitation of ARTS-derived peptides.** HeLa or COS-7 cells were transfected with pEBG-XIAP-BIR3 or pEBG-cIAP1 or pEBG empty vector. Cell lysates were incubated with the indicated peptides for 3h, followed by GST-pull down using glutathione beads for additional 2h. After three 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 0.2x10^6 cells/well in hypotonic media (0.5ml RPMI medium+0.5ml DDW) containing fluorescein-labeled peptides. After 4h the media was replaced with complete RPMI medium containing the peptides, and incubation was continued for additional 4h. Cells were harvested and washed once with PBSx1. Cells were suspended in equal volumes of PBSx1 and seeded on slides pre-coated with poly-L-lysine (Sigma). 4% paraformaldehyde was used to fix the cells for 30min, followed by two repeated washes with PBSx1. Permeabilization was performed using 0.1% TritonX-100 and 0.1% sodium citrate in DDW for 5min. Staining with DAPI (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 1µM fluorescein-labeled 9mer peptides (Pep1, Pep2, and Pep3 or without peptide additions) as is described in previous section. TUNEL (terminal deoxynucleotidyltransferase–mediated deoxyuridine triphosphate nick end-labeling) assay (In Situ Cell Death Detection Kit; Roche, #12-156-792-910) was performed 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 1-2 × 10⁶ cells per well. Following overnight incubation, 100nM 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 20h prior to cell harvesting. Cells were spun at 1000 rpm for 10 min, washed once at RT in 1×PBS solution, and treated with 80μl of lysis buffer (kit #BF1100, R&D) for 30min on ice. Protein concentration of cell lysates was determined by the BCA Protein Assay Kit (Pierce) according to manufacturer’s instructions. 100μg total protein lysate was used to analyze caspase-3 activity using the Carboxyfluorescein FLICA (FAM-DEVD-FMK) Apoptosis Detection Kit (Immu-nochemistry Technologies, LLC). FL600 Microplate Fluorescence Reader (Bio-Tek, USA) was used to examine the fluorescent readout of the samples.
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Results

**ARTS can preferentially kill cancer cells**

Over-expression of ARTS alone is sufficient for induction of apoptosis in several tumor cell lines (18, 23, 28). Expression of ARTS is frequently lost in Acute Lymphoblastic Leukemia (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 suggest 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 performed 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 to transfection with control vector (Figure 1A left panel, Figure 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 (Figure 1A right panel, Figure 1B). These results suggest 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 (Figure 2AI and (24). IAP antagonists in Drosophila - Reaper, Hid and Grim, use a conserved N-terminal IAP Binding Motif (IBM) for binding and induction of apoptosis (19, 20). This IBM is necessary and sufficient for IAP inhibition and it is conserved amongst all other known IAP-antagonists, including mammalian Smac and Omi/HtrA2 (13, 14, 21, 40). 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, 41). Although Sept4_i1 shares 83% amino acid identity with ARTS, it does not contain the 27mer C-terminal domain of ARTS (ARTS-CTD) and cannot induce apoptosis (28). Therefore, we reasoned that this unique ARTS-CTD may be important for its pro-apoptotic function and IAP-binding. To test this hypothesis, we investigated whether the 27mer 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 (Figure 2AII). This construct was able to promote apoptosis in leukemia K562 and cervical carcinoma HeLa cells as efficiently as the full-length ARTS (Figure 2BI, 2BII). In addition, ARTS-CTD construct specifically reduced the levels of XIAP but not cIAP1 in these cells (Figure 2BII). Similar results exhibiting specific reduction of XIAP but not cIAP1 were seen following
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transfection of full length ARTS in HeLa cells (22). This suggests 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 27mers bear no similarity with other IAP-binding proteins and lack any detectable IBM consensus sequences, it appears that ARTS contains a novel IAP-binding motif, which we will here forth refer to as ARTS-IAP binding motif (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, NMR analysis of ARTS-CTD bound to BIR3/XIAP revealed that the last third of ARTS-CTD is particularly important for that binding (42). To investigate which of the residues in AIBM could substitute the function of the full length ARTS protein, we subdivided the 27mer AIBM into three non-overlapping 9mer peptides (ARTS 248-256, ARTS 257-265 and ARTS 266-274), termed Pep1, Pep2, and Pep3 respectively (Figure 3A). These peptides were labeled with fluorescein as well as the 27mer AIBM (ARTS 248-274). Since the 27mer AIBM construct could bind to BIR3/XIAP, it served as a positive control in this assay (Figure 2A). The ability of the peptides to bind to BIR3/XIAP was examined. Incubation of the fluorescein-labeled 9mer peptides with COS-7 cells expressing BIR3/XIAP-GST resulted in efficient uptake of these peptides without any further treatment (Figure 4A). Moreover, as shown in Figure 3B, co-immunoprecipitation 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 (Figure 3BII). In addition, we found that Pep3 can also bind to cIAP1, although to a lesser extent than to BIR3/XIAP (Supplementary Figure S3).

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

In order 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 three fluorescein-labeled 9mer peptides were able to penetrate K562 cells with high efficiency (Figure 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 (Figure 4A). Pep3 exhibited the strongest ability to potentiate apoptosis in K562 leukemia cells (Figure 4B), with an EC50 of approximately 200nM (Supplementary Figure S2). Similar results were obtained using cell mortality assays (Figure 4C, Supplementary Figure S2). Pep3 was also most potent in promoting caspase-3 activity (Figure 5A), caspase-9 activity (Figure 5B) and in reducing XIAP levels (Figure 5C). Similar results were obtained when using CCRF-CEM T-cell leukemia cell line (Figure 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 (Figure 4-6). The ability of AIBM derivatives to bind XIAP seems to be linked with their pro-apoptotic activity, as a mutated Pep3 (H248A Pep3) with impaired binding to BIR3/XIAP (42) had compromised cell killing activity (Supplementary Figure S2). In all these assays, Pep3 was the most potent inducer of cell death (Figures 5-6), with highest ability to reduce XIAP levels in both CCRF-CEM and K562 cells (Figure 5E, 6B). Importantly, the AIBM derived Pep2 and Pep3-induced apoptosis involved activation of caspase-9 and caspase-3, and reduction of XIAP levels (Figure 5B, 5C, 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 three 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 non-responsive (data not shown). Therefore, Pep3 does not appear to cause general, unspecific cell death. This suggests that, despite their small size, AIBM-based 9mer peptides retain at least some of the pro-apoptotic activity of full-length ARTS and may therefore specifically kill cancer cells, by a similar, if not identical mechanism. Collectively, our experiments define the 27mer 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 provide 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 physiological 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 towards apoptosis (9). In addition, XIAP-mutant mice are protected against Eµ-Myc-driven lymphoma due to increased apoptosis of pre-malignant lymphocytes (9, 43). Conversely, loss of ARTS function has been implicated in hematopoietic 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 anti-cancer drugs have focused on specifically antagonizing XIAP (44). These approaches include anti-sense 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).

Over-expression 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 (Figure 1). Moreover, certain cancer cell lines such as human glioblastoma (T98G), prostate carcinoma (DU145) and pancreatic cancer cell line (PANC1) were completely non-responsive 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 outer membrane of mitochondria (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 (Figure 2). Since the 27 residues residing at the extreme C-terminus of ARTS show no detectable sequence
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similarity to any known motif and have a composition entirely distinct from the IBM, we conclude that it contains a novel IAP binding motif, that we term ARTS-IAP-Binding-Motif (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 which are needed for a better, more efficient binding. A somewhat similar situation is seen for the IBM. Although the pro-apoptotic function of Smac requires a conserved four 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 5th 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 (42). 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 (Figures 3B, 4-6). In addition, two 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, 42). Based on these studies it appears that the C-terminus of ARTS is highly disordered. Moreover, these studies indicate that peptides derived from the C-terminal domain of ARTS are intrinsically disordered and lack significant secondary structure (27, 42). Pep3 was found to be the most potent activator of caspase-9 and caspase-3 (Figure 5-6). Pep2 showed variable potency in killing cancer cells (Figures 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 down-regulation of XIAP levels (Figures 5C, 6C, Supplementary Figure S1) in a caspase independent manner (Supplementary Figure 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 (Figures 4, 5A-B, 6A-B). ARTS was shown to bind to other IAP family members such as cIAP1 (22). Similarly, while Pep3 shows highest binding to BIR3/XIAP, it also binds to cIAP1, although to a lesser extent (Supplementary Figure 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.
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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 preferentialy targeting XIAP (Figures 5C, 6B). These results provide 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 over-expressing XIAP.
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Reference List


ARTS-based peptides promote apoptosis of cancer cells


ARTS-based peptides promote apoptosis of cancer cells

Figure legends

Figure 1: Exogenous ARTS induces apoptosis in ARH77 - multiple myeloma cells but not in normal lymphocytes. A. ARH77 human multiple myeloma cells (A - right) or human normal lymphocytes isolated from healthy donors (A - left) were transfected with ARTS or empty vector (top panel) using AMAXA nucleofector system. In situ cell death detection assay – TUNEL (bottom panel) was performed 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.

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 co-transfected 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 panel) show that ARTS is expressed in considerable amounts in these transfected cells. Results of a GST-pull down (GST-PD) assay (right panel) shows 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 co-transfected 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 panel) show that ARTS-CTD is expressed in considerable levels in these transfected cells. GST-pull down (GST-PD) assay (right panel) 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 27mer 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 to 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.

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 three non-overlapping 9mer peptides (ARTS 248-256, ARTS 257-265 and ARTS 266-274), termed Pep1, Pep2, and Pep3 respectively. B. Pep2 and Pep3,
AIBM-derived 9mer peptides, show binding to GST-BIR3. BI. Peptides spanning the 27 residues of AIBM and three consecutive 9mer peptides: Pep1, Pep2 and Pep3 were tested for their binding to BIR3/XIAP. *In vitro* binding assay was performed using purified GST-BIR3/XIAP and fluorescein-labeled peptides. Co-immunoprecipitation 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 3h, followed by GST-pull down using glutathione beads for additional 2h. After three washes, fluorescence emission was measured using a fluorimeter reader (Enspire 2300 multilabel reader, Perkin Elmer). Relative fluorescence units representing relative binding is 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).

**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 9mer peptides (Pep1-fl, Pep2-fl and Pep3-fl) for 8h. TUNEL assay was performed to measure apoptosis rate. Though all three peptides were efficiently taken up by the leukemia cells (A - green), Pep3 exhibited the highest apoptosis rate, Pep2 showing mild apoptotic effect (A-middle panel), while Pep1 had no apoptotic effect. This suggests that these peptides exhibit a specific differential effect on these cancer cells rather than non-specific 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, while 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.

**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, Pep3) for 24h. 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 24h. 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 24h with either Pep1, Pep2 or Pep3 or with...
ARTS-based peptides promote apoptosis of cancer cells

scrambled peptide which had no effect on the cells. XIAP levels were determined by Western blot analysis. Densitometry analyses were performed using TotalLab TL100 graphic software, measurements are presented relative to actin levels, which served as loading control (mean±SEM, n=4).

Figure 6: AIBM–derived 9mers, Pep2 and Pep3 induce apoptosis in CCRF-CEM T-cell leukemia cells. A. CCRF-CEM T-cell leukemia cells were incubated with AIBM-derived 9mer peptides (Pep1, Pep2, Pep3) for 24h, followed by Western blot analyses. Scrambled peptide was used as a control. Apoptosis was detected by determining the levels of phosphorilated 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 induces caspase-9 and caspase-3 cleavage and activation. CCRF-CEM cells were incubated with AIBM-derived peptides for 24h. 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 24h. XIAP levels were determined by Western blot analysis. Densitometry analyses were performed using TotalLab TL100 graphic software, measurements are presented relative to actin levels, which served as loading control (mean±SEM, n=4).
ACKNOWLEDGMENTS

We thank Colin Duckett for generously providing us GST-BIR3/XIAP construct, used in this ms. We are very grateful to Juliana Kagan and Bavat Bornstein for technical assistance and to Hermann Steller for critical reading of this manuscript. This work was supported by funds from BSF (US Israel Binational Science Foundation) grant #2003085 (to S.L), ISF (Israel Science Foundation), Grant #1264/06 (to S.L), grant from Israel Cancer Association (ICA) (to S.L), and by generous contributions from the Charles Wolfson Charitable Trust, England and by Ms. Helen Steyer and Mr. Tommy Steyer, USA.
Figure 1

A

<table>
<thead>
<tr>
<th>Normal lymphocytes</th>
<th>ARH77 Multiple myeloma</th>
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<tbody>
<tr>
<td>Vector</td>
<td>ARTS</td>
</tr>
<tr>
<td>Anti-ARTS Dapi</td>
<td>Vector</td>
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<tr>
<td>TUNEL Dapi</td>
<td>ARTS</td>
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</table>

B

![Graph showing TUNEL positive cells (%)](image)

- Vector
- ARTS

Normal lymphocytes: 10%
ARH77 Multiple myeloma: 60%

p < 0.0005
Figure 2

**AI** ARTS

**Lysates**
- GST-BIR3+Myc-ARTS
- GST-BIR3+Myc-control
- GST-control+Myc-ARTS
- GST-control+Myc-control

**GST-PD**
- GST-BIR3+Myc-ARTS
- GST-BIR3+Myc-control
- GST-control+Myc-ARTS
- GST-control+Myc-control

**ARTS**
- [Image of ARTS antibody]

**ARTS-CTD**
- [Image of ARTS-CTD antibody]

**BI**

**K562 cells**
- Vector
- ARTS
- ARTS-CTD

**H2AX**
- [Image of H2AX]

**Actin**
- [Image of Actin]

**BII**

**HeLa cells**
- Vector
- ARTS
- ARTS-CTD

**ARTS**
- [Image of ARTS]

**AIBM**
- [Image of AIBM]

**XIAP**
- [Image of XIAP]

**cIAP1**
- [Image of cIAP1]

**cCASP3**
- [Image of cCASP3]

**Actin**
- [Image of Actin]
**Figure 3**

A

**ARTS derived (AIBM) peptides**

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<td>YGPSLRLLA</td>
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<td>Pep2 (ARTS 257-265)</td>
<td>PPGAVKGTG</td>
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<td>Pep3 (ARTS 266-274)</td>
<td>QEHQGQGCH</td>
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BI

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GST-BIR3  

anti GST  

BII

![Graph showing relative binding for different peptides](chart.png)
Figure 4

A

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B

C

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p < 0.0005

p = 0.001

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Figure 5

A  K562 cells

B

C  K562 cells

XIAP/Actin
Figure 6

A  CCRF-CEM cells

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B

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C

<table>
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**XIAP/Actin**

![Graph showing densitometry units for Control, Pep1, Pep2, and Pep3.](Graph.png)
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.

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