ARTS-based anticancer therapy: taking aim at cancer stem cells

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Apoptosis related protein in TGF-β signaling pathway (ARTS/Sept4) is here forth referred to as ARTS, was originally found to promote apoptosis induced by TGF-β, but later was shown to promote apoptosis induced by a wide variety of apoptotic stimuli. In vitro and in vivo studies revealed that ARTS induces apoptosis, at least in part, through direct binding and antagonizing XIAP. High levels of XIAP are found in many types of cancers and often correlate with poor prognosis. ARTS was shown to function as a tumor-suppressor protein in human patients and mouse-tumor models. In particular, Sept4/ARTS-deficient mice have increased tumor susceptibility and contain increased numbers of stem cells (SCs) and progenitor cells, apparently owing to their resistance towards apoptosis. Based on these results we propose that loss of proapoptotic ARTS may act as the “first hit” initiating tumorigenesis in two distinct ways. First, loss of ARTS-mediated apoptosis leads to increased numbers of normal SCs. Elevated numbers of normal SCs may lead to increased cancer risk due to higher numbers of cellular targets available for transforming mutations. Second, after these SCs acquire additional transforming mutations and become cancer stem cells (CSCs), they are more likely to survive in the absence of ARTS owing to increased resistance toward apoptosis. A combination of these two mechanisms, over time, is expected to significantly increase tumor risk. Because CSCs appear to share phenotypic markers with normal SCs, targeting the signaling pathways that affect normal SC development and maintenance can serve as a useful approach towards true eradication of cancer. In this article we describe the role of ARTS in apoptosis and cancer, with focus on its potential role as a CSC marker and as a potential target for anticancer and anti-CSC therapy.

Apoptosis pathways, activators & inhibitors
Programmed cell death by apoptosis is important for regulating cell numbers and maintaining tissue homeostasis [1,2]. The main executioners of apoptosis are caspases, a family of proteases harboring a cysteine residue at their active site that preferentially cleave substrates after aspartate [3-5].

The apoptotic process is tightly controlled through the action of both activators and inhibitors of caspases [6-8]. Inhibitor of apoptosis (IAP) proteins are a major family of caspase inhibitors [9,10]. All IAP proteins contain at least one BIR domain. BIR domains can directly interact with caspases and inhibit their apoptotic activity [6,7,10,11]. Thus far, eight IAP proteins have been identified in mammals: NAIP, cIAP1, cIAP2, XIAP, MLIAP, ILP2, survivin and BRUCE/Apollon [9,12]. Some of these proteins, namely XIAP, cIAP1, cIAP2, MLIAP and ILP2, also contain a RING domain that bestows E3-ubiquitin ligase activity on these proteins [13-15]. XIAP, the best studied IAP, contains three BIR domains and can directly inhibit caspases-3, -7 and -9 [16-19]. There are two main pathways leading to caspase activation in mammalian cells [20]. The mitochondrial pathway (intrinsic pathway) and the extrinsic pathway activated through death receptors mainly in cells of the immune system. Caspase activation in the mitochondrial pathway is executed by two different modes of action (Figure 1). On the one hand, caspases are activated by the release of Cytochrome C (CytoC), leading to formation of a holoenzyme complex known as the apoptosome. CytoC released from the mitochondria binds to APAF-1 to activate procaspase-9 [21-23]. Second, mitochondrial factors such as SMAC/DIABLO, Omi/Htra2 and ARTS [24-27] acting as IAP antagonists, bind to IAP proteins in the cytosol, release these caspases from their inhibition by the IAP proteins and promote their activation (Figure 1). XIAP also contains an E3-ubiquitin ligase activity that promotes caspase-3 ubiquitination and its subsequent proteasome-mediated degradation [14]. IAP antagonists in Drosophila as well as SMAC/DIABLO and

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Omi/HtrA2 in mammalian cells use a short, conserved N-terminal sequence (AVPI) termed IAP Binding Motif (IBM) used for IAP-binding and inhibition [7,24–26,28,29]. The crystal structure of a XIAP/SMAC complex confirmed that SMAC binds the same IBM-binding grooves as the caspases [30]. The SMAC IBM is also very similar to the IBM of caspase-9 [31].

**ARTS promotes apoptosis via a distinct mechanism that differs from other IAP-antagonists**

ARTS is a mitochondrial protein that promotes apoptosis through binding to XIAP [27,32–34]. ARTS is derived by differential splicing from the human Septin gene Sept4 [33,35]. 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 oncogenesis [36–38]. ARTS is exceptional both in terms of its mitochondrial localization and its proapoptotic function, not shared by any other known Septin family member [39]. Moreover, ARTS promotes apoptosis via a mechanism distinct from all other known IAP antagonists. First, ARTS does not contain the canonical IBM found in most other IAP antagonists including SMAC/DIABLO, and it binds to XIAP via a unique sequence that we term ARTS–IBM (AIBM) [40]. Second, ARTS binds to both BIR1 and BIR3 domains in XIAP [41,42]. Yet, ARTS binds to distinct sequences within BIR3, which are not bound by other IAP-antagonists such as SMAC.
and Omi/HtrA [41]. Third, ARTS appears to initiate the mitochondrial apoptotic pathway upstream of CytoC and SMAC [43]. ARTS–XIAP complex is formed as quickly as 15–30 min after induction of apoptosis, significantly before the release of SMAC and CytoC from mitochondria, which occurs hours later [43]. Furthermore, the translocation of ARTS from the mitochondria to the cytosol is required for the on-time release of CytoC and SMAC from the mitochondria, as knockdown of ARTS in HeLa cells inhibits the release of both CytoC and SMAC [45]. Finally, SMAC selectively reduces the levels of c-IAP1 and c-IAP2 but not that of XIAP [44], and SMAC-based IAP antagonists have the ability to induce degradation of cIAPs, but not XIAP [45,46]. cIAP degradation by SMAC, occurs through NF-kB activation, and TNF-α-dependent apoptosis [45]. By contrast, ARTS appears to promote apoptosis through direct binding and degradation of XIAP, and ARTS inhibits XIAP-induced NF-kB activation [42]. Collectively, it appears that ARTS functions via a distinct mechanism to promote caspase activation and tumor suppression.

### Anticancer therapies targeting XIAP

High levels of IAP proteins are found in many types of cancers [47–49] and it often correlates with poor prognosis [50–52]. Therefore targeting IAP proteins presents a promising approach for developing novel anticancer drugs [48,53–55]. XIAP is considered to be the most potent inhibitor of caspases in vitro and elevated levels of this protein are found in a wide variety of human tumors [47,48,56,57]. Conversely, because mice deficient for XIAP are viable [58], the physiological function of XIAP in vivo has remained unclear. However, it was later demonstrated that loss of XIAP function causes elevated caspase-3 levels and sensitizes certain primary cells towards apoptosis [14]. In addition, XIAP-mutant mice are protected against Eµ-Myc-driven lymphoma owing to increased apoptosis of premalignant lymphocytes [14,59]. Several approaches for developing anticancer drugs have focused on specifically antagonizing XIAP [60–62]. These approaches include ASOs or RNAi-based technologies selectively inhibiting expression of XIAP [49,63–66]. In addition, small molecule XIAP-antagonists were designed and tested in clinical trials [45,46,53,54,56,67–71]. Small-molecule XIAP inhibitors de-repress downstream caspases [72]. Targeting XIAP has been shown to sensitize non-small-cell lung carcinoma to γ-irradiation and human ovarian and prostate cancer cells to chemotherapeutic agents in vitro [73–75]. In addition, inhibition of XIAP induced apoptosis and enhanced sensitivity towards chemotherapy in human prostate cancer cells [75]. Inhibition of XIAP with an antisense oligonucleotide (ASO) delayed tumor growth in a lung cancer xenograft model [76] and XIAP ASO induced apoptosis preferentially in CD34+38- cells in a Phase I/II study of patients with relapsed/refractory acute myeloid leukemia (AML) [77].

**Designing ARTS-based anticancer therapy: targeting cancers which exhibit loss of ARTS as well as cancers overexpressing XIAP**

In recent years, IAP proteins have emerged as promising targets for cancer therapy and several small-molecule IAP antagonists have been developed and are currently being evaluated in clinical trials [45,46,48]. All currently available chemical IAP-antagonists are IBM-derivatives (reapet/SMAC mimetics) with very similar properties. These compounds initially designed to target XIAP, were found to preferentially induce degradation of cIAPs but not XIAP, thereby stimulating TNF-α production and NF-kB activation leading to inflammatory side effects in patients.

Evidence for the physiological role of ARTS as an IAP-antagonist and a tumor suppressor protein came both from human and mouse studies. Expression of ARTS is absent in lymphoblasts of more than 70% of childhood acute lymphoblastic leukemia (ALL) and lymphoma patients [78, Unpublished Data]. Similarly, it was recently revealed that Sepr4/ARTS deficient mice develop spontaneous hematopoietic tumors [79]. This suggests that ARTS functions as a tumor suppressor protein in vivo and plays a particular important role in generation of hematopoietic cancers. The tumor suppressor function of ARTS seems to be linked to its role as an XIAP-antagonist, since these Sepr4/ARTS-null mice exhibit elevated XIAP protein levels and increased resistance to cell death [79]. Importantly, the tumor and apoptosis phenotypes of Sepr4/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 [79]. Altogether, ARTS specifically targets XIAP, therefore ARTS-based agonists will be distinct from all known IBM-derived IAP antagonists, which are currently developed as anticancer drugs. Moreover, since ARTS does not act through inducing TNF-α, it is unlikely to involve inflammatory side effects. In addition, ARTS-based compounds are expected to target...
a wide range of cancer types by being particularly effective both against tumors exhibiting loss of ARTS, as well as for tumors expressing high levels of XIAP. These features provide a window of therapeutic opportunity for ARTS to selectively target cancer cells with minimal affects on healthy cells, which contain normal levels of both ARTS and XIAP.

Cancer stem cells: resistance to apoptosis through increased XIAP levels

Defects in apoptosis can result in the expansion of a population of transformed malignant cells. Many properties of tumors are also characteristic of stem cells (SCs) [80–83]. In particular, cancer SCs (CSCs) probably derive from normal tissue SCs or early progenitors that already possess self-renewal and unlimited proliferation potential [80,84]. Moreover, the long lifespan of normal SCs compared with short-lived differentiated progenitors or terminally differentiated cells is expected to facilitate the accumulation of genetic aberrations, which lead to cancer formation [84,85]. There is growing evidence that apoptosis plays an important physiological role in restricting the numbers of normal SCs and preventing the emergence of CSCs [86]. Evidence for resistance to apoptosis in the CD133⁺ fraction of glioma SCs was shown when compared with the CD133⁻ fraction [87]. Elevated levels of XIAP are associated with resistance to chemotherapy [63,88,89]. High levels of XIAP were found in CD34⁺/CD38⁻ AML SCs [90], and high levels of IAP proteins have been described in CD133⁺ population in glioblastoma [87]. In addition XIAP ASO achieves target knockdown and induced apoptosis preferentially in CD34⁺38⁻ patients with relapsed/refractory AML [77]. The relevance of the regulation of mitochondrial apoptosis in SCs has been further demonstrated by the effective sensitization of glioblastoma-initiating cells to γ-irradiation-induced apoptosis via inhibition of XIAP [91,92].

Taking into consideration that Sept4/ARTS-null mice exhibit increased numbers of SCs with elevated levels of XIAP and increased resistance to apoptosis, it appears that loss of ARTS is at least one way in which SCs can acquire increased resistance to apoptosis.

Cancer SC markers

The concept of a small subset of SCs that could initiate tumors was extensively described in a wide range of cancers such as breast [93], brain [94], glioblastoma multiforme [95], colon [96], ovary [97] and lung cancer [98].

The pursuit to indentify CSC markers that could be targeted for therapeutic purposes has led to several surface markers suggested to be enriched in the CSC population. In many types of solid tumors, such as ovarian cancer, breast cancer, glioblastoma, colon cancer, lung cancer and rectal cancer, cell populations exhibiting CSCs have been enriched making use of single or multiple cell surface markers, such as CD133⁺ (in ovarian cancer, colon cancer, lung cancer, glioblastoma, rectal cancer), CD44⁺/CD24low (in breast cancer), CD133⁺ or CD44⁺/CD117⁺ (in ovarian cancer) [84,99–105].

Importantly, Shmelkov et al. confronted the view that CD133 is a marker of CSCs in colon cancer. They show that CD133 expression is not restricted to SCs, and that both CD133⁺ and CD133⁻ metastatic colon cancer cells initiate tumors [106]. In addition, olfactomedin-4 has been suggested as a CSC marker for colorectal cancer cells. [107], Arachidonate 12-lipoxygenase was suggested as a marker for prostate CSCs [108] and ALDH activity was proposed to serve as a marker of CSCs in head and neck squamous cell carcinoma [109,110]. Several CSC markers have been suggested for indentifying human hepatocellular carcinoma SCs. These include EpCAM [111] and CD13, a cell surface marker specific to semiquiescent hepatocellular carcinoma SCs [112].

The cell surface markers CD34⁺/CD38⁻ were found to enrich the population of AML cells. These markers are the same markers used for the isolation of hematopoietic SCs (HSC) yet were shown to have leukemia initiating capacity when transplanted in immunodeficient (NOD/SCID) mice [113]. Interestingly, despite examining 85 different potential melanoma CSC markers, there was no single marker that could distinguish between tumorigenic from nontumorigenic melanoma cells [114].

In human samples isolated from healthy donors, ARTS was normally expressed in CD34⁻ HSCs as well as in mature lymphocytes [78]. This suggests that the absence of ARTS in leukemic blasts was not simply the result of an incomplete cellular differentiation, but that it is rather associated with their malignant state. Interestingly, ARTS/Sept4-null mice contain increased numbers of HSCs and hematopoietic progenitor cells [79]. It appears that these elevated numbers of stem and progenitor cells in ARTS/Sept4-null mice are responsible for the increased incidence of hematopoietic tumors in these mice.

Taken into consideration that ARTS seems to be particularly important for regulating...
apoptosis in SCs, it is possible that ARTS may serve as a marker to distinguish normal SCs from CSCs.

**Stem cells & cancer**
The first to demonstrate the ability of cells to transfer malignancy were Furth and Kahn (1937) who were able to inoculate inbred mice with cells derived from a leukemia arising in the same inbred strain (113). They identified that only a small number, approximately 5%, of inoculations resulted in successful transplantation. Since then there has been increased interest in CSC research, improving experimental models in order to uncover the ‘stemness’ of CSCs. At least two main theories describing the origin of CSCs are currently debated; one suggests that CSCs represent differentiated cells that re-initiate their ‘stem’ features as part of, or following, malignant transformation. The other theory suggest that CSCs are mature SCs maintaining their ‘stem’ features while undergoing a malignant change [116]. Association between the emergence of cancer and increased numbers of SCs is seen in cases of myelodysplasia followed by the development of both ALL and AML (115,117).

However, according to the hierarchical model of CSCs, best demonstrated in AML, only a small fraction of SCs become CSCs, since during differentiation ‘downstream’ in the hierarchy, most cancer cells lose their tumorigenic capacity. Only CSCs that underwent irreversible epigenetic or genetic changes, and are characterized by specific markers, can transfer the disease when transplanted into immunocompromised NOD/SCID mice. Importantly, this CSC model does not apply to generation of melanoma CSCs, since several studies have shown that melanomas contain relatively large populations of tumorigenic cells. These populations of melanoma tumorigenic cells exhibited phenotypic heterogeneity that was not hierarchically organized, and underwent reversible phenotypic changes that were not associated with loss of tumorigenic potential [114,118,120].

Regardless of the CSC model suggested, it seems to be a consensus view that if SCs could be identified and their genetic abnormalities characterized, specific targeted therapy could be designed. Two recent studies involving ALL highlight the genetic and phenotypic heterogeneity of the leukemia SCs. Anderson et al. examined a series of pediatric ALL cases in which the ETV6–RUNX1 gene fusion was an early or initiating genetic lesion. Using multiplexed FISH analysis they identified a progressive accumulation of up to eight genetic changes in single cells [121]. Their data suggest dynamic patterns of subclonal development that are non-linear with a variable branching architecture. Serial transplantation in immunodeficient mice of leukemia propagating cells also showed heterogeneous genetic alternations, reflecting the diversity of subclones and their varied proliferative capacities. In parallel, Notta et al. reported very similar findings consistent with a nonlinear, branching, multiclonal model of leukemogenesis in BCR–ABL lymphoblastic leukemia samples [122]. They found that individual patient samples at diagnosis were composed of genetically diverse subclones that were related through a complex evolutionary process. These subclones also vary in their xenograft growth properties and leukemia-initiating-cell frequency [122].

**Developing ARTS-based anticancer therapy: a new approach for targeting CSCs**

(t12:21) Tel/AML1 is the most common chromosomal translocation in childhood ALL occurring in 25% of patients [123,124]. This translocation is often detected at birth yet, not all children bearing this translocation develop...
leukemia [125,126]. Expression of ARTS is lost in approximately 70% of ALL and lymphoma patients [78,79; Unpublished Data]. Although it is in comparison a relatively small-scale study, the numbers are significant and striking. Moreover, the fact that approximately 30% of ARTS/Sept4 deficient mice develop spontaneous neoplasia as compared with no incidence of tumors seen in wt littermates [79] points to an important role that ARTS may play in initiation of leukenogenesis. Sept4/ARTS-deficient mice have increased numbers of HSCs and hematopoietic progenitor cells, as indicated by both the use of markers and transplantation experiments testing for the presence of functional SCs by reconstituting the hematopoietic system of lethally irradiated recipient mice [79]. Importantly, no increase in cell proliferation was found in Sept4/ARTS-null mice, suggesting that the elevated numbers of functional SCs are owing to impaired SC apoptosis. HSPCs from Sept4/ARTS-null mice were significantly more resistant toward apoptosis than their wild-type counterparts and showed a robust increase in true clonogenic cell survival [79]. This suggests that ARTS functions as a tumor suppressor that regulates HSPC pool size by inducing apoptosis of superfluous SCs. According to this model, loss of proapoptotic ARTS function may act as the first hit initiating tumorigenesis in two distinct ways. First, loss of ARTS-mediated apoptosis leads to increased numbers of normal HSPCs. Elevated numbers of normal HSPCs could lead to increased cancer risk due to the presence of the number of cellular targets available for transforming mutations (Figure 2) [81–83,127]. Second, after these SCs acquire transforming mutations and become CSCs, they are more likely to survive in the absence of ARTS due to increased resistance toward apoptosis (Figure 2). A combination of these two proposed mechanisms, over time, is expected to significantly increase tumor risk. Consistent with this model, loss of ARTS in ALL patients was specific and related to its proapoptotic function as levels of the other, nonapoptotic splice variant of the Sept4 gene (Sept4_i1/HS/PNUTL2) remained intact [78]. Moreover, in mice, Sept4/ARTS function was specific for cell death of HSPCs in the hematopoietic compartment and loss of Sept4/ARTS led to long-term survival of HSPCs. Possible cooperation of loss of ARTS with other tumor-promoting events such as with (12,21) (TEL AML1), the most common translocation in childhood ALL may occur leading to leukemogenesis. Interestingly, six out of the 33 ALL patients in our study contained the t(12,21) (TEL AML1) translocation in addition to loss of ARTS [78]. Furthermore, Sept4/ARTS null mice exhibited increased numbers of HSCs and accelerated tumor development in an Eµ-Myc background, attesting for additional functional cooperation with the c-Myc gene in lymphomagenesis. This collaboration is very similar to what has been described previously for overexpression of antiapoptotic proteins such as Bcl-2 [128–130].

Traditionally, cancer therapeutics have been optimized towards the majority of cells present

Executive summary

- ARTS (Sept4 i2) is a mitochondrial proapoptotic protein. Upon induction of apoptosis, ARTS translocates from the mitochondrial outermembrane to the cytosol where it promotes caspase activation prior to the release of Cytochrome C and SMAC/DIABLO.
- ARTS promotes caspase activation and apoptosis by directly binding and antagonizing XIAP.
- The mechanism by which ARTS antagonizes XIAP is distinct from all other known inhibitor of apoptosis (IAP) antagonists.
- ARTS was shown to function as a tumor suppressor protein in human and mouse studies.
- Apoptosis plays an important physiological role in restricting the numbers of normal stem cells (SCs) and preventing the emergence of cancer SCs.
- High levels of IAP proteins are found in several types of cancers. Thus, targeting IAP proteins presents a promising approach for developing novel anticancer drugs.
- Elevated levels of XIAP are associated with resistance to chemotherapy and are found in SCs from various cancer tissues.
- The tumor suppressor function of ARTS seems to be linked to its role as an XIAP-antagonist, since Sept4/ARTS-null mice have an increased rate of spontaneous tumors, exhibit elevated XIAP protein levels and are more resistant to cell death. 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.
- ARTS seems to be particularly important for regulating apoptosis in SCs. Therefore, it is possible that ARTS may serve as a marker to distinguish normal SCs from cancer SCs.
- ARTS-based compounds are expected to target a wide range of cancer types by being particularly effective against both tumors exhibiting loss of ARTS, as well as for tumors expressing high levels of XIAP. These features provide a window of therapeutic opportunity for ARTS to selectively target cancer cells with minimal affects on healthy cells, which contain normal levels of both ARTS and XIAP.
in a tumor. However, in order to completely eradicate a tumor and prevent regrowth and/or metastases, it may be necessary to efficiently target the CSC compartment. Advances in our understanding of how CSCs escape cell death are likely to provide rational approaches to generate a new and improved class of anticancer drugs that selectively kill CSCs.

Conclusion & future perspective

The CSC hypothesis is an attractive model that explains several properties of metastatic tumors, but considerable controversy remains because of challenges to unequivocally identify CSCs. With continued advances in SC research, new sets of markers should emerge to visualize and isolate CSCs. One major feature that may distinguish normal SC from CSCs is acquired resistance towards apoptosis. In this regard, loss of ARTS expression may be a critical functional and diagnostic event common to many types of cancer. In the coming years, it will be important to critically investigate the association between cancer, apoptosis and SCs, and this will have important implications for both basic research and the clinic. For one, progress in this area will shed new insights into the origin of CSCs. Moreover, a better understanding of when and how ARTS is silenced during tumorigenesis will facilitate the early identification of cancerous cells, provide new markers for the clinic, and influence the development of new therapeutic strategies. With an increasingly detailed understanding of the precise mechanism by which ARTS induces apoptosis, it should be possible to develop small-molecule mimics that provide highly efficient and specific targeting of XIAP. If ‘ARTS-mimetics’ are particularly effective in targeting CSCs, they would provide both a powerful research tool to investigate the role of CSCs in the origin of metastases, and ultimately provide more effective treatment of patients suffering from metastatic disease.

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