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Expert Opinion

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Human toxin-based recombinant immunotoxins/chimeric proteins as a drug delivery system for targeted treatment of human diseases

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Introduction: The development of specific immunosuppressive reagents remains the major goal in the treatment of human diseases. One such approach is the use of recombinant immunotoxins/chimeric proteins, composed of targeting and killing moieties, fused at the cDNA level. Most of these 'magic bullets' use bacterial or plant toxins to induce cell death. These toxins are extremely potent, but they also cause severe toxicity and systemic side effects that limit the maximal doses given to patients. Moreover, being of non-human origin, they are highly immunogenic, and the resulting neutralizing antibody production impairs their efficacy.

Areas covered: This review describes recombinant immunotoxins/chimeric proteins composed of the classical delivering, cell-targeting molecules, fused to highly cytotoxic human proteins capable of generating an intense apoptotic response within the target cell. This review focuses on the new 'Human Killing Moieties' of these targeted proteins and describes recent progress in the development of these promising molecules.

Expert opinion: Human toxin-based immunotoxins/chimeric proteins for the targeted delivery of drugs are still in their early stages of development. However, they are certain to advance in the very near future to become an extra weapon in the everlasting war against human diseases, mainly cancer.

Keywords: apoptosis, cancer, chimeric proteins, recombinant immunotoxins, single chain antibody, targeting

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1. Introduction

One of the major goals in modern medicine is the design of targeted therapeutic molecules that can selectively kill and eliminate specific pathogenic cell populations without harming neighboring healthy cells. This concept was in fact conceived by Paul Ehrlich, who over a century ago suggested that drugs could be bound to specific delivery vehicles that would carry them specifically to their site of action. These carriers were termed 'magic bullets', which theoretically could cure all diseases, providing their pathologies were known.

Recombinant immunotoxins/chimeric proteins are an expanding, new and important class of such targeted therapeutic agents. The designs of these

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Article highlights.

- Recombinant immunotoxins/chimeric proteins based on the classical plant or bacterial toxins are highly immunogenic and cause severe general toxicity and systemic side effects.
- Human toxin-based recombinant immunotoxins/chimeric proteins are composed of the classical delivering, cell-targeting molecules, namely, antibodies or cytokines/growth factors/hormones, fused to highly cytotoxic human proteins capable of generating an intense apoptotic response within the target cell.
- The 'killing moiety' is an endogenous human protein, namely a proapoptotic protein of the apoptotic cascade (Bax, Bik, Bak, Bid/tBid, caspases), granzymes, endonucleases, TRAIL, FASL, RNase or kinase.
- Being of human origin, these human-based chimeras are expected to display reduced immunogenicity in human recipients.
- As target cells are killed through apoptosis, systemic damage is less likely.
- These new molecules still possess some limitations, such as poor penetration into solid tumors and some nonspecific toxicity, caused by the fact that most target antigens are expressed to some extent on normal tissues.
- Human toxin-based immunotoxons/chimeric proteins are expected to become a new, extra weapon in the battle against human diseases, mainly against cancer.

This box summarizes key points contained in the article.

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constructs typically involve a 'cell-targeting' delivery motif, genetically fused to a 'killing moiety' composed of a highly toxic class of proteins or enzymes capable of ruthlessly attacking critical cellular machinery, once delivered successfully to the cytoplasm of the target cell. Initial development of this class of constructs typically contained recombinant single-chain antibodies (to form immunotoxins) or recombinant growth factors (to form chimeric proteins) as the 'cell-targeting' delivery motif, fused to highly cytotoxic plant or bacterial toxins that posses the 'killing moiety'. This review describes second-generation immunotoxins/chimeric proteins composed of the classical cell-targeting molecules fused to highly cytotoxic human proteins capable of generating an intense apoptotic response within the target cell [1]. This review focuses on the new 'Human Killing Moieties' of these new targeted proteins.

2. The delivery system: the 'cell-targeting moiety' of immunotoxins/chimeric proteins

The delivery moiety of recombinant immunotoxins/chimeric proteins is a ligand derived from the immune system. Although the classic delivery ligand from the immune system is the antibody, growth factors are also proteins of immunologic interest, and growth factors or cytokines fused to toxins were intensively used and developed in recombinant immunotoxins/chimeric proteins.

2.1 Antibodies as 'cell-targeting' delivery moiety of immunotoxins

With the advent of hybridoma technology as pioneered by Kohler and Milstein [2], it was possible to produce monoclonal antibodies of defined antigen specificity. A major breakthrough in the field of antibody engineering was the generation of antibody fragments as recombinant proteins in the periplasm of *Escherichia coli* [3]. The genes encoding the variable regions of monoclonal antibodies were cloned into *E. coli* expression vectors in order to produce antibody fragments, which preserve the binding specificity of the parental hybridoma antibodies [4]. These single-chain antibodies (scFvs) are a minimal form of functional antibodies. Initially, recombinant immunotoxins utilized scFvs for the targeted delivery of the toxin. These were made up of the light and heavy chains of an antibody connected by a 15-amino acid flexible peptide [5].

An example is immunotoxin LMB-2 (anti-TacFv-PE38), which contains the Fv of an antibody (anti-Tac) directed at the α-subunit of the interleukin-2 receptor (IL-2R), fused to a truncated form of Pseudomonas exotoxin A (PE38, see later). Although LMB-2 and a few other immunotoxins were sufficiently stable for clinical use [6], many single-chain immunotoxins were not, because the disulfide bond that normally stabilizes the Fv region is removed to make the scFv, thus the light and heavy chains of many scFvs can dissociate and bind to other dissociated Fvs, leading to aggregation and loss of activity.

This instability was overcome by designing a very stable Fv in which the peptide linker of the scFv is replaced by a disulfide bond inserted in the framework region of the Fv. Using the crystal structures of various antibodies, Brinkmann and colleagues determined which two amino acid residues in the light and heavy chains of the Fv are in a location that allows them to be replaced by cysteine residues that combine to form a stable cystine connecting the two chains of the Fv [7]. It is also possible to use protein engineering to make stable single-chain immunotoxins by mutating residues that lie in the region where the light and heavy chains of the Fv are in contact [8,9].

Many immunotoxins with disulfide- stabilized Fvs (dsFvs) have been constructed, and several have been evaluated in clinical trials. The first to enter the clinic was directed at Her2/neu expressed on breast cancer cells [10]. Another is BL22, which had produced many complete remissions in hairy-cell leukemia [11]. A variety of recombinant immunotoxins have been developed over the years utilizing scFv or dsFv of antibodies directed against different antigens, such as CD19 [12], CD25 [13-15], CD22 [11, 16-18], CD30 [19], CD40 [20], CD7 [21], LeY [22,23], erbB2 [10,24], EGF-receptor [25,26], mesothelin [27], and others, expressed on various cancers. However, none of these

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'classical' recombinant immunotoxins was ever approved for clinical use in humans.

2.2 Cytokines, growth factors and hormones as the 'cell-targeting' delivery moiety of chimeric proteins

Although immunotoxins based on antibodies or fragments of antibodies as their cell-targeting delivery moiety acted as potent killing agents, they still caused nonspecific toxicity and, at sufficiently high concentrations, they damaged normal cells that did not express the specific target antigen. Researchers speculated that smaller molecules such as cytokines, growth factors (GF), or hormones could achieve the desired specificity in targeting cancer cells, based on the fact that cancer cells have more GF receptors than normal cells and are usually involved in an autocrine, paracrine GF exertion loop, allowing them to proliferate without control. In patients, several hundreds to thousands of these receptors are typically expressed per malignant cell. These include receptors for cytokines, interleukins, hormones, and so on. The constructs containing those small molecules as their cell-targeting delivery moiety were termed chimeric proteins.

A variety of cytokines, growth factors and hormones were used as the cell-targeting delivery moiety of chimeric proteins, including IL-2 [28-30], IL-3 [31], IL-4 [32,33], IL-13 [34,35], EGF, TNF-α [25,26], GM-CSF [36], transferrin [37], GnRH [38-42] and others. Most of these ligands were fused to the classical bacterial PE or diphtheria (DT) toxins and tested in preclinical studies. The first and only chimeric protein approved by the FDA for use in human patients was DAB389IL-2 (ONTAK), which is indicated for the treatment of cutaneous T-cell lymphoma [43-45]. The molecule, consisting of a truncated form of the bacterial protein DT fused to IL-2 as the cell-targeting delivery moiety, proved that chimeric proteins can be useful pharmaceutical agents. ONTAK is also used for the treatment of other neoplasms.

Another example of a delivery ligand is the hormone GnRH. Also termed luteinizing hormone-releasing hormone (LHRH), GnRH is a decapeptide synthesized by the hypothalamic neurons and secreted in a pulsatile manner into the hypophysioportal circulation by means of the portal vessels. As GnRH is involved in many hormone-dependent cancers, and analogues are used to treat them, the author and other groups have used this hormone/peptide as the cell-targeting delivery domain to target specifically malignant cells. In the author's laboratory, a number of GnRH-based chimeric proteins were constructed using an analogue of the decapeptide GnRH (GnRH–trp6; tryptophan was substituted for glycine in the sixth position) as the delivery domain, fused to PE, producing the GnRH–PE66 and GnRH–PE40 chimeric proteins. Significant growth inhibition and death of a wide variety of cancer cell lines, both from hormone-responsive and non-responsive tissues, was detected on treatment with our chimeric proteins. Pathological studies revealed that all the responsive cell lines were of the adenocarcinoma type, whereas non-adenocarcinoma cell lines and other malignancies were not affected. Comparable to the cell lines, counterpart primary tissues taken from human patients showed growth inhibition when treated with the GnRH-based chimeric protein [38]. In an *in vivo* nude-mouse colon adenocarcinoma xenograft model, the GnRH–PE66 chimeric protein inhibited formation of the tumors as well as tumor development once the growths were established [39,40]. Moreover, the author and co-workers' studies revealed for the first time that a very large group of cancers, both hormone-dependent and hormone-independent, all defined as the adenocarcinoma type, respond to GnRH-based chimeric proteins and, most probably, express the GnRH-receptor [46,47].

It should be pointed out that most of the recombinant immunotoxins/chimeric proteins are developed for cancer treatment; however, this drug delivery system can be applied to any human disease for targeted treatment. The author's laboratory evaluated several chimeric proteins for the targeted treatment of allergy responses [48,49], multiple sclerosis [50], and more.

3. What to deliver for targeted human therapy: the 'killing moiety' of recombinant immunotoxins/chimeric proteins

3.1 The classical bacterial/plant toxins

Classical immunotoxins/chimeric proteins carry potent plant toxins or bacterial proteins capable of killing cells. Plant holotoxins, also referred to as class II ribosome-inactivating proteins, contain both binding and catalytic domains, and include ricin, abrin, mistletoe, lectin and modeccin. Hemitoxins, also called class I ribosome-inactivating proteins, contain only catalytic domains and include pokeweed antiviral protein (PAP), saporin, Bryodin 1, bouganin and gelonin [51]. Plant toxins have been shown to prevent the association of elongation factor EF1 and EF2 with the 60S ribosomal subunit by removing the base of A4324 in 28S ribosomal RNA [52,53]. Ricin also removes the neighboring base G4323 [52]. Toxin cytotoxicity is optimal when the catalytic domain alone translocates to the cytosol [54,55]. Attempts to fuse plant toxin fragments to cell-targeting delivery moieties to make recombinant toxins have not resulted in molecules suitable for drug development. The difficulty has been either in premature separation of the toxin and delivery moiety before cell binding, or, after internalization, lack of separation of toxin and delivery moiety before translocation to the cytosol [56-58]. However, more recently, a few recombinant immunotoxins based on gelonin [59] or bouganin [60] were engineered with a protease (such as furin)-sensitive linker, to improve intracellular toxin release, and demonstrated efficacy both *in vitro* and *in vivo*. Nevertheless, introduction of the furin site did not always improve the activity of the immunotoxin [60].

The ability of delivery moieties to separate predictably from the killing domain only after internalization is an important

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feature of recombinant toxins [61] and is a unique feature provided by the bacterial toxins PE and DT, the two most commonly used bacterial toxins in the field of recombinant immunotoxins/chimeric toxins.

Both PE and DT enzymatically modify EF2 in the cytosol [62-64]. Both catalyze the adenosine diphosphate (ADP) ribosylation of residue His699 of EF2, which is post-translationally modified to a diphthimide residue [65-67]. Both toxins are produced as single-chain proteins containing a catalytic domain that is fused to the binding domain through a central translocation domain, which facilitates transfer of the catalytic domain into the cytosol. In each case, the toxin is proteolytically cleaved within the translocation domain, and a disulfide bond holds the two fragments together until it is reduced.

To improve specificity, toxins to be fused to delivery moieties have deletions of their binding domains to prevent their binding to normal cells. The most common truncated form of DT is DT388 or DAB389, containing the first 388 amino acids of DT [68-70]. The most common truncated form of PE is PE38, composed of amino acids 253 – 364 and 381 – 613 of PE. To allow the ADP-ribosylating domain to translocate to the cytosol without the delivery moiety, the delivery moiety is placed at the amino terminus of PE and at the carboxyl terminus of DT [71].

3.2 Drawbacks of using bacterial and plant toxins

In spite of the promise shown by bacterial and plant toxin-based immunotoxins/chimeric proteins, they pose several obstacles that limit their wide clinical application [72]. Though the toxin incorporated into the immunotoxins/chimeric protein is truncated, and large parts of the binding and translocation subunits are removed from its sequence, some of this ability remains and causes nonspecific toxicity and side effects. Thus, every immunotoxin/chimeric protein displays some degree of nonspecific toxicity and at high concentrations it damages normal cells that do not express the specific target antigen. These toxin-based recombinant proteins affect mainly hepatocytes, as reflected by abnormal liver function tests, or the vascular endothelium, resulting in the vascular leak syndrome (VLS) [73]. The nonspecific toxicity is probably a result of the easy access and the very rapid nonspecific uptake of proteins by hepatocytes. The damage to the liver, kidney or vascular endothelium could either be caused by direct toxicity or might be secondary: macrophages taking up these molecules could be stimulated to release cytokines, causing injury to the liver, kidney and blood vessels.

Another major drawback in the clinical application of these molecules is the immune response they elicit, mainly towards the toxin moiety. Plant and bacterial toxins are highly immunogenic and, being large molecules, are difficult to humanize. A prominent example is DT-based immunotoxins/chimeric proteins; as most people in developed countries have been vaccinated against DT [74,75], it leads to neutralization of the activity in the serum.

To overcome these problems, various strategies have been tried. Notable among them are the administration of immunosuppressive agents such as cyclophosphamide and cyclosporine and monoclonal antibodies such as rituximab, a polyethylene glycol modification of the toxin, and site-directed mutagenesis of the toxin to generate less immunogenic variants [76]. One such strategy was to identify B-cell epitopes in PE and to introduce mutations in a PE-based recombinant immunotoxin targeting the CD22 (HA22-LR-6X), in which most B-cell epitopes were removed by deleting domain II and mutating six residues in domain III of PE. This mutated immunotoxin had low immunogenicity, low nonspecific toxicity and high antitumor activity in mice [77]. A DNA–protamine-based immunotoxin has also been developed [78]. Owing to the limited success achieved with these strategies in animal models and clinical studies, efforts are being made to generate a new class of immunotoxins/chimeric proteins in which the 'killing moiety' is an endogenous protein of human origin, namely a proapoptotic protein, RNase or kinase, mainly geared to inducing apoptotic cell death.

3.3 Proapoptotic proteins used for targeted immunotoxins/chimeric proteins

Apoptosis, programmed cell death, is an essential process aimed at removing unneeded cells both during embryonic development and in tissue homeostasis. It is a well-defined biochemical and morphological process that ends with the formation of apoptotic bodies, fragmented DNA-containing vesicles that are easily removed by phagocytosis. This 'clean' process avoids the inflammatory response that results from leakage of the cellular contents to the extracellular environment, as occurs in necrosis [79]. The apoptotic process can be activated through two major pathways: the extrinsic pathway, via death receptors on the cell surface; or the intrinsic pathway, activated by cellular stress. The tight control over this process is extremely important. Oversensitivity of the cells can lead to degenerative diseases and ischemic damage, while defects that render the cells resistant to death stimuli can lead to tumor formation or autoimmune diseases [79].

3.3.1 The Bcl-2 Family

The Bcl2 family has a crucial role in the regulation of apoptosis and it regulates the mitochondrial pathway [80-82]. This process is characterized by cytochrome *c* release from the mitochondrial intermembrane space by means of the Bax/Bak channel to the cytosol, owing to permeabilization of the outer mitochondria membrane. In the cytosol, cytochrome *c* engages the apoptotic protease-activating factor-1 (Apaf-1), and forms the apoptosome that leads to the activation of the initiator caspase 9 and eventually to cell death [83]. The proteins that belong to this family can be classified as either anti-apoptotic or proapoptotic. The balance between these two groups will determine the cell fate – survival or commitment to apoptotic death. All the Bcl2 protein members contain at least one of four Bcl2 homology (BH) domains. The

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antiapoptotic Bcl2 protein and its close relatives have four Bcl2 homology (BH1-BH4) domains and all block apoptosis. These proteins usually integrate within the outer mitochondria membrane (OMM) but can also be found in the cytosol or in the endoplasmic reticulum (ER) membrane. The proapoptotic proteins can be divided into effector proteins and BH3-only proteins. The members of the effector group contain four BH domains like the antiapoptotic proteins and promote apoptosis by forming pores in the outer membranes of the mitochondria. The mammalian BH3-only protein family at present comprises eight members, all of which promote apoptosis when overexpressed [84]. These proteins share little sequence homology apart from the BH3 motif, and are regulated in distinct ways.

3.3.1.1 *Bax, Bik, Bak*

The very first chimeric protein constructed with an apoptotic moiety as the trigger of selective cell death was IL-2-Bax, constructed by the author's laboratory [85]. To validate the use of proapoptotic proteins as the killing moiety in chimeric proteins, the author and co-workers had chosen a well-known target, the IL-2R targeted by its ligand – the human IL-2. A classical bacterial-based chimeric protein such as IL-2-PE had already been reported to eliminate successfully activated T cells, both *in vitro* and *in vivo* [86-99]. In the IL-2-Bax chimeric protein, the killing domain is Bax, the proapoptotic protein of the Bcl-2 family. IL-2-Bax increased the population of apoptotic cells in a variety of target T-cell lines as well as in human fresh PHA-activated lymphocytes in a dose-dependent manner and had no effect on cells lacking IL-2R expression [85,100].

Next, the author's laboratory constructed some GnRH-based chimeric proteins fused to proapoptotic proteins, Bax, Bik and Bak of the Bcl-2 family [101]. These proteins were able specifically to target and kill adenocarcinoma cells, similar to the specific activity of GnRH–PE chimeric proteins. Bax and Bak were also fused to various domains of the Fc portion of the human IgE for targeted elimination of human mast cells [102].

3.3.1.2 *Bid and tBid*

Bid is a proapoptotic BH3-domain-only member of the Bcl-2 family that is thought to induce programmed cell death by stimulating the release of other proapoptotic factors (cytochrome *c*, apoptosis-inducing factor and procaspase 9) from the mitochondria [103,104]. Full-length Bid, which is normally presented in the cytosol, is cleaved by activated caspase 8, among other caspases, into a 15.5 kDa C-terminal fragment (tBid), which translocates to the mitochondria and causes cytochrome *c* release [105]. As Bid is recognized as an intracellular link connecting the extrinsic and intrinsic apoptotic pathways, the application of Bid to kill tumor cells was suggested to be an efficient strategy for treating malignant diseases [106]. Shan *et al.* [107] introduced an scFV fusion gene construct, composed of a human epidermal growth factor receptor-2 (HER2)-specific single-chain antibody, domain II of Pseudomonas exotoxin A [10] and the carboxy terminal fragment of Bid to HER2-positive osteosarcoma cells. Shan *et al.* demonstrated that the e23sFv-PEAII-Bid1-60 molecule selectively recognized and killed HER2-overexpressing osteosarcoma cells *in vitro*. Subsequently, the authors introduced the e23Fv-PEAII-Bid1-60 gene into BALB/c athymic mice bearing HER2-positive osteosarcomas using injections of liposome-encapsulated vector. Expression of the e23Fv-PEAII-Bid1-60 gene suppressed tumor growth, significantly prolonged animal survival and inhibited metastasis, thereby suggesting it may represent a competitive approach to treating HER2/neu-positive osteosarcoma [107].

In a continuous study, a furin cleavage site from DT was introduced into the molecule. e23sFv-TD-tBid selectively binds to HER2-positive cells and induces apoptotic cell death *in vitro* and *in vivo*. The therapeutic value of e23sFv-TD-tBid was also shown by its cytotoxic effects on primary patient-derived breast tumor cells but not on endothelial cells [108].

3.3.2 *Caspases*

So far 15 caspases have been identified, and they were numbered sequentially at the time they were characterized. The term caspases indicates their function: cysteine proteases that cleave after aspartate [109]. Caspases are present in the cytosol in an inactive form – zymogen. This inactive protein contains several domains: a pro-domain of variable length, heterodimerization domains, a large subunit (20 kDa) and a small subunit (10 kDa). Caspase activation results in cleavage between the large and small subunits, and between the large subunit and the pro-domain, giving rise to an active heterotetramer. The cleavage sites between the two subunits contain critical aspartate residues and, hence, are potential caspase substrate sequences, forming a hierarchy by which one caspase can activate another. The caspases are divided into three subgroups according to function. Although caspases 1, 4, 5, 11, 12 and 13 can cause apoptosis when overexpressed, their primary function is to mediate the immune response. The rest of the caspases are divided into initiators (caspases 2, 8, 9 and 10) and effectors or executioners (caspases 3, 6 and 7).

One of the main caspases is caspase 3, a 32 kDa protein (3 kDa pro-domain, 17 kDa large subunit, 12 kDa small subunit). Caspase 3 is the main executioner of apoptosis and operates at the heart of the process. On activation, it cleaves numerous target proteins, thus influencing multiple key processes in the cell. At the same time, direct activation of caspase 3 circumvents the major checkpoints of the apoptotic pathway (Bcl2 family proteins, apoptosome formation, etc.) and because it has intrinsic catalytic activity, it is also independent of the activity of other proteins. These characteristics make caspase 3 an excellent candidate to act as a killing moiety of immunotoxins/chimeric proteins.

The use of caspase 3 as immunocasp-3 was first reported by Jia *et al.* [110]. This molecule comprises a single-chain anti-erbB2/HER2 antibody with an NH2-terminal signal sequence, a Pseudomonas exotoxin A translocation domain

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and a constitutively active caspase 3 molecule. The idea was that cells transfected with the immunocasp-3 gene would express and secrete the chimeric protein, which would then bind to HER2-overexpressing tumor cells. Subsequent cleavage of the constitutively active capase-3 domain from the immunocasp-3 molecule and its release from internalized vesicles would lead to apoptotic tumor cell death. To test this strategy, Jia *et al.* transduced human lymphoma Jurkat cells with a chimeric immunocasp-3 gene expression vector and showed that they not only expressed and secreted the fusion protein, but also selectively killed tumor cells overexpressing HER2 *in vitro*. Injection of the transduced Jurkat cells led to tumor regression in a mouse xenograft model because of continuous secretion of immunocasp-3 by the transduced cells. The growth of HER2-positive tumor cells in this model was inhibited by somatic as well as intratumor injection of immunocasp-3 expression plasmid DNA, indicating that the immunocasp-3 molecules secreted by transfected cells have systematic antitumor activity [110]. The immunocasp-3 was suggested as an anticancer treatment either as a cell-based therapy or as a DNA vaccine. This molecule was also found to be potent against human gastric cells expressing HER2, both *in vitro* and *in vivo*, in human gastric cancer xenografts [111].

The anti-HER2 antibody as a cell-targeting delivery moiety was also utilized to target caspase 6 (immunocasp-6) in an osteosarcoma lung metastasis mouse model. Expression of the immunocasp-6 gene not only significantly prolonged the animal's survival, but also greatly inhibited tumor metastasis [112]. It should be pointed out that in these studies with caspase 3 or 6 as the killing moiety, the cancer cells were transduced with the gene of the immunocasp rather than using the recombinant protein for treatment.

In the author's laboratory caspase 3 was used to target specifically activated T cells expressing IL-2R in the form of chimeric proteins. IL-2-caspase 3 was tested for its ability to ameliorate clinical symptoms of acute murine experimental colitis, using the dextran sodium sulfate (DSS)-induced mouse model. The author and co-workers' studies demonstrated that treatment with IL-2-caspase 3 dose-responsively ameliorated DSS-induced colitis in mice, represented by a beneficial effect on the clinical symptoms of disease. Treatment with IL-2-caspase 3 also decreased neutrophil and macrophage infiltration of the inflamed tissue. Moreover, in addition to the preventive capability of IL-2-caspase 3, it was shown to be therapeutic in a model where treatment begins at the stage of continuing-active acute disease [113]. The author and co-workers also evaluated the effects of IL-2-caspase 3 on the immune system, including its effect on regulatory T (T_{reg}) cells, as well as the effects of another IL-2-based fusion protein (ONTAK, DT-IL-2) [114].

In these studies it was demonstrated that at a high dose, both IL-2 fusion proteins protected against bleeding, shortening and necrosis of the colon. However, unlike mice treated with IL-2-caspase 3, administration of ONTAK was associated with weight loss and severe lymphopenia, with a balanced ($CD4^+$ and $CD8^+$) loss of 55% of splenocytes. Moreover, while ONTAK depleted the fractions of $CD4^+CD25^+FoxP3^+$ T_{reg} cells, IL-2-caspase 3 spared this subset of cells [114].

IL-2-caspase 3 was also tested on experimental autoimmune encephalomyelitis (EAE), a T-cell-mediated disease. The author and co-workers' data show that IL-2-caspase 3 promoted cell-specific apoptosis both *in vitro* and *in vivo*. Cell lines preferentially expressing the IL-2R -chain and encephalitogenic lymphocytes derived from EAE-induced mice were highly sensitive to the chimeras' activity. This was demonstrated by increased DNA fragmentation and annexin labeling together with reduced specific T-cell proliferation in response to IL-2-caspase 3 treatment. Furthermore, IL-2-caspase 3 treatment of EAE-induced mice caused a significant delay in disease onset together with a reduction in disease burden, depending on the time that treatment begun [115]. Caspase 3 was also fused to GnRH peptide, producing GnRH-caspase 3 for the targeted treatment of adenocarcinomas. **Table 1** and **Figures 1** and **2** illustrate the activity of caspase 3-based chimeric proteins, fused to different delivery moieties, in two animal models, demonstrating the therapeutic potential of these new chimeric proteins.

3.3.3 Granzymes

3.3.3.1 Granzyme B

Granzyme B (GzmB) is used by cytotoxic lymphocytes as a molecular weapon for the defense against virus-infected and malignantly transformed host cells. It belongs to a family of small serine proteases that are stored in secretory vesicles of killer cells. After secretion of these cytolytic granules during killer cell attack, GzmB is translocated into the cytosol of target cells with the help of the pore-forming protein perforin. GzmB carries a protease specificity similar to caspase 8, and once delivered it activates major executioner apoptosis pathways. As GzmB is very effective at killing human tumor cell lines that are otherwise resistant to many cytotoxic drugs, and because GzmB of human origin can be recombinantly expressed, its use as part of a 'magic bullet' in tumor therapy is a very attractive idea. Recent reviews by Kurschus and Jenne [116] and by Rosenblum and Barth [117] describe the various GzmB-based immunotoxins targeted to a variety of antigens, including gp240, VEGF-receptor, Lewis Y antigen, HER2, CD64 and CD22, expressed on different tumor cells.

The therapeutic application of GzmB-containing immunotoxins is, however, associated with two major problems. First, the high isoelectric point of GzmB results in strong off-target binding and accumulation of GzmB in various body compartments [118-121]. These adverse properties of GzmB can be corrected with a few mutations. The real bottleneck and most critical issue, however, is the delivery of GzmB from endosomal compartments to the cytosol. There are several effective translocation domains, but all are derived from plants, bacteria, or viruses and are immunologically not tolerated. The optimal translocation domain or

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Table 1. Effect of IL-2-caspase 3 treatment on EAE clinical course*.

	Treatment from day 10		Treatment from day 13	
	PBS	IL-2-caspase3	PBS	IL-2-caspase 3
Mean severity‡	1.3 ± 0.3	0.5 ± 0.2¶	1.6 ± 0.4	1.6 ± 0.4
Cumulative score§	11.8 ± 2.8	3.8 ± 1.7	17.5 ± 4.6	18.0 ± 2.9
Disease onset (days)	15.3 ± 0.6	18.2 ± 0.7¶	15.3 ± 0.9	15.4 ± 0.9
Incidence	11/16	6/14	6/8	5/5

*Treatment of EAE C57BL mice with IL-2-caspase 3 [15 μg/day (i.v.)] or PBS started on day 10 or day 13 after disease induction. Treatment period was 14 days in all experimental groups. Each of the results represents the mean ± s.e. of two separately performed experiments.

‡Mean severity: mean of the daily disease score throughout the experiment.

§Cumulative score: number of days animal was sick × clinical score.

¶Statistically significant ($p < 0.05$) differences between the control PBS and the IL-2-caspase 3-treated groups [115].

EAE: Experimental autoimmune encephalomyelitis.

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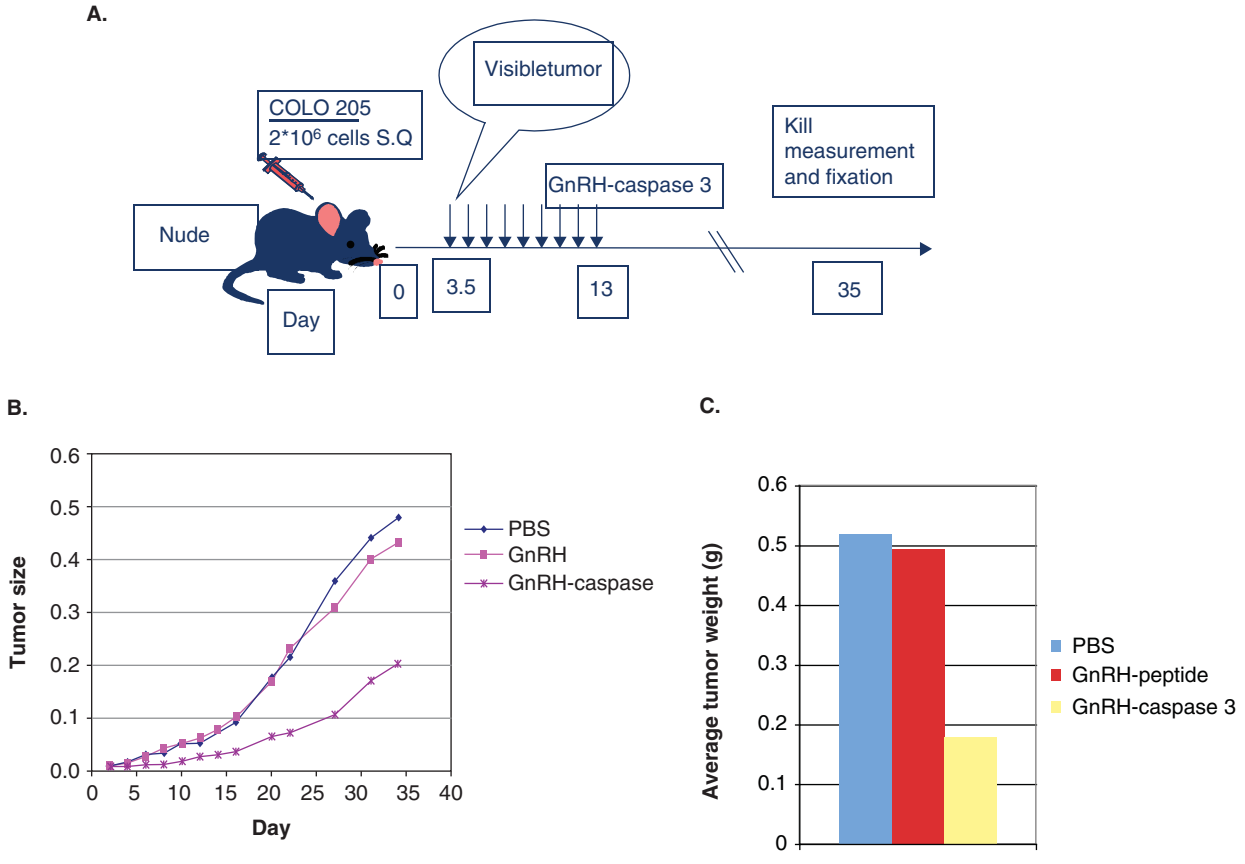

Figure 1. GnRH-caspase 3 chimeric protein inhibits colon adenocarcinoma tumor growth. A. Study design. **B.** Average tumor size in cubic centimeters as a function of time after implantation. The experimental was treated from day +3. **C.** Average tumor volume and weight at the experimental end point. The treated tumors were 2.9 times smaller in weight and volume than those in the control group.

membrane-penetrating peptide should have none or little immunogenic potential, should preferably be of human origin or rationally designed, and should only be active after endocytosis at the lower pH of the endosomal/lysosomal compartment. Guided by a highly specific targeting antibody, the immunoconjugate should be endocytosed, the targeting moiety at the N terminus removed by an endogenous protease and the activated GzmB efficiently translocated to the cytosol with the help of an optimized translocation peptide. This new concept of targeted therapeutics, however, is still at an early developmental stage and requires much more effort to solve some major problems.

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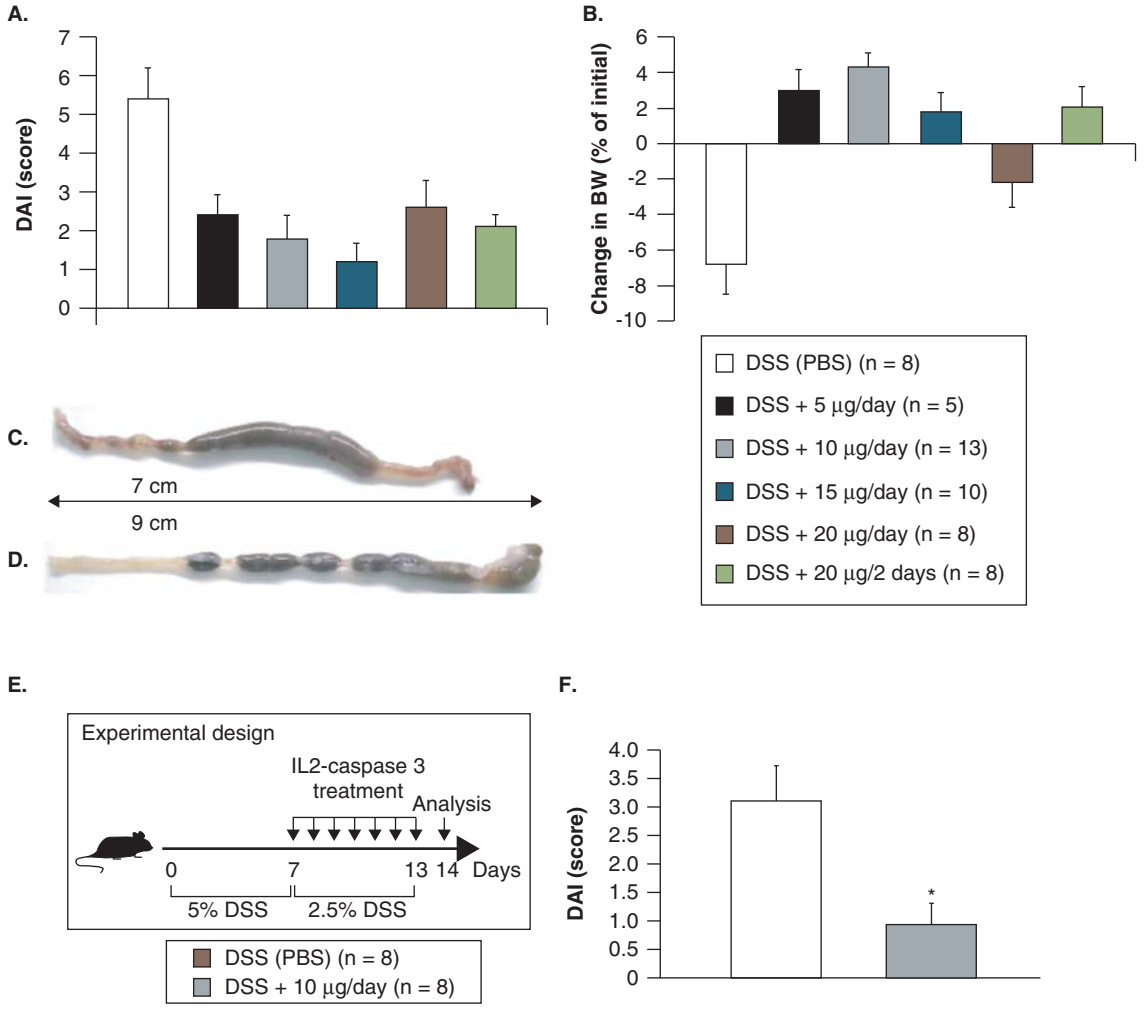

Figure 2. Therapeutic potency of IL-2-caspase 3 targeted treatment in a murine experimental model of inflammatory bowel disease. A – D. IL-2-caspase 3 ameliorates DSS-induced colitis in a dose-dependent manner. DSS-exposed (5%) mice were treated with various doses of IL-2-caspase 3. The effects on (**A**) DAI and (**B**) BW are presented. The differences between a colon obtained from a non-treated mouse, with typical ulcerations, bloody stool and diarrhoea (**C**), versus a colon from a mouse treated with 15 mg/(mouse day) IL-2-caspase 3 (**D**) are evident. Data represent the mean with the s.e.m. of at least two separate experiments. There were at least five mice per group per experiment. **E, F.** IL-2-caspase 3 has therapeutic properties in DSS-induced colitis. **E.** After disease induction for 7 days with 5% DSS, the amount of DSS in the drinking water was reduced to 2.5% and mice began to receive treatment with IL-2-caspase 3 (10 µg/day, i.v.). **F.** The effects on DAI [113].

*$p < 0.05$ of non-treated versus treated mice (Student *t* test for unpaired data).

BW: Body weight; DAI: Disease activity index; DSS: Dextran sodium sulfate.

3.3.3.2 *Granzyme A*

Granzyme A is a serine protease stored in the cytoplasmic granules of killer lymphocytes. When these lymphocyte cells encounter their target, an immunological synapse is formed. The granules migrate towards the synapse and their contents, including various granzymes, are released and delivered into the target cell [122,123]. Following granzyme A's entry into the cell, it causes mitochondrial damage, reactive oxygen species elevation and subsequent translocation of the endoplasmic reticulum-associated multi-protein complex, SET, from the cytoplasm to the nucleus. In the nucleus, among its many actions, granzyme A cleaves SET protein, thus releasing granzyme A-activated DNase, nm23-H1, which initiates DNA damage. In this way, granzyme A's delivery to the cell induces caspase-independent cell death. The author's laboratory took advantage of this specific characteristic to overcome multidrug resistance (MDR), one of the main problems of conventional anticancer therapy whereby cells acquire resistance to structurally and functionally unrelated drugs following chemotherapeutic treatment. The most well-characterized cause

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of MDR is the overexpression of a 170 kDa membrane glycoprotein known as P-glycoprotein (Pgp), which belongs to a superfamily of ATP-binding cassette transporters. These transporters, through an ATP-dependent mechanism, actively efflux a wide variety of chemotherapeutic drugs used to treat cancer out of the cells. Thus, they lower the drug concentration inside the cells, rendering them resistant. Most chemotherapeutic drugs trigger cell death by means of apoptosis. At apoptosis onset, a sequence of events leads to the activation of caspases by proteolytic cleavage. In its classical form, apoptosis can occur only when caspases, in particular caspase 3, are activated. To overcome MDR, a new chimeric protein, IL-2 granzyme A (IGA), was constructed, using IL-2 as a targeting moiety and granzyme A as a killing moiety, fused at the cDNA level [124]. It was shown that the IGA chimeric protein enters the target-sensitive and MDR cancer cells overexpressing IL-2 receptor and induces caspase 3-independent cell death. Specifically, after its entry IGA causes a decrease in the mitochondrial potential and triggers translocation of nm23-H1, a granzyme A-dependent DNase, from the cytoplasm to the nucleus, where it causes single-strand DNA nicks, thus causing cell death. Moreover, IGA is able to overcome MDR and kill cells resistant to chemotherapeutic drugs. The author believes that overcoming MDR with targeted molecules such as IGA chimeric protein, which causes caspase-independent apoptotic cell death, could be applied to many other resistant types of tumor using the appropriate targeting moiety. Thus, this new class of targeted molecules could open up new vistas in the fight against human cancer [124].

3.3.4 Endonucleases

3.3.4.1 DNA fragmentation factor 40

DNA fragmentation is a typical morphology seen in apoptotic cells. Three research groups have isolated a candidate human nuclease responsible for the fragmentation. This nuclease is activated on treatment of cellular lysates with active caspase 3. The nuclease is known as the DNA fragmentation factor (DFF), caspase-activated DNase (CAD) or CPAN [125-127]. In eukaryotic cells, DFF40 is bound to its inhibitor DFF45 [128]. In addition to its inhibitory action, DFF45 acts also as a chaperone. Whereas DFF40 is translated, DFF45 binds to the polypeptide chain being formed via its N terminus and helps DFF40 fold properly. When the entire polypeptide chain has been translated, DFF45 stays bound to DFF40, therefore acting as its inhibitor. Usually, in the absence of DFF45, DFF40 does not fold properly and aggregates into an inactive enzyme. Two of the caspase 3 substrates are DFF45 and its splice variant DFF35. When an apoptotic signal activates caspase 3 it cleaves DFF45 and releases DFF40 from inhibition, thus allowing it to enter the nucleus and degrade DNA. The outcome of this apoptotic cascade is one of the most common morphologic features of apoptosis: DNA fragmentation. In the author's laboratory DFF40 was genetically fused to GnRH as the targeting domain, to produce GnRH-DFF40 [129]. GnRH-DFF40 shows DNase activity *in vitro*. It was found that this chimeric protein can target and kill adenocarcinoma cells. Such death occurs by means of apoptotic pathways, resulting in an increase in the sub-G1 population, DNA fragmentation, terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL)-positive cells, and morphology typical of apoptotic cells. These apoptotic events involve the mitochondria as confirmed by cytochrome *c* depletion and caspase 9 and caspase 3 activation. Preliminary *in vivo* results showed that treatment of colon adenocarcinoma xenografts in nude mice with the new chimeric protein caused a reduction in tumor weight [129]. However, GnRH-DFF40 was difficult to produce, owing to the 11 cysteine residues within the DFF40 protein.

3.3.4.2 AIF

Apoptosis-inducing factor (AIF) is a mitochondrial flavoprotein with NADH oxidase activity that has a vital function in healthy cells but is also an important mediator of caspase-independent programmed cell death in stressed and damaged cells. Mahmud *et al.* [130] generated a truncated AIF derivative (AIF100) that lacks the mitochondrial import signal of the protein. Bacterially expressed AIF100 was functionally active and induced cell death on microinjection into Vero cells accompanied by clear signs of apoptosis. For specific targeting to tumor cells, AIF100 was genetically fused to the scFv(FRP5) antibody fragment that recognizes the ErbB2 (HER2) receptor tyrosine kinase. The authors showed that recombinant AIF specifically targeted to human cancer cells and delivered into the cytosol has potent cell killing activity, suggesting this molecule as an effector suitable for the development of humanized immunotoxin-like molecules [130].

3.3.5 Tumor necrosis factor-related apoptosis-inducing ligand

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily and is involved in the elimination of cancer cells and virus-infected cells by T cells and natural killer cells [131,132]. TRAIL is expressed as a homotrimeric type 2 transmembrane protein or as its proteolytic product, which is soluble TRAIL (sTRAIL). TRAIL has been found to bind to five receptors: death receptor 4 (DR4/TRAIL-R1), death receptor 5 (DR5/TRAIL-R2), decoy receptors DcR1 and DcR2 and also the soluble receptor osteoprotegrin [133]. The binding of TRAIL to DR4 or DR5 recruits caspase 8 via Fas-associated protein with death domain (FADD), leading to the formation of a death-inducing signaling complex and subsequent caspase-mediated apoptosis [134,135]. TRAIL activity is modulated mainly by its low-affinity binding to the decoy receptors, which lack the death domain [136]. TRAIL has been found to show selective toxicity towards tumor cells [137], presumably owing to the overexpression of TRAIL receptors in tumor cells or the high rate of expression of decoy receptors in normal cells. These properties led to the development of several therapeutics targeting TRAIL receptors. These include recombinant sTRAIL, agonistic antibodies and gene therapy agents that express TRAIL.

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The efficacy of antibody-based therapies is often hampered as the tumor cells adopt tactics such as target antigen shedding and masking to escape immune surveillance. Target antigen expression may be downregulated as a result of therapy. In this context, therapeutic strategies that bring about bystander effects are important. A promising strategy that has been developed uses TRAIL-based immunotoxins that target cancer cells overexpressing growth factor receptors and bring about the bystander effect. The bystander effect is based on the principle that targeted tumor cells are not only eliminated, but also exploited to convey a therapeutic effect towards neighboring tumor cells that lack expression of the target antigen. Three chimeric proteins, scFv54–sTRAIL, scFv425–sTRAIL and scFvCD7–sTRAIL, which target epithelial glycoprotein-2, epidermal growth factor receptor and CD7-positive cancer cells, respectively, have been developed [50, 138-139]. All three of the proteins, after binding to their target cells, could effectively get rid of nearby target cells lacking the target antigen by crosslinking the agonistic TRAIL receptors on them. This useful feature makes sTRAIL-based immunotoxins worthy of consideration as a substitute for existing targeted therapies.

3.3.6 *Fas-ligand*

Fas-ligand (FASL) is an effector molecule involved in the death pathway of apoptosis induction. To exploit this property, several FASL agonists such as anti-FAS antibodies and multimeric soluble forms of FASL were developed. In spite of their tumoricidal activity towards cell lines and primary tumors, their clinical application in humans was not possible as they were found to be lethal to mouse models [140,141]. It was discovered that oligomeric, multimeric and aggregated forms of FASL in recombinant soluble FASL (sFASL) preparations led to systemic toxicity, whereas a homotrimeric form showed toxicity only on selective targeting to a tumor cell [142]. Hence, fusion proteins using this form of sFASL were tried. sFASL was genetically fused to anti-CD7 scFv [143] and was found to be highly toxic to $CD7^+$ T-cell acute lymphoblastic leukemia (T-ALL) and acute myeloblastic leukemia [16] cell lines.

3.3.7 *RNases*

Based on the immunotoxin principle, immunoRNases have been proposed in which the toxin moiety of immunotoxins is replaced by a non-toxic RNase. An immunoRNase is in fact an immuno-pro-toxin, as it can travel in the bloodstream without any damages to cells devoid of the targeted component while selectively targeting specific cell populations. Once internalized by the target cell, the RNase moiety will exert its RNA degrading activity, which will readily lead to cell death. By choosing a human RNase, and a human antibody fragment as immune moiety, an immunoRNase would be not only non-toxic, but also non-immunogenic. As for the possible inhibitory action of the cytosolic RNase inhibitor, exerted on all non-toxic vertebrate RNases, it can be countered by flooding the cytosol with high levels of immunoRNase, which will neutralize the RNase inhibitor, or by using RNases resistant to the inhibitor. Ribonucleases constitute a large superfamily spread across several species. Human RNases are encoded by at least eight different genes, out of which five (RNases 1 – 5) have been identified at the protein level. RNases of human origin have been utilized in immunotoxin constructs, including human pancreatic RNase1 [144-148], RNase2 or eosinophil-derived neurotoxin (END) [149], RNase3 or eosinophil cationic protein (ECP) [149], and RNAse5 or angiogenin [150-152].

Human RNase-based immunotoxins could be used to circumvent the problems of nonspecific toxicity and immunogenicity associated with bacteria- or plant-based toxins. Although most of them are less potent *in vitro* in comparison with their plant or bacterial counterparts, they seem to be less toxic to animals and may selectively kill target cells *in vivo* at safer doses. The rate of protein synthesis inhibition by ricin- and diphtheria toxin-based conjugates increases proportionally to the square root of the toxin concentration. This might lead to a reduced log kill of target cells. In RNase conjugates there is a linear relationship between the rate of protein synthesis inhibition and toxin concentration, which is advantageous for attaining an increased log cell kill.

3.3.8 *Kinases*

Death-associated protein kinase 2 (DAPK2) is a calcium/calmodulin-regulated proapoptotic serine/threonine kinase, which acts as a tumor suppressor. DAPK2 is downregulated in Hodgkin's lymphoma-derived tumor cell lines and its inactivation might stem from promoter-region hypermethylation. To determine whether selective reconstitution of DAPK2 catalytic activity in these cells could induce apoptosis, Tur and co-workers [153,154] produced a chimeric protein comprising a human CD30 ligand conjugated to a human DAPK2 calmodulin-deletion mutant. Thus, recombinant immunokinase DAPK2-CD30L has a constitutive kinase activity with enhanced proapoptotic function. This immunokinase chimeric protein inhibited cell proliferation and induced apoptotic cell death specifically in CD30/DAPK2-negative tumor cell lines, and significantly prolonged overall survival in a disseminated Hodgkin's lymphoma xenograft SCID mouse model [153,154]. These proof-of-concept studies provided the first demonstration of therapeutic strategies based on the restoration of a defective tumor-suppressing kinase activity by a new class of recombinant immunotherapeutic molecules.

4. Conclusions

The results obtained with all these apoptotic protein-based immunotoxins are encouraging. As all of the above-mentioned immunotoxins/chimeric proteins are of human origin, they are expected to display reduced immunogenicity in human recipients. Besides, as target cells are killed through apoptosis induction, systemic damage is less likely. The

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apoptotic cells shrink and condense, whereas the organelles and plasma membrane maintain their integrity. The dead cells are rapidly engulfed by macrophages and are eliminated before any of their contents can leak outside to cause a systemic response. Thus, targeted apoptosis-inducing immunotoxins/chimeric proteins should open new avenues in the battle against human diseases.

5. Expert opinion

With progress in molecular biology, completion of the human genome project and our advanced understanding of a variety of biological fields, many long-standing dreams in the realm of medicine regarding the targeted delivery of various drugs to cells are coming close to fruition. This has been achieved partly by using recombinant immunotoxins/chimeric proteins based on human highly cytotoxic proteins that, once delivered into the target cells, produce a natural apoptotic death.

Using this 'magic bullet' strategy of targeted recombinant immunotoxins/chimeric proteins for drug delivery, there is a hope of ameliorating the side effects caused by nonspecific treatments such as chemotherapy and radiotherapy, as well as of recombinant immunotoxins/chimeric proteins based on bacterial or plant toxins. Furthermore, by using human proapoptotic proteins as the killing moiety, two important advantages are achieved: first, using human proteins instead of bacterial or plant toxins, the risk of dose-dependent toxicity and immunogenicity is reduced; and second, these apoptosis-based chimeric proteins induce apoptotic cell death, thus preventing the spillage of cellular contents into the extracellular matrix and avoiding inflammatory responses.

As more cell surface molecules are recognized and used as targets for immunotoxin/chimeric protein therapy, combinations of these molecules with monoclonal antibodies can also be used. The 'multiple weapons' approach, combining several chemotherapy agents and monoclonal antibodies, is widely used in oncology. Using multiple agents, each disturbing a different cellular mechanism, helps to prevent the development of resistance to treatment, which occurs when a subgroup of the malignant cells finds a way to overcome a specific agent's activity. The same approach can be implemented when using immunotoxins/chimeric proteins. In this case, resistance can develop if the cells downregulate the expression of the target antigen in response to treatment. A combination of two or more immunotoxins/chimeric proteins, each targeting a different cell surface antigen, might overcome this obstacle.

Nevertheless, the recombinant immunotoxins/chimeric proteins based on human apoptotic proteins as a delivery system still possess some limitations. For example, < 0.001% of any injected biological reagent ever reaches its solid tumor target and therefore numerous laboratories have been trying to overcome this limitation by exploring the use of: i) specific gene therapy to localize treatment more effectively; and ii) adoptive immunotherapy with cancer-specific T cells, which can access *in vivo* tumors in a unique way. Another limitation that still exists with all targeted immunotoxins/chimeric proteins is the problem of nonspecific toxicity, caused by the fact that most target antigens are expressed to some extent on normal tissues.

Although human toxin-based immunotoxins/chimeric proteins for the targeted delivery of drugs are still in their early stages, they are certain to advance in the very near future to become a new weapon in the everlasting war against human diseases, mainly cancer.

Declaration of interest

The author states no conflict of interest and has received no payment in preparation of this manuscript.

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