Minireview

Naturally occurring, tumor-specific, therapeutic proteins

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Abstract

The emerging approach to cancer treatment known as targeted therapies offers hope in improving the treatment of therapy-resistant cancers. Recent understanding of the molecular pathogenesis of cancer has led to the development of targeted novel drugs such as monoclonal antibodies, small molecule inhibitors, mimetics, antisense and small interference RNA-based strategies, among others. These compounds act on specific targets that are believed to contribute to the development and progression of cancers and resistance of tumors to conventional therapies. Delivered individually or combined with chemo- and/or radiotherapy, such novel drugs have produced significant responses in certain types of cancer. Among the most successful novel compounds are those which target tyrosine kinases (imatinib, trastuzumab, sinutinib, cetuximab). However, these compounds can cause severe side-effects as they inhibit pathways such as epidermal growth factor receptor (EGFR) or platelet-derived growth factor receptor, which are also important for normal functions in non-transformed cells. Recently, a number of proteins have been identified which show a remarkable tumor-specific cytotoxic activity. This toxicity is independent of tumor type or specific genetic changes such as p53, pRB or EGFR aberrations. These tumor-specific killer proteins are either derived from common human and animal viruses such as E1A, E4ORF4 and VP3 (apoptin) or of cellular origin, such as TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) and MDA-7 (melanoma differentiation associated-7). This review aims to present a current overview of a selection of these proteins with preferential toxicity among cancer cells and will provide an insight into the possible mechanism of action, tumor specificity and their potential as novel tumor-specific cancer therapeutics.

Keywords: apoptin, apoptosis, cancer, HAMLET, MDA-7, oncolytic viruses, TRAIL

Experimental Biology and Medicine 2011; **236**: 524–536. DOI: 10.1258/ebm.2011.011004

Introduction

Cancer is a leading cause of death worldwide. Multiple genetic mutations lead to cellular derailment and hence malignancy. However, because of the intricacy and unpredictability of these alterations and their overall effect on cellular signalling pathways, cancer treatment remains nebulous. Surgery, radiotherapy and standard chemotherapy remain the mainstay of treatment, but because of their high failure rate novel therapies are needed, making use of the knowledge derived from molecular, cellular and systems biology studies of tumor development and proliferation.[1–3] Indeed, unravelling such aberrant molecular processes has already begun to shed some light in the generation of novel anticancer therapies.[4,5]

In general, apoptosis is of fundamental importance in the human body. It starts as early as during embryonic development and maintains tissue homeostasis in adults. The physiological role of apoptosis has been extensively described in relation to the immune system[6] and the nervous system.[7] Apoptosis and mitosis need to be closely regulated and kept in balance. An imbalance in favor of mitosis and uncontrolled growth can lead to tumorigenesis. Recently, a group of viral and cellular proteins have been described that selectively induce apoptosis in tumor cells. This concept of tumor-selective cell death has attracted the attention of researchers worldwide with promising results.[8]

In this report, we will review the basic molecular mechanisms of apoptosis as well as the three-way relationship between apoptosis, cancer and viruses. The principle of tumor-selective killing by these proteins will be discussed.

Molecular mechanisms of apoptosis, an overview

Apoptosis involves a complicated chain of molecular events. Here we have summarized the key elements of this intricate

process with particular emphasis on the key mediators that regulate its outcome.

The regulators: mitochondria, Bcl-2 family of proteins and p53

Mitochondria and their interaction with the Bcl-2 family of proteins play a key role in the regulation of apoptosis. Apoptosis requires the release of proapoptotic proteins from the mitochondrial intermembrane space, as a result of modifications in the integrity of its outer membrane. Such events are controlled by the Bcl-2 protein family, which is composed of antiapoptotic and proapoptotic subfamilies. Broadly speaking, the antiapoptotic members stabilize the outer mitochondrial membrane and inhibit the release of intermembrane proteins while proapoptotic members do just the opposite. The destabilization of the membrane may be achieved by the formation of outer membrane permeability transition (PT) pores that lead to loss of the mitochondrial transmembrane potential and allow leakage of proteins such as cytochrome *c*, Smac/Diablo and HrA2/omi. Proapoptotic proteins of the Bcl-2 family such as Bax and Bak are directly involved in this process inducing the opening of these pores. The released cytochrome *c* associates with APAF-1, dATP and procaspase-9 to form the apoptosome complex which activates effector caspases. Once the cell has committed to die, apoptosis-inducing factor (AIF), endonuclease G (Endo G) and caspase-activated DNAse (CAD) are also released from the mitochondria. Both AIF and Endo G are caspase-dependent.[9] AIF translocates to the nucleus causing DNA fragmentation[10] as does EndoG where it cleaves nuclear chromatin to produce oligonucleosomal DNA fragments (stage I condensation of chromatin).[11] CAD once released from the mitochondria is cleaved by caspase-3 and translocates to the nucleus where it leads to oligonucleosomal DNA fragmentation (stage II condensation of chromatin).[12]

All proapoptotic proteins contain the BH-3 motif necessary for dimerization with other proteins of the Bcl-2 family. However, proteins that possess BH3 only are unable to cause mitochondrial membrane permeability and commit a cell to apoptosis like their multidomain family members. They sense and relay stress signals and indirectly activate their more potent proapoptotic members; Bad, for instance, can promote cell death in its phosphorylated form by heterodimerizing with the antiapoptotic Bcl-2 and Bcl-X(L) through its BH-3 domain and rendering them inactive.[13,14] Serine phosphorylation of Bad, however, leads to its sequestration in the cytosol by 14-3-3, a phosphoserine-binding molecule. Finally, another two BH-3 only members worth mentioning are the p53-induced Puma and Noxa. Puma exerts its proapoptotic effect by increasing Bax expression,[15] whereas Noxa interacts with antiapoptotic Bcl-2 family members resulting in activation of caspase-9.[16]

The transcription factor and tumor suppressor p53 and its other family members p63 and p73 are important in mediating the apoptotic process.[17] Mutations in the *p53* gene are found in a number of human malignancies suggesting that it plays a major role in tumorigenesis. p53 has a protective role in cells exposed to stress stimuli by either initiating cell cycle arrest or, if necessary, apoptosis. Normally, p53 is negatively regulated by ubiquitination and subsequent degradation through interaction with MDM-2, an oncogenic E3 ligase.[18,19] p53 can be activated by three main mechanisms. Ionizing radiation gives rise to double-stranded DNA breaks that lead to p53 phosphorylation. This decreases its affinity for MDM-2.[20] It can also become activated secondary to oncogenic growth signals (e.g. Ras and Myc activation) that lead to p14ARF-mediated sequestration of MDM-2.[21,22] Thirdly, chemotherapeutic agents, ultraviolet light and protein kinase inhibitors promote ATR and casein kinase II-mediated phosphorylation of MDM-2.[23] Upon activation, p53 stimulates the transcription of proapoptotic genes such as FAS/CD95,[24] Noxa[25] and Puma,[26] as well as apoptosis-inducing factor-1 (APAF-1).[27]

The initiators and executors: the caspases

The classic apoptotic pathway is conveyed by the caspases, a family of cysteine proteases. The main ones are the initiators (caspase-2, 8, 9 and 10) and effectors (caspase-3, 6 and 7), although others exist without as yet known roles in the regulation of apoptosis.[28] Caspases reside as inactive proenzymes in the cytosol, where they become activated by cleavage of their N-terminal, and the initiators transduce upstream death signals to effector caspases. Some caspases also become activated when they aggregate with other caspases. From the caspase family, capsase-3 is considered to be the most important executioner. It specifically promotes DNA fragmentation by activating CAD.[29] This protein is normally kept inactive in the cell nucleus by binding to its negative regulator, inhibitor of CAD (ICAD).[30] ICAD is cleaved by caspase-3 and therefore liberates CAD. Cytoskeletal re-organization and disintegration of the cell into apobodies is also promoted by caspase-3.[31] Under normal cellular conditions activated caspases are controlled by the inhibitors of apoptosis (IAPs) protein family. Upon apoptosis, however, IAPs are neutralized by the active presence of Smac/DIABLO[32] and Omi/HtrA2.[33]

The pathways

The two principal pathways of apoptosis are the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The extrinsic pathway is of importance in the immune system and involves binding of a death ligand to a receptor of the tumor necrosis factor (TNF) superfamily[34] with its subsequent trimerization and recruitment of adaptor proteins (e.g. FADD [Fas-associated death domain], TRADD [TNF Receptor-Associated Death Domain]) to their cytosolic death domains. To date, the best characterized death receptors and their corresponding ligands are the FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5.

The adaptor proteins, such as FADD, that bind the death domain homodimers, recruit via their own death effector domain procaspase 8 and form the death signalling complex. Two procaspase-8 molecules induce proteolytic autoactivation. The then activated caspase-8 may directly

activate effector capsases or alternatively cleave the proapoptotic Bid which activates mitochondria and therefore links the extrinsic to the intrinsic pathway. The cytosolic protein c-FLIP can bind to both FADD and caspase-8 and inactivate them.[35,36]

This intrinsic pathway is activated by stimuli that trigger the release of proapoptotic proteins directly from the mitochondria. Examples of these include hypoxia, DNA damage and cellular stress, Ca^{2+} fluctuations, nitric oxide, fatty acids and proteases.[37] All these stimuli lead to the formation of PT pores in the mitochondria causing changes to the inner mitochondrial membrane that result in the opening of PT pores through the Bcl-2 family as described above.

T lymphocytes have cytotoxic effects on tumor cells via the extrinsic pathway and the FasL/FasR interaction.[38] However, the cytotoxic effects can be achieved via another pathway that involves the secretion of perforin, a transmembrane pore-forming molecule, with the subsequent release of serine proteases granzyme A and B (including other components) that travel through the pores formed to enter the target cell. Apoptosis is then induced via granzyme A or granzyme B.[39] Granzyme A activates caspase-independent pathways by inducing single-stranded DNA damage and blocking the protection of chromatin and DNA structure.[40,41] Granzyme B, on the other hand, can directly activate the executioner caspase-3, which in turn activates the CAD, but it can also directly cleave the ICAD as well as other factors.[42] In addition, granzyme B also cleaves Bid and therefore induces the release of cytochrome *c*, effectively utilizing the mitochondrial pathway to enhance its apoptotic effect.[42]

The last stage of apoptosis is the uptake of apoptotic cells by phagocytosis. This process is initiated by externalization of phosphatidylserine on the surface of apoptotic cells. The mechanism is not well understood even though Fas, caspase-8 and caspase-3 have been shown to be involved in studies of oxidative damage stress erythrocytes. In addition, the process of phosphatidylserine externalization during a caspase-independent pathway has been shown for T lymphocytes.[43,44] Phosphatidylserine on the outer leaflet of apoptotic cells facilitates non-inflammatory phagocytic recognition, allowing for early uptake and disposal of apoptotic cell debris.[45]

A three-way relationship: apoptosis, cancer and viruses

Apoptosis is crucial in removing cells that have acquired genetic damage or specific abnormalities. Cancer cells arise because of their ability to form mutations that make them escape from the prophylactic apoptosis signals.[46] Classic examples of this are the over-expression of Bcl-2 protein as a result of chromosomal translocation as seen in breast cancer and B-cell lymphoma,[47] and the non-functioning mutations or absent p53 that is known to be involved in numerous malignancies.[48]

Viruses have been shown to be closely linked with cancer by modulating apoptosis processes to their advantage. Oncogenic viruses are those viruses that promote the expression of oncoproteins that in turn interfere with growth inhibitory cellular signalling pathways, rendering the cells tumorigenic. The best studied viruses include polyoma viruses (e.g. SV40), herpes viruses (e.g. Epstein–Barr virus), and adenoviruses, human papilloma viruses (HPV) and hepatitis viruses.

SV40 produces a large T-antigen that interferes with p53 as well as RB family members. It also produces small *t*-antigen (st) protein that blunts the effect of the protein phosphatase PP2A family;[49] this family of proteins is vital for cell homeostasis and lower levels of expression have been associated with certain cancers.

An interesting group of oncogenic viruses are the adenoviruses that possess both pro- and antiapoptotic proteins. Early region proteins E1A and E1B have been shown to be involved in cellular transformation when only present together.[50] The apoptotic ability of E1B 55K is manifested by blocking p53. However, its counterpart E1B 19K acts as a general apoptosis inhibitor, acting on various parts of the apoptosis pathway. Paradoxically, E1A on its own is toxic and proapoptotic by inducing a DNA damage response that culminates in the degradation of Bcl-2 family members (e.g. mcl-2) and the accumulation of p53.[51] However, this apoptotic potential is suppressed by the E1B proteins. Furthermore, the Ras oncogene forms an integral part of the E1A pathway, demonstrating a parallel between apoptosis and transformation.[52] Additionally, the early region E4Orf4 of human adenovirus type 2 has been shown to possess tumor-selective apoptosis function. However, further studies are necessary to understand the function of this protein in regulating cell death and survival.[53] The oncogenic HPV virus encodes proteins with transforming ability. For instance, E6 protein has been shown to cause p53 degradation and hence loss of p53 functions. In fact, p53 is a common target of oncogenic viruses which further emphasizes its importance in the maintenance of cellular integrity.[54] The importance of the dual effect of HPV E6 and E7 proteins in the development of cancer was initially shown in cervical tumors where inhibition of p53 by E6 resulted in interference with the cell cycle regulation, leading to undue cellular proliferation and hence cancer.[55]

From a different perspective, naturally occurring and engineered viruses have been shown to possess oncolytic activity.[56] Oncolytic viruses can be defined as those that propagate selectively in tumor cells and kill them or suppress their activity, leaving normal cells unharmed. These viruses therefore have important therapeutic potential against cancer cells. Further research is also focusing on virus-induced tumor survival pathways that change into an apoptotic one by causing cellular stress; understanding how this occurs can lead to further putative therapeutic targets. It is clear that the intracellular environment and chromosomal instability in a malignant cell is somehow favored by these viruses. It has been suggested that the intracellular defence mechanism in transformed cells makes them susceptible to killing by replicating viruses. Another suggestion has been that malignant cells express higher levels of viral receptors on their surface, making them more sensitive to infection.[57]

A well studied oncolytic virus is Parvovirus-H1 (PH1). Animal models showed tumor regression with no

side-effects,[58] whereas another study demonstrated promising results when used in combination with a low-dose chemotherapeutic agent in rats with pancreatic tumors (gemcitabine).[59] Once again, there were fewer side-effects compared with chemo-monotherapy. It is worth noting that phosphorylation of this virus's major non-structural protein, NS1, by cellular kinases is important for the ability of PH1 to kill tumor cells, further supporting the importance of the cellular environment of cancer cells in modulating the viral toxicity.[60] These observations highlight the apoptosis paradox, where tumor cells on the one hand escape apoptosis and proliferate. On the other hand, they possess a physiological death pathway that can be triggered by oncolytic viruses. In the following sections, a selection of proteins that have shown strong evidence of tumor-selective cytotoxicity will be described and the mechanism of their tumoricidal activities will be discussed in more detail.

Apoptin

Apoptin is a small protein expressed in the chicken anemia virus that has been shown to be the cause of selected apoptosis in infected tumor cells.[61,62] To date, a number of research groups have reported that over 70 different tumor cell lines are sensitive to apoptin-induced apoptosis, whereas normal cell lines in general remain unaffected.

The cellular mechanisms responsible for apoptin activation remain unclear; a series of observations in human cells have indicated that apoptin acts in numerous ways in the cell. Initial insight into the diverse mechanism of action of apoptin can be gained through examination of its primary structure. Apoptin is a protein composed of 121 amino acids and does not seem to have any sequence homology with other known cellular proteins.[63] It is rich in prolines, an amino acid that distorts the secondary structure of the protein, resulting in an intrinsically unstructured protein.[64] *In vitro* studies of purified recombinant apoptin have shown that the N-terminus hydrophobic proline-rich region of apoptin interacts non-covalently to form multimers of 30–40 monomers.[64,65] This is also the case when the protein is expressed *in vivo* and Leliveld *et al.*[64] have shown that apoptin multimers remain biologically active. The C-terminal of the protein, on the other hand, contains a bipartite nuclear localization sequence as well as a putative nuclear export sequence, which regulates the nucleocytoplasmic shuttling of the protein, a key characteristic of apoptin.[66,67] A small isoleucine-rich stretch is required for self-association but also for the binding of other partners, such as promyelocytic leukemia protein. Interestingly, apoptin contains a number of Ser and Thr amino acids that act as phosphorylation sites allowing interactions with kinases as well as an SH3-binding domain.[68]

Mechanisms of action

How apoptin induces cell death is not clearly understood, and cumulative data so far indicate that apoptin plays variable roles in the apoptotic pathways. A fundamental characteristic of apoptin's mechanism of action is that it induces p53-independent apoptosis in human cells.[69] Danen-Van Oorschot *et al.*[70] have shown that caspase activation is required for apoptin-induced cell death; yet apoptin does not seem to be involved in the extrinsic death receptor pathway. Instead it activates the intrinsic pathway, with the associated release of cytochrome *c* from mitochondria to the cytosol. In addition, tumor cells devoid of Apaf-1 were strongly protected from apoptin-induced apoptosis.[71] Interestingly, the involvement of the pro- and antiapoptotic Bcl-2 proteins in the presence of apoptin is debatable, as different groups have shown contrasting data.[71,72]

A number of studies have demonstrated that nuclear localization of apoptin in tumor cells is required for induction of apoptosis, whereas in normal cells, the protein is localized preferentially in the cytoplasm where it is unstable and rapidly degraded. Experimental data have shown that nuclear accumulation of apoptin is regulated by a CRM-1 (chromosome region maintenance 1)/exportin 1 nuclear export signal that is blocked by phosphorylation of Thr-108 at the C-terminus in apoptin by an unknown kinase.[73,74] In addition, a truncated mutant of apoptin consisting of amino acids 1–69, as expected, is located in the cytoplasm but it has some proapoptotic activity.[66] The above are indications that apoptin has two separate independent death domains, the N-terminal, consisting of amino acids 1–69 and the C-terminal consisting of amino acids 80–121. Furthermore, an apoptin mutant T108E that mimics constitutive phosphorylation appears to be able to enter the nucleus and to kill untransformed cells,[75] indicating that phosphorylation is important both for the protein to enter the nucleus and also to initiate apoptosis. Phosphorylation seems therefore to be a key regulatory pathway for the mechanism of apoptin-mediated cell death and much attention has been focused on identifying the kinase(s) responsible. Recently, Maddika *et al.*[76] have identified the cyclin-dependent kinase 2 (CDK2) as a mediator for apoptin phosphorylation. CDK2 is activated by the abnormal phosphatidylinositol 3-kinase (PI3-Akt) pathway which co-localizes with apoptin in the nucleus. Interestingly, components of the PI3-Akt pathway have been identified as interaction partners for apoptin.[77,78] In a recent study, Jiang *et al.*[79] have clearly demonstrated the role of protein kinase C (PKC) isozymes, in particular PKCβ, in apoptin phosphorylation and its apoptosis-inducing function in the multiple myeloma cancer model. These studies highlight that not only apoptin but also its regulatory factors such as tumor-specific kinases have important potential for the development of cancer-specific therapeutics.

Tumor necrosis factor-related apoptosis-inducing ligand

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a type-II transmembrane protein and a member of the TNF superfamily of cytokines. It functions as a modulator of innate and adaptive immunity but also plays a key role in the extracellular regulation of apoptosis, acting as an immune surveillance mechanism against cancers.[80,81] Because of this antitumor activity, TRAIL has become an important therapeutic target for cancer.[82] *In vitro* and

in vivo studies have demonstrated TRAIL-specific tumor cytotoxicity, while combinatorial treatment with low-dose chemotherapeutic agents has led to further significant therapeutic responses.[83,84] Conversely, TRAIL resistance secondary to deficiencies in the apoptotic machinery has been associated with enhanced tumor growth and metastasis.[80,84] The molecular pathways of TRAIL, TRAIL resistance and its pharmacological reversal are discussed below.

TRAIL molecular pathways and resistance

TRAIL promotes the apoptotic process through its homotrimeric binding to the membrane death receptors 4 and 5 (DR4/TRAIL-R1 and DR5/TRAIL-R2) and the subsequent recruitment of adaptor proteins, FADD and initiator capsases. TRAIL generally activates the extrinsic apoptotic pathway; however, as discussed in the previous section, it can also activate the intrinsic apoptotic pathway via the BH3 only protein Bid. Nonetheless, which pathway prevails is dependent on the cell type and its respective intracellular milieu. Apart from its interaction with the death receptors, TRAIL interacts with another set of receptors: the decoy receptors (DcR1/TRAIL-R3 and DcR2/TRAIL-R4), which cannot convey the apoptotic signal. These lack or contain a truncated death domain, thus failing to transduce the TRAIL-mediated apoptotic signal. The death receptors, as discussed previously, play a crucial role in the transmission of the apoptotic signal and their interaction with decoy receptors can cause resistance and tumor survival. Surface expression of DR4 and DR5 has been shown to be affected by a number of mechanisms in cancer cells, such as epigenetic silencing, loss of function mutations and decreased surface transport.[86–88] Post-translational modifications of DRs also seem to play a critical role in the sensitization of tumor cells to TRAIL. *O*-glycosylation or *S*-palmitoylation has been shown to be important for death receptor clustering and their localization to lipid rafts, respectively.[89,90] These mechanisms promote efficient TRAIL-induced signal transduction and it is postulated that if inhibited, resistance can occur. Moreover, any imbalance between the levels of decoy receptors and DRs on the cell surface could lead to resistance.[91,92] An imbalance in the death-inducing signalling complex components (i.e. c-FLIP/caspase-8 ratio) could have a similar effect, as demonstrated in many cancer cells with high levels of c-FLIP or low levels of caspase-8.[93,94] TRAIL resistance can also occur at the mitochondrial level by the increase of prosurvival Bcl-2 members or a deficiency in the proapoptotic Bax that could block the subsequent release of proapoptotic factors such as Smac/Diablo from mitochondria.[95] Similarly, the increased expression of IAP members (e.g. XIAP, survivin) leads to decreased caspase activity, cell survival and TRAIL resistance.

Other mechanisms of TRAIL resistance involve signal transduction pathways that occur in parallel to TRAIL-induced pathways. Moreover, these pathways could partly explain how the TRAIL response could be switched from apoptotic to proliferative. This proliferative effect has been described for glioma and small cell lung cancer lines, Bcl-XL-over-expressing Colo357 mice with pancreatic duct carcinoma and *ex vivo*-treated blast cells from leukemia patients.[96,97] Firstly, the prosurvival phosphoinositide 3-kinase (PI3K)–Akt axis is able to block TRAIL-induced apoptosis by increasing the expression of antiapoptotic factors c-FLIP, XIAP and Bcl-2.[98] Secondly, the nuclear factor kappa B (NF-κB) family of dimeric transcription factors have been implicated in the regulation of the cellular response to TRAIL; however, their precise role remains unclear.[99] The mitogen activated protein (MAP) kinase signalling pathways (ERKs, JNKs and p38-MAPKs) have also been implicated in regulating the cellular response to TRAIL. ERK activation has been shown to be prosurvival in HeLa cervical carcinoma cells and inhibition of this pathway sensitizes cells to TRAIL-induced death.[100] Similarly, Mucha *et al.*[101] have shown that JNK signalling could confer TRAIL resistance in cancer cells; inhibition of this signalling pathway promoted TRAIL-induced apoptosis in hepatocellular carcinoma but not in normal cells. In contrast though, other studies have demonstrated the opposite: activation of JNK sensitizes the tumor cells to TRAIL-induced apoptosis.[102] These data suggest that a balance between various molecules and signalling pathways will dictate either the apoptotic or proliferative effect of the cytokine.

Tackling TRAIL-mediated apoptosis resistance pharmacologically

Recent insight into the mechanisms of TRAIL resistance has led to the development of TRAIL-mediated apoptosis sensitizing drugs; these have shown promising results in the management of TRAIL-resistant tumors when used in combination with TRAIL. The upregulation of death receptors by these sensitizing compounds is one mode of action that has been described. Shenoy *et al.*[103] have recently shown that LY303511, the inactive analogue of PI3K inhibitor LY294002, enhanced the sensitivity of SHEP-1Nb cells to TRAIL-mediated apoptosis by upregulating DR4 and DR5 in a MAPK-dependent fashion. The same group also highlighted the importance of reactive oxygen species (ROS) production in TRAIL sensitization by demonstrating limited death receptor upregulation in the presence of catalase, an H_2O_2 scavenger. Similarly, drugs such as sulforaphane and curcumin have been shown to sensitize hepatoma and renal carcinoma, respectively, to TRAIL via a ROS-mediated upregulation of DR5, while pretreatment with antioxidants (*N*-acetyl cysteine and peroxiredoxin II) decreased the DR levels.[104,105] Another group of drugs, the histone deacetylase inhibitors (e.g. trichostatin A, sodium butyrate) have also been shown to sensitize tumor cells to TRAIL by increasing mRNA levels and the subsequent surface expression of DR5.[106] The further group of drugs to consider in this category is proteasome inhibitors that are already in clinical use for the management of multiple myeloma. One of these, bortezomib (Velcade, PS-341), has been shown to upregulate DR5 and also prevent caspase-8 degradation.[107] Another such compound, MG132, induces DR5 expression in prostate cancer cells in a C/EBP homologous protein (CHOP)-dependent manner.[108] In glioma cells, however, DR5 upregulation is

achieved in a CHOP-independent but JNK-dependent way.[109] Finally, MG132 also sensitizes tumor cells to TRAIL through ROS production which leads to p53-mediated DR5 upregulation, a process inhibited by N-acetylcysteine (NAC) and cellular glutathione.[110] Lipid rafts have been postulated to sensitize cells to TRAIL-mediated apoptosis through death receptor clustering and re-distribution.[111] This has been shown pharmacologically with quercetin and its derivative, LY303511, in colon adenocarcinoma and HeLa cells, respectively, even in the presence of limiting anti-DR5 antibody for the latter.[112,113] Cyclooxygenase 2 inhibitors and aplidin, an antitumor agent, also seem to increase TRAIL sensitivity through lipid raft-mediated clustering of DR5.[114] Conversely, pretreatment of cancer cells with cholesterol extracting agents such as nystatin and methyl-beta-cyclodextrine have shown to disrupt lipid rafts, block DR redistribution and hence TRAIL sensitization.[115] TRAIL-sensitizing drugs are also known to work by downregulating c-FLIP through a number of mechanisms. Withaferin A, for instance, can regulate both c-FLIPs and c-FLIPL transcriptionally in a ROS-mediated, NF-κB-dependent manner. This activity has been shown to be hampered in the presence of NAC.[116] Moreover, quercetin decreases c-FLIP at the post-transcriptional level via proteasomal degradation. Interestingly, even though the endogenous ligand of peroxisome proliferator-activated receptor gamma (PPARgamma), 15d-PG(2) can stabilize DR5 mRNA and enhance TRAIL-induced apoptosis, treatment with PPARgamma agonist or synthetic ligand rosiglitazone results in selective downregulation of c-FLIPs.[117] IAPs are known to be upregulated in cancers and are important drug targets. Knockdown models of XIAP and all three IAPs (c-IAP-1, c-IAP-L, XIAP) in colon and prostate carcinoma cells, respectively, demonstrated increased sensitivity to TRAIL-mediated apoptosis.[118,119] TRAIL has also been used in combination with XIAP inhibitors, inducing apoptosis in pancreatic carcinoma cells.[120] TRAIL is also known to downregulate antiapoptotic Bcl-2 proteins. The soy isoflavonoid drug, daidzein, specifically downregulates Bcl-2 and thus selectively induces death upon combination with TRAIL in glioblastoma cells but not in human astrocytes.[121] The BH3 mimetic, ABT-737, sensitizes pancreatic cancer cells to TRAIL-induced apoptosis by releasing proapoptotic Bim and Bak from their prosurvival counterpart.[122] However, treatment of colon cancer cells over-expressing Bcl-2 with another Bcl-2 inhibitor, HA 14-1, promotes Bax re-distribution and subsequent cytochrome *c* release, thus reversing TRAIL resistance.[123] On a final note, a novel approach to activating the TRAIL pathway in a tumor-selective manner, even in the presence of sensitizing compounds, involves the recently developed specific multivalent DR5-selective synthetic peptides.[124]

Where are we now and the future

In conclusion, TRAIL targeted therapies, either alone or in combination with novel and conventional therapeutics, have shown promising results in phase I clinical trials. Combinatorial therapy of TRAIL with chemotherapeutic agents such as R-roscovitine, for instance, has shown to increase the antiapoptotic activity of TRAIL in thyroid cancer.[125] Importantly, TRAIL has shown very little or no toxicity in normal cells and TRAIL treatment is well tolerated, offering TRAIL as a promising tumor-selective cancer therapeutic. The initial concerns about its toxicity were attributed to the use of tagged TRAIL, whereas in its untagged version no toxicity to normal cells has been shown.[126] Indeed, initial reports in phase I and II trials have shown few side-effects with nausea, fatigue and leucopenia being the commonest. Currently, a number of phase II clinical studies using TRAIL monotherapy or combined with anticancer therapeutics are underway.[127] The pharmacological strategies that are being used include the administration of recombinant human TRAIL (Apo2L/AMG951), death receptor activating antibodies (mapatumumab/NGS-ETR1 and lexatumumumab/NGS-ETR2/AMG655) and the adenoviral-mediated delivery of the TRAIL coding sequence in tumor cells.[128] The TRAIL pathway is considered as one of the most promising non-genotoxic cancer-specific therapy with six completed and 25 ongoing clinical trials. However, it is worth noting that most of the available data from these studies involve tumors of a poor prognostic character. Suffice it to say that the outcome of TRAIL therapy in less aggressive and advanced tumors remains unknown. The use of adjuvant TRAIL-based therapy is considered to be a promising future therapeutic.

MDA-7

Melanoma differentiation associated-7/interleukin-24 (MDA-7/IL-24) protein has been classified as a member of the interleukin family due to the presence of the conserved interleukin signature motif.[129] This is another tumor suppressor protein, the expression level of which has been shown to be decreased in advanced melanomas.[130] By contrast, *in vivo* over-expression of the protein results in tumor cell death, as shown in a broad spectrum of cancer cells.[131] However, what makes this an interesting protein to study in relation to cancer is the fact that when over-expressed in normal cells, there was no effect on cell viability or growth.[131]

The pathway by which MDA-7/IL-24 induces cancer-specific apoptosis has been extensively investigated. These studies have shown that the protein is involved in the regulation of the endoplasmic reticulum (ER) stress following the binding of the protein to an HP70 family chaperone, BiP/GRP78, where the latter becomes inactivated.[132] This binding eventually leads to the phosphorylation of eukaryotic initiation factor 2 (eIF2) and therefore to a general suppression of protein expression, particularly of antiapoptotic proteins, such as Bcl-XL, MCL-1 and c-FLIP.[133,134] A similar response/effect is achieved by the binding of the cytokine to the double-stranded RNA-dependent protein kinase.[135] This binding induces activation of the kinase, resulting in the activation of its downstream targets, such as eIF2.[136] On the other hand, the cytokine has been shown to promote the proapoptotic Bax and Bak gene expression, which play critical roles in cell death.[134] BAX and BAK protein activation

eventually lead to mitochondrial dysfunction and cell death.[137] Furthermore, MDA-7 has been implicated in inducing cell death with the contribution of ceramide, a promoter of apoptosis and a key mediator of ER stress pathway. Cancer cells infected with a replication incompetent adenovirus (Ad-*mda-7*) were shown to have selectively increased levels of ceramide. Sauane *et al.*[138] have shown the correlation between ceramide and the cytokine and that ceramide is required for inducing cell death by MDA-7. The same study demonstrates that MDA-7 induced the inactivation of the antiapoptotic molecule Bcl-2, a downstream protein in the ceramide-mediated survival pathway. Another mechanism by which MDA-7 specifically induces apoptosis is via oxidative stress. This occurs when MDA-7, as delivered by adenoviral infection in prostate cancer cells, promotes the production of ROS followed by mitochondrial dysfunction (also promoted by MDA-7, as explained earlier).[139] When the Bcl-2 and Bcl-XL antiapoptotic molecules are expressed, the ROS production is also inhibited, as is eventually apoptosis. Interestingly, MDA-7 has been shown to activate the Fas/TRAIL pathways resulting in tumor-selective apoptosis. Studies in melanoma cells have demonstrated that MDA-7 induces the secretion of interferon-beta, which subsequently leads to interferon regulatory factor (IRF-1) regulation and Fas/TRAIL activation. [140]

All the different characteristics of MDA-7 make this protein an attractive antitumor agent. The phase I clinical trial in advanced carcinomas and melanomas, involving the repeated intratumoral injection of a replication incompetent serotype 5 adenovirus that expresses MDA-7 (Ad-*mda-7*), demonstrated that MDA-7 is well tolerated while showing significant evidence of cancer-specific apoptosis.[141,142] It was also evident that infection with Ad-*mda-7* resulted in increased levels of MDA-7 protein and apoptosis in cancer cells distant to the administration site. These results indicate that secreted MDA-7 has a 'toxic bystander' effect on uninfected tumor cells.[143,144] In addition, this study supported the immunomodulatory properties of the cytokine, by showing increased serum levels of IL-6, IL-10 and TNF-α with increasing populations of CD3+ and CD8+ T-cells. Nevertheless, resistance of tumor cells to MDA-7 has been an issue particularly with pancreatic and colorectal cancer cells,[145,146] and therefore studies that focus on combinational therapy appear to be more promising. So far, combinational therapy using MDA-7 with Trastuzumab (Herceptin) in breast cancer cells have shown significantly increased antitumor activity compared with the controls, something that may improve the treatment options for this type of cancer.[147,148] Using Ad-*mda-7* in combination with radiation[134,149] and chemotherapy[134] in mice bearing glioblastoma (GBM) cells showed that the presence of the cytokine could prolong the survival of the animals by multiple mechanisms of action. Strategies to overcome resistance of cancer cells to Ad-*mda-7* are crucial in designing the appropriate combinational therapeutic strategy, particularly as this agent is being evaluated in phase II clinical trials for multiple cancers. In addition, identification of biomarkers, which may allow prediction of the response or resistance to MDA-7-mediated therapy, would be helpful in selecting the appropriate mono- or combinational therapy.

Human α-lactalbumin made lethal to tumor cells

Human α-lactalbumin made lethal to tumor (HAMLET) cells, human milk-derived molecular complex consisting of partially unfolded α-lactalbumin and five molecules of oleic acid, kills tumor cells in an apoptotic-like fashion.[150] This tumoricidal activity was first discovered by Hakansson *et al.*[151] when studying the role of milk fractions on bacterial attachments of lung carcinoma cells. Nearly a decade later, Fast *et al.*[152] showed that the binding of α-lactalbumin to oleic acid was associated with calcium release followed by the partial conformational change of the protein. Remarkably this partial unfolding of protein and its subsequent binding to fatty acids confers tumor-selective cytotoxicity. Such biochemical events suggest alternative ways by which proteins can diversify their functions and substrate interactions.[153]

Initially, HAMLET's tumor-specific cytotoxicity was shown *in vitro* with normal cells being resistant to its effect.[154] The therapeutic potential of HAMLET was confirmed by two pilot *in vivo* studies for the local treatment of skin papillomas and bladder cancer using intravesicular instillation of the complex.[150,155] In these studies the papillomatous skin tumor disappeared completely and eight out of nine bladder cancer patients showed tumor regression (reviewed in Noteborn[156]). Recent studies of the effects of HAMLET on mouse bladder carcinoma models have also shown promising results with a dose-dependent decrease of tumorigenic cells, a delay in cancer development and a selective uptake of HAMLET by the bladder tumors.[157] Importantly, no major toxic side-effects have been documented for *in vivo* use.[158]

To date, the mechanisms of tumor cell death remain unclear. It is thought that it might involve tumor-specific shuttling mechanisms, allowing for HAMLET's internalization and subcellular localization.[159] However, there is insufficient experimental data to validate this hypothesis. HAMLET seems to translocate preferentially to tumor cell nuclei and specifically interact with histones, causing chromatin disruption, nuclear condensation and therefore loss of transcription.[160] Similarly to apoptin,[161] this affinity for nuclear structures could underpin the mechanism of apoptosis induction in tumor cells. Yet, a recent study on HAMLET's bovine counterpart (BAMLET) has shown how it accumulates in the endolysosomal compartment of tumor cells and induces early leakage of cathepsins into the cytosol followed by the activation of proapoptotic protein Bax, providing some insight into the intricate mechanisms of HAMLET-induced tumor cell death.[162] Based on these studies, it has been speculated that because HAMLET has a widespread interaction with several crucial organelles, a number of cell death pathways may potentially be activated in parallel. Nonetheless, treatment with HAMLET destabilizes mitochondrial membranes leading to cytochrome *c* release, phosphatidyl serine

exposure and low-grade caspase response.[163] Additionally, HAMLET creates a state of unfolded protein overload with subsequent activation of 20S proteasomes, which contribute to cell death.[164] It is important to note that although some HAMLET-treated cells undergo morphological changes similar to those of apoptosis, neither caspase inhibition with the pan-caspase inhibitor zVAD-fmk nor Bcl-2 over-expression or altered p53 status appear to alter tumor cell sensitivity/resistance to HAMLET.[154] Moreover, antibodies blocking FAS/CD95 receptor pathways have been unable to inhibit HAMLET-induced cell death.[154]

Macroautophagy has been proposed as a potential mechanism for HAMLET-induced cell death. This is a lysosomal catabolic pathway where cytosolic molecules and organelles are degraded and if necessary recycled.[165] This process involves upstream autophagic stimuli that promote the formation of autophagosomes that eventually fuse with lysosomes. The exact role of macroautophagy in cell death/survival is a matter of debate with some believing that it is an adaptive stress response in the dying cell to promote survival.[166] Others, however, have described this process as a cell death pathway.[167] Interestingly, dying HAMLET-treated cells show features characteristic of macroautophagy.[168] Moreover, HAMLET-induced cell death was reduced when macroautophagy was inhibited, indicating the importance of this process in HAMLET-induced cytotoxicity. Similarly, it has been shown that cancer cells possess altered macroautophagy pathways as opposed to their normal counterparts, which might partly explain the observed tumor-selective activity of HAMLET.[169]

One of the drawbacks of therapeutic application of HAMLET is the need for local administration and therefore a restricted spectrum of tumors can be targeted by this agent. Further studies are needed to unravel both the molecular mechanisms of the apoptosis-like cell death induced by HAMLET as well as alternative production and delivery systems to exploit its potential as an effective and tumor-specific therapeutic agent.

Table 1 Clinical treatment approach for apoptin, TRAIL, MDA-7 and HAMLET

Protein	Origin	Treatment approach	Clinical stage	References
Apoptin	Chicken anemia virus	Recombinant apoptin (TAT−, PDT4−) Combinational therapy Non-replicative adenovirus	Preclinical	72,170–173
TRAIL	Human cells	Recombinant TRAIL Agonistic monoclonal antibodies Death receptors Gene transfer therapy	Phase I and II	124,127
MDA-7	Human cells	Viral delivery Recombinant MDA-7 (GST−) Plasmid DNA	Phase I	140,174,175
HAMLET	Human breast milk	*In vitro* refolded HAMLET	Phase I and II	150,154,168

TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; HAMLET, human α-lactalbumin made lethal to tumor cell; MDA-7, melanoma differentiation associated-7

Discussion

Cancer remains one of the major causes of death worldwide, accounting for over 13% of all human deaths per year. Recent insight into cancer cell biology has led to the pharmaceutical development of novel targeted anticancer drugs. The application of several novel compounds in clinical trials has proved very promising. However, successful treatment of cancer with targeted therapies is still the exception and limited to malignancies for which specific genetic, epigenetic or metabolic causes have been identified.

This review has sought to assess the therapeutic potential of a group of proteins from both cellular and viral origins with cancer-selective cytotoxicity, as summarized in Table 1. Remarkably, these proteins appear to function regardless of the genetic background and type of cancer cells and can therefore have therapeutic potential for a wide range of cancer types. Discovery of the mechanism by which these proteins, including apoptin, TRAIL, MDA-7 and HAMLET, selectively kill cancer cells has highlighted the presence of common pathways shared among different tumor types. Several possible mechanisms such as activation of specific kinases, altered DNA damage repair response, distorted post-translational protein modification, and differences in the cytoplasmic or internal membrane structure and organization have been proposed as the underlying mechanisms for the tumor selectivity of such proteins. However, the precise mode of their tumor selectivity remains largely unknown. Similarly, tumor-selective killing by oncolytic viruses has been extensively studied with a view of their application in cancer therapeutics. The oncolytic viruses' activity also relies on common changes in cancer cells such as active signalling and stress pathways, loss of cell cycle control mechanisms and impaired DNA damage repair mechanisms. The emerging knowledge of the mode of tumor selectivity of such proteins not only offers novel tumor-specific therapeutics, but could also provide important clues for stratification of the strategies for sensitizing cancer cells to novel and targeted therapies. Undoubtedly, further research is needed to understand the mechanistic basis of the activity of these proteins and to develop tools to exploit their selectivity for therapy.

Author contributions: All authors participated in the design, writing and editing of the manuscript.

ACKNOWLEDGEMENTS

We would like to thank the CRUK and Rosetrees Trust for funding the apoptin studies.

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