

Review

Human Cytolytic Fusion Proteins: Modified Versions of Human Granzyme B and Angiogenin Have the Potential to Replace Bacterial Toxins in Targeted Therapies against CD64+ Diseases

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Received: 19 November 2013; in revised form: 23 January 2014 / Accepted: 28 January 2014 /

Published: 19 February 2014

Abstract: Targeted therapies for the treatment of cancer, but also inflammation and autoimmune diseases will reduce major side effects accompanied with conventional treatment modalities. The immunotoxin concept uses bacterial or plant toxins, coupled to antibodies or natural ligands targeting cancer cells. Initially, immunotoxins suffered from drawbacks like nonspecific cytotoxicity. Even the third generation of immunotoxins comprised of truncated antibodies and modified effector molecules experienced clinical set-backs due to immune responses. Long-term treatment of cancer and non-life-threatening chronic inflammatory diseases requires their complete ‘humanization’. This lead to evaluating human cytolytic fusion proteins (hCFPs), based on human apoptosis-inducing proteins. Lacking an endogenous translocation domain dramatically reduces the cell-death inducing capacity of such proteins. Here, we report on optimizing hCFPs, based on the anti-CD64 single chain variable fragment H22(scFv), specifically eliminating CD64+ macrophages and malignant progenitor cells. We replaced the bacterial toxin in

H22(scFv)-ETA' with the pro-apoptotic human granzyme B or angiogenin. Translocation was promoted by a sophisticated adapter containing a membrane transfer peptide (MTD) flanked by endosomal and cytosolic cleavable peptides, thus achieving *in vitro* cytotoxic activity comparable to bacterial immunotoxins. We demonstrate for the first time that optimized hCFPs, based on granzyme B or angiogenin, can compete with classical ETA-based immunotoxins.

Keywords: immunotherapy; immunotoxin; humanization; CD64; granzyme B; angiogenin; endosomal release

1. Introduction

Paul Ehrlich's idea of a "magic bullet" to deliver a compound exclusively to kill and eliminate disease-causing organisms has become feasible through the development of monoclonal antibodies (mAbs). Coupling mAbs or fragments thereof to toxic molecules led to the introduction of antibody drug conjugates (ADCs) and immunotoxins (ITs), thus offering an alternative therapeutic strategy for the specific treatment of multiple malignant disorders and inflammatory diseases without the undesirable side effects of conventional treatment modalities. Within ADCs mAbs are chemically conjugated to a highly toxic drug or radionuclide by a linker influencing their stability as well as the proper release of the toxic payload into the targeted cancer cell. After U.S. Food and Drug Administration (FDA) approval of brentuximab vedotin (Adcetris®), in 2011, and trastuzumab emtansine (Kadcyla®), in 2013, several companies are ramping up development of a burgeoning pipeline [1]. However, secure attachment of the toxic payload to the mAb still remains a critical challenge. Within ITs, the chemical drug is replaced by a naturally-occurring toxin (initially of bacterial or plant origin) fulfilling its death-inducing mission by the inhibition of protein synthesis or modification of signal transduction pathways, leading to apoptosis. Early-generation ITs still comprise full-length mAbs chemically linked to native toxins from bacteria or plants, and these suffered from drawbacks such as nonspecific binding and uptake into non-target cells (reflecting the presence of the toxin's natural cell-binding domain), inefficient tumor penetration (due to their large size), a complex manufacturing process, and immunogenicity (reflecting their non-human origin) [2]. These technological and pharmacological disadvantages have been addressed by the development of second and third generation ITs comprising genetic fusions of variable antibody fragments (scFv) or native ligands and truncated bacterial or plant protein toxins. Many of these fusion proteins have already been tested successfully *in vitro* and *in vivo* with some even undergoing clinical trials [3], and one has been approved by the FDA for the treatment of cutaneous T cell lymphoma (denileukin diftitox or Ontak®) [4]. However, most ITs failed in the clinic due to their immunogenicity, which is particularly relevant because of the long-term treatment required for cancer, involving multiple doses of each IT. Complete humanization is therefore necessary for clinical development [5,6].

A number of innovative strategies addressing the problem of immunogenicity have been introduced, including the co-application of immunosuppressive drugs [7,8], PEGylation of the toxic domain [9], and the removal of major B-cell and T-cell epitopes by site-directed mutagenesis [10,11]. Antibody

engineering techniques allow human anti-mouse antibody (HAMA) responses to be reduced by generating chimeric antibodies in which murine constant domains are replaced with human counterparts [12] or complementary determining region (CDR) grafting in which the antigen-binding sequences are grafted into a human antibody skeleton [13]. State-of-the-art humanization can be achieved by creating genetically modified mice or rats expressing fully human antibody sequences or screening human scFv/antibody libraries [14]. Natural human ligands can also be used as targeting components [5,15–17].

Complete humanization can be achieved by replacing bacterial or plant toxins with human effector molecules, such as pro-apoptotic enzymes, ribonucleases (RNases), and death receptors. This fourth generation of ITs are generally described as human cytolytic fusion proteins (hCFPs). Several hCFPs have demonstrated specific activity and efficiency *in vitro* and in preclinical studies [6,18]. Nevertheless, their cytotoxic potential lags behind third generation ITs containing plant or bacterial toxins, and further research has shown that endosomal escape is the major bottleneck for hCFPs efficacy [19]. Cytosolic release is necessary to bring human enzymes into contact with their substrates because they lack the natural translocation domain found in bacterial and most plant toxins. The cytosolic availability of hCFPs can be increased without losing specificity by co-administering membrane destabilizing/endosomolytic agents, such as the 4-aminoquinoline derivate chloroquine, the plant glycoside saponin, and retinoic acid [17,20–23]. Another approach is to separate the binding and effector domains using a multifunctional, synthetic adapter containing cell penetrating peptides (CPPs) [24,25]. This article compares hCFPs modified with such adapters to classical ITs, focusing on diseases associated with the upregulation of CD64.

2. CD64 in Cancer, Chronic Inflammation, and Autoimmune Diseases

CD64 is the only high-affinity Fc γ receptor (Fc γ R) in humans. It binds human IgG1, IgG3, and IgG4 with high affinity, but has no affinity for IgG2 [26]. In addition to high-affinity binding, CD64 displays several properties not shared with the other three Fc γ Rs: Fc γ RII (CD32), Fc γ RIII (CD16), and neonatal Fc γ R. These are (1) the ability to bind and internalize monomeric IgG [27], and (2) constitutive expression solely on macrophages, monocytes and their progenitors [28–31]. CD64 is upregulated on cancer cells of the monocyte/macrophage lineage such as acute myeloid leukemia (AML) cells [32,33], as well as on macrophages in chronic inflammatory diseases such as rheumatoid arthritis [34–36], inflammatory bowel disease [37–39], and systemic lupus erythematosus [40,41]. These unique properties and expression profiles mean that CD64 is a suitable target molecule for selective therapy. Interestingly, although Fc γ Rs are expressed on both human and murine immune cells, there are significant species-dependent differences between these receptors [31]. To circumvent this issue, transgenic mice expressing human Fc γ Rs have been generated to enable their use as *in vivo* preclinical models [42,43].

2.1. Acute Myeloid Leukemia (AML)

AML is a heterogeneous group of malignancies in which hematopoietic stem cells of the myeloid lineage proliferate in an unregulated manner and therefore disturb the balance of blood cell production [44]. Those malignant progenitor cells fail to differentiate and accumulate in the blood and bone

marrow [45], where they suppress the hematopoiesis of normal blood cells. There are two common classification systems used to distinguish between AML subgroups. The French-American-British (FAB) system is based on the predominating cell type and its degree of maturity [46], whereas the World Health Organization (WHO) system is based on clinical symptoms, giving prognostic information [47].

Chemotherapy is still the standard treatment for AML. A combination of cytarabine (AraC) and anthracyclines is administered for induction therapy, followed by a high dose of AraC for consolidation therapy in patients younger than 60 [48]. However, only 60%–80% of the patients achieve complete remission (CR) after induction therapy, defined as <5% of the malignant cells remaining detectable in the bone marrow, and 50%–70% of patients relapse within three years [48]. If AML recurs due to repopulation by residual cells and/or the development of multi-drug resistance [49], irradiation and stem cell transplantation are further treatment options. However, most patients are >65 years old, and these types of therapies are connected with an elevated risk of complications in comorbid diseases [50]. The elimination of minimal residual tumor cells after chemotherapy could improve the survival of AML patients [51]. A promising tool to achieve this is targeted tumor therapy [52], which has a lower risk of severe side effects because it discriminates between malignant and normal cells [53], in contrast to chemotherapy [54].

CD64 is a promising antigen for the specific treatment of AML. The expression of this receptor is induced on myeloid cells by cytokines such as interferon gamma (IFN γ) [30]. CD64 is found on AML cells, especially subtypes M4 and M5 (FAB classification) [33,55], but neither on pluripotent stem cells nor on CD34+ hematopoietic progenitor cells [56]. Furthermore, CD64 is expressed to some extent in acute promyelocytic leukemia (APL, M3 subtype) and chronic myelomonocytic leukemia (CMML) [57]. CD64 is an attractive target for AML therapy because its expression is restricted to malignant AML cells, its density on the cell surface is upregulated by IFN γ , and it is internalized rapidly.

2.2. Macrophages and Inflammation

Inflammation is a complex and dynamic physiological process, which requires well-balanced and controlled interactions among diverse cells. Macrophages are essential for both the initiation and resolution of inflammation in response to infections and skin damage, and their functions include the production of pro-inflammatory cytokines and cytotoxic compounds, the secretion of growth factors to promote the activity of other cells contributing to the resolution of inflammation, fighting invading microbes, and scavenging cell debris. These factors are produced at different times by different types of macrophages. Several recent reports have made clear that there is no ‘generic’ macrophage, but the term refers to a diverse population of versatile cells that share some common features. As macrophages can adapt to the changing cytokine milieu and also influence the milieu by producing an array of soluble mediators, they are considered to be functionally plastic and flexible and are described as polarized rather than activated [58]. Mirroring the Th1/Th2 dichotomy, polarized macrophages were initially subdivided into the M1 and M2 types.

M1 polarization, also described as classical activation, occurs in response to IFN γ produced by innate and adaptive immune cells, alone or in concert with bacterial lipopolysaccharide (LPS) [59]. Natural killer (NK) cells produce IFN γ in response to stress or a first encounter with pathogens as part

of the innate immunity, thus, polarizing macrophages towards M1. In this state, macrophages show a higher capacity for antigen presentation [60,61], and possess enhanced microbicidal and tumoricidal activity mediated by the production of superoxide anions, oxygen radicals, and nitrogen radicals that confer direct toxicity [62–64].

Whereas M1 macrophages represent the one extreme of polarization, the other extreme is termed M2 polarization or alternative activation [65]. During the past decade, this term has been expanded to encompass a more heterogeneous cell population [66,67]. Together, the diversity of polarized macrophages illustrates how different physiological processes might be accurately regulated by distinct macrophage subtypes depending on the environmental conditions. However, the imbalance and/or dysregulation of macrophage-controlled processes are strongly associated with pathological outcomes, including several autoimmune diseases, impaired wound healing, metabolic disorders, and cancer [68–73]. Certain macrophage subsets sustainably maintain the cytokine milieu by autocrine regulation, leading to the persistence of these cells for an inappropriate duration. In addition, T cells may also be activated, promoting the inflammatory status. To allow the specific targeting of macrophages and to interrupt the self-reinforcing circle of inflammation, a surface molecule was required as a target entrance mediator. Screening revealed that CD64 is upregulated on macrophages in several chronic diseases, including atopic dermatitis during the chronic phase [74].

3. Targeting CD64

3.1. Classical Immunotoxins

The first antibody specific for human CD64 was the full-length mouse-derived mAb M22 developed by Medarex, Inc. The M22 sequence was subsequently humanized by CDR grafting to generate the full-length mAb H22 [75]. Both M22 and H22 specifically bind, cross-link and modulate the expression of CD64 [76–78]. Specific elimination of CD64+ target cells has been achieved using M22 and H22 chemically coupled to bacterial and plant toxins (see below). Subsequent re-formatting to a scFv produced M22(scFv) and H22(scFv), which were suitable for genetic fusion to protein toxins, producing ITs that were both technologically and pharmacologically superior.

The chimeric immunotoxin M22(scFv)-ETA', comprising M22(scFv) genetically fused to a deletion mutant of *Pseudomonas* exotoxin A (ETA'), selectively killed CD64-overexpressing leukemic cell lines *in vitro* and induced apoptosis in *ex vivo* primary leukemic cells from a patient with CD64+ AML [77]. After the development of the humanized version H22(scFv)-ETA', potent antitumor activity was demonstrated against myeloid tumor cells promoting significantly prolonged overall survival in AML xenograft animals [79].

The specific elimination of CD64+ macrophages by the immunoconjugate H22-RicinA, resolving chronic skin inflammation in transgenic mice within 24 h, demonstrated the first evidence of successful CD64 targeting in non-cancer related applications [80]. In addition, histological analysis showed that other inflammatory cells disappeared from the site of inflammation, and clinical parameters such as vascular leakage and physiological temperature were restored. The same group also reported that treatment with H22-RicinA inhibited the progression of arthritis in a rat model and efficiently killed activated macrophages from synovial fluid obtained from human rheumatoid arthritis

patients [36]. Although H22-RicinA was effective in resolving local chronic skin inflammation in mice [80], its large size (~220 kDa) and heterogeneity hindered its clinical development. Later on, the H22(scFv)-ETA' fusion protein has been successfully used to treat chronic inflammatory diseases successfully in pre-clinical animal models [81,82].

The performance of CD64-targeted fusion proteins can also be improved by promoting its internalization. Although cross-linking is not required for the internalization of CD64, it does enhance the efficiency of internalization [28,83]. Therefore, the bivalent construct H22(scFv)2-ETA' was engineered in order to bind two CD64 molecules simultaneously on the cell surface, and this proved more efficacious *in vitro* and in a transgenic mouse model of chronic cutaneous inflammation [81]. The therapeutic approach of targeting macrophages was then transferred to an ischemia-reperfusion rat model, where treatment with H22(scFv)-ETA' was shown to preserve renal function and morphology, and ameliorate ischemia-induced kidney injury [82].

Although the elimination of activated macrophages allowed the effective treatment of inflammatory diseases, this strategy needs to be considered with caution in the context of other macrophage-associated diseases. For example, during pulmonary tuberculosis in mice, macrophages were shown to have a dual function. Therefore, although non-specific depletion of all macrophages using liposomes improved the clinical outcome, the specific elimination of activated macrophages impaired resistance to infection, reflected by the presence of enhanced mycobacterial outgrowths [84]. Therefore, it is important to ensure the specific and selective targeting of appropriate macrophage populations without adversely affecting those with beneficial functions.

As ITs containing bacterial and plant toxins cause nonspecific cytotoxicity, immunogenicity and other undesired side effects, their clinical deployment is mostly limited to the treatment of life-threatening diseases, such as cancer. However, the new generation of fully human cytolytic fusion proteins may overcome these disadvantages and increase the range of potential target diseases, including CD64+ chronic inflammatory and autoimmune disorders where there is a significant unmet clinical need.

3.2. "Humanization" of Immunotoxins Using Granzyme B and Angiogenin

As discussed above, targeting CD64 in inflammatory and malignant disease models has provided proof of principle for therapeutic approaches combining the antibody fragment H22(scFv) and a cell-death-inducing molecule. Thus far, H22(scFv) fused to ETA' has been shown to prolong survival significantly in a disseminated AML tumor model and to reduce the number of inflammatory macrophages in a transgenic mouse model.

ETA is a virulence factor secreted by *Pseudomonas aeruginosa*, which causes a wide range of infections. The remarkable cytotoxicity of ETA (a single molecule can kill a cell) makes it an ideal candidate toxin for targeted therapies [85,86]. ETA is 613 amino acids in length with three functional domains [87]. The cellular binding domain (Ia) promotes cellular uptake by receptor-mediated endocytosis. After proteolytic processing of the holotoxin by endosomal furin activity, the translocation domain (II) facilitates endosomal release and transfers the catalytic A domain (III) to the transreticular Golgi apparatus. Subsequent cellular sorting mediated by the internal KDEL-like sequence REDL leads to a retrograde transport from the cis-Golgi to the endoplasmic reticulum and

finally translocation to the cytosol. Once released into the cytosol, the catalytic A domain inactivates eukaryotic elongation factor 2 (eEF2) by ADP ribosylation of the diphthamide residue (a post-translationally modified histidine residue) and thus inhibits protein synthesis. The mechanisms of ETA uptake and toxicity are described in detail by Weldon and Pastan [85].

Despite its potency, native ETA cannot be deployed in the clinic because it is taken up by non-target cells resulting in unwanted side effects such as vascular leak syndrome (VLS), which is characterized by increased vascular permeability leading, in the worst cases, to multiple organ failure [88]. ETA can be detoxified by replacing the natural cell-binding domain with a targeting ligand, thus truncated versions of the toxin (e.g., PE38, PE40, or ETA') are paired with antibodies to redirect its cytotoxicity towards specific cells. This approach has been successful *in vitro*, in preclinical studies and in a number of clinical trials [3,85,86,89]. Even with these modifications, the bacterial origin of ETA means that the resulting ITs are immunogenic and possess residual nonspecific toxicity, meaning that high-dose therapy and multiple treatment regimens cannot be used [90,91]. Tumor cells can also evolve resistance against ETA, e.g., if mutations occur in the genes required for diphthamide synthesis thus abolishing the functional acceptor for ADP ribosyl groups, as recently observed in a phase I clinical trial in leukemia patients undergoing treatment with HA22, a recombinant immunotoxin comprising the PE38 variant of ETA fused to a CD22-specific scFv [92].

We therefore replaced the bacterial toxin within the H22(scFv)-ETA' construct with human effector molecules to generate a new generation of immunotoxins (hCFPs). We demonstrated the specific cytotoxicity of hCFPs based on granzyme B *in vitro* [93,94] and *ex vivo* [93], and achieved similar results for hCFPs based on angiogenin [25].

Granzyme B is a human immunoprotease stored in the cytotoxic granules of NK cells and cytotoxic T cells [95–98]. It is one of the most important effector molecules for the immune surveillance of virus-infected and transformed tumor cells [99] and is exceptional among the apoptosis-inducing enzymes used in immunotherapeutic approaches because it can induce apoptosis at multiple levels using different pathways [96,97,99]. Granzyme B is therefore more likely to overcome anti-apoptosis mechanisms that evolve in tumor cells and this makes it a potent and reliable inducer of apoptosis in hCFPs [100]. The potential of granzyme B in cancer therapy has been demonstrated by coupling it to antibodies and ligands that target CD64, gp240, ErbB2/Her2 (human epidermal growth factor receptor 2), hLHR (human luteinizing hormone receptor), LeY (Lewis Y antigen), EGFR (epidermal growth factor receptor), and CD30 [100–102]. Granzyme B-based hCFPs have been proven successful in *ex vivo* experiments [93] and in a xenograft mouse model [102].

Human angiogenin (RNase 5) is a member of the ribonuclease A superfamily which is usually involved in angiogenesis, but also plays a role in inflammation, wound healing, and antimicrobial defense. Angiogenin is often found in and/or around tumors, where it may promote vascularization [103]. When fused to a tumor-specific antibody, angiogenin exerts cytotoxic activity following receptor-mediated endocytosis by degrading tRNA and, thus inhibiting protein synthesis [104]. The pro-apoptotic activity of angiogenin has been demonstrated by targeting CD64 and CD30 overexpressed on Hodgkin lymphoma cells [15,105].

4. Comparative Efficacy of Human Effector Domains and Bacterial Toxins in Targeted Therapy

Granzyme B and angiogenin are only two examples of numerous human effector domains that have been investigated during the last decade [5,6,18,19]. A common feature of all human effector domains used thus far to construct hCFPs is their moderate cytotoxicity compared to bacterial toxins. However, the direct quantitative comparison of IC₅₀ values is difficult because different cell-based methods can be used to determine cytotoxic effects. Cytotoxicity assays are generally conducted in multiwell plates and are based on the conversion of a substrate into a colored, fluorescent or luminescent product, which represents the metabolic or enzymatic activity of viable cells in a manner directly proportional to the viable cell number. The cytotoxic activity of classical ITs and hCFPs is usually monitored by colorimetric cell viability assays based on the reduction of tetrazolium compounds to a colored formazan product, e.g., MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), MTS (3-(4,5-dimethylthiazol-2-yl-5)-3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium, or the more sensitive reagent XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) [106,107]. Alternative assays are based on the production of ATP, or on the activity of caspases. Direct quantitative comparisons among these assays should be interpreted with caution [108].

Cell-based assays generally produce more variable results than *in vitro* assays because internal viability controls or standard curves are rarely prepared. The comparability of IC₅₀ values calculated from dose response curves is therefore influenced by a variety of factors, including dose and exposure times, cell concentrations, the type of endpoint read-out (biochemical, fluorescent, or bioluminescent detection), variability in the cell cycle and cultivation time, and differences in the sensitivity of cell lines cultivated in different laboratories using different media and components that influence receptor expression and formazan production [109,110]. Some medium components such as serum albumin can reduce tetrazolium salts thus leading to the underestimation of cytotoxic effects [111–114]. Particular care must be taken if different cell lines expressing the same receptor are used for comparative functional analysis because XTT metabolism is not consistent among cell different lines [110,115].

In this review, we take the opportunity to exemplarily compare the IC₅₀ values of hCFPs containing granzyme B or angiogenin against corresponding ITs based on ETA. Table 1 lists hCFPs and ITs targeting CD64, EGFR, LeY (expressed on several human tumor types), as well as the breast-cancer-specific receptor ErbB2/Her2 and the Hodgkin lymphoma target CD30. The IC₅₀ values show that ETA is much more potent than the human effector domains *in vitro*, regardless of the target antigen. In-house investigations targeting CD64 overexpressed on the AML cell line U937 revealed that H22(scFv)-ETA' and M22(scFv)-ETA' are ~12–120-fold more cytotoxic than equivalent constructs containing granzyme B. These observations were confirmed by the comparison of constructs in which truncated ETA or granzyme B were genetically fused to tumor growth factor alpha (TGF α) or the ErbB2-specific antibody fragment scFv(FRP5). The bacterial toxin was ~11–175-fold more toxic against the epidermoid carcinoma cell line A431 even in the presence of chloroquine which promotes the endosomal release of the human effector domain. The PE38 version of ETA was 2.3–82-fold more cytotoxic than granzyme B towards the cancer cell lines MCF-7, SK-BR3 and L540cy, when genetically fused to the B3 antibody fragment targeting the Lewis Y carbohydrate antigen, or Ki4(scFv) targeting CD30.

In contrast to granzyme B, hCFPs based on angiogenin generate similar IC_{50} values to truncated variants of ETA. Our published IC_{50} values for Ang-CD30L and H22(scFv)-Ang are within the same order of magnitude as those published earlier for the corresponding ETA' constructs. Nevertheless, these tendencies could not be reproduced by further internal investigations in comparative XTT viability assays using identical assay conditions and therefore providing more reliable data (see below). A third example, a fusion and a conjugate of angiogenin and EGF, showed IC_{50} values of 40 nM and 70 nM, respectively. Using the same target receptor (EGFR) and the same cell line (A431) but a different ligand (TGF α), the bacterial construct was ~200-fold more cytotoxic. Although these constructs do not provide the perfect comparison, they indicate that angiogenin is less cytotoxic than ETA.

These data indicate that the specific cytotoxicity of targeted therapeutics does not depend solely on the affinity of the cell-binding moiety for surface markers, the availability of receptor molecules on the cell surface of target cells and the internalization efficiency, but also significantly on efficient translocation of the cytotoxic domain into the cytosol. In contrast to human enzymes, bacterial toxins are evolutionarily tuned to enter mammalian cells. The transfer of these skills onto human effector domains is therefore a key goal in the development of efficacious hCFPs.

Table 1. Comparison of immunotoxins (ITs), based on truncated ETA, and human cytolytic fusion proteins (hCFPs), based on granzyme B or angiogenin.

Target	Cell line	Construct	IC_{50}	Reference
CD64	HL-60	M22(scFv)-ETA'	0.17 nM	[77]
	U937	M22(scFv)-ETA'	0.18 nM	[77]
		H22(scFv)-ETA'	0.14 nM	[81]
		Gb-H22(scFv)	1.7–17 nM	[93]
		H22(scFv)-Ang	0.2 nM	[25]
EGFR	A431	TGF α -ETA (T-ETA)	0.02 nM	[116]
		GrB-TGF α (GrB-T)*	3.5 nM	[116], [17]
		EGF-Ang	40 nM	[117]
		Ang EGF**	70 nM	[118]
ErbB2/Her2	A431	scFv(FRP5)-ETA (5-ETA)	0.5 nM	[116]
		GrB-scFv(FRP5) (GrB-5)*	5.8 nM	[116], [17]
LeY	SK-BR3	B3-PE38	42 nM	[119]
		B3-GzmB	98 nM	[119]
	MCF-7	B3-PE38	1.7 nM	[119]
		B3-GzmB	140 nM	[119]
CD30	L540cy	Ki4(scFv)-ETA'	0.04–0.08 nM	[120]
		Gb-Ki4(scFv)	2.5 nM	[102]
		CD30L-ETA'	0.4 nM	[121]
	L540	Ang-CD30L	0.23 nM	[15]

*Chloroquine present, ** chemically conjugated. Abbreviations: EGFR: epidermal growth factor receptor; ErbB2/Her: human epidermal growth factor receptor 2; LeY: Lewis Y antigen; Gb/GrB/GzmB: granzyme B; Ang: angiogenin; ETA/ETA'/PE38: truncated versions of Pseudomonas exotoxin A; EGF: epidermal growth factor.

5. Optimization of Endosomal Release Using Multifunctional, Cleavable Adapters

Drugs that interact with intracellular targets must be delivered efficiently across the hydrophobic plasma membrane. More than 95% of protein-based drugs that demonstrate efficacy *in vitro* have poor pharmacokinetic properties that limit their therapeutic potential because they are neither small nor lipophilic enough to pass through biological membranes. Biopharmaceuticals, such as ITs, are therefore targeting cell surface receptors and are taken up by receptor-mediated endocytosis into clathrin-coated pits. The first intracellular barrier they encounter is the endosomal membrane, which promotes the retention of proteins within endosomes. The maturation of endosomes involves acidification, fusion to lysosomes and subsequent enzymatic degradation (endosomal/lysosomal degradation pathway), and this represents one of the major bottlenecks limiting the biological activity of receptor-targeting protein drugs. Research has, therefore, focused on strategies to promote the release of biopharmaceuticals from early endosomes [122,123]. Various strategies have been developed, including the use of carrier systems, such as liposomes, cationic polymers and nanoparticles, or the linkage of protein-based drugs to short peptides that facilitate membrane translocation [122,124,125].

Unlike biopharmaceuticals, infectious agents, such as bacteria, viruses and bacterial toxins, possess inherent mechanisms that can bypass cellular membrane barriers either by direct transcytosis into the cytoplasm or escape from the endosome following receptor-mediated endocytosis [126–128]. The latter mechanism is exploited by so-called AB exotoxins, such as ETA and diphtheria toxin, which consist of an enzymatically active/toxic component (A) and a cell binding component (B). Once internalized, bacterial toxins avoid lysosomal degradation by executing sophisticated endosomal escape mechanisms [129]. These include pore formation, pH-buffering and insertion into the endosome lipid bilayer, all of which result in the disruption of the endosomal membrane [130]. Several bacterial toxins make use of a retrograde transport to the ER via the Golgi apparatus prior to the translocation of the enzymatically active domain to the cytosol. This alternative way of intracellular routing enables an escape from the endosomal/lysosomal degradation pathway [85]. In contrast to ITs based on bacterial toxins and plant type-II ribosome inactivating proteins (RIPs), hCFPs based on human effector domains or ITs based on plant type-I-RIPs lack an intrinsic translocation function, so they are vulnerable to endosomal trapping.

A number of strategies overcoming these drawbacks have been explored during the last decade. Many proteins and peptides derived from pathogens have already been shown to increase the bioavailability of diverse untargeted macromolecules (such as proteins and nucleic acids) by facilitating receptor-independent cellular uptake [130]. These include cell penetrating peptides (CPPs)—also called protein transduction domains (PTDs) or membrane transfer peptides (MTPs)—a group of small peptides that are either rich in basic amino acids and exhibit a net positive charge, or possess an amphipathic alpha-helical structure that allow direct interaction with biological membranes to promote the direct transcytosis of larger molecules. Prominent examples of CPPs include the fusogenic HA2 domain of influenza virus hemagglutinin [131], the transduction domain derived from human immunodeficiency virus 1 (HIV-1) transacting activator of transcription (TAT) [132], and bacterial transduction domains derived from ETA [133] and diphtheria toxin [134]. Genetically engineered PTDs or fully synthetic peptides with similar activity have been used extensively for the

successful intracellular delivery of biologically-active molecules [135]. Nevertheless, the receptor-independent uptake mechanisms of conventional CPPs do not mediate selective delivery and are therefore unsuitable for targeted therapy, with a few exceptions [136–138].

Both selectivity and cytosolic availability are necessary for the efficient activity of ITs, so effective transduction of the endosomal membrane is required following receptor-mediated endocytosis. One strategy to address the endosomal release of effector molecules lacking endogenous translocation domains has been the insertion of PTDs between the binding and effector domains [122,139]. In 2001 the concept of a multifunctional adapter flanking a MTP with a cytosolic cleavable peptide (CCP) and an endosomal cleavable peptide (ECP) was described for the first time by Keller *et al.* [24]. Two years later, the same group published more detailed experiments on the effect of this adapter on the optimization of an IT based on EGF and the plant type-I-RIP Saporin [140]. The sophisticated composition of the adapter, combining both functional and cleavable elements, ensured both the efficient transport of the toxic cargo from the endosome into the cytosol and the prevention of its release from the cytosol to protect non-target cells. The concept of an adapter-mediated toxin uptake can be summarized as follows: an endogenous endosomal enzyme cleaves the ECP, so that the MTP is only exposed after uptake via receptor-mediated endocytosis. Following endosomal cleavage of the ECP, the MTP promotes translocation of the effector molecule across the membrane. Once in the cytosol, cleavage of the CCP releases the effector molecule. Removing the MTP prevents further membrane translocation and therefore the potential non-specific impact of the effector molecule on surrounding, healthy cells [24,140]. While significantly increasing the cytotoxic activity the cleavable elements of the adapter markedly influence serum stability of the hCFPs. Hence, depending on the administration route of ITs or hCFPs, the adapter composition and its influence on the serum stability should be considered carefully. Modification of the adapter by depletion of the ECP vulnerable for extracellular furin activity fully restored serum activity, but diminished the adapter effect on the cytotoxic potential [25]. The use of optimized adapter compositions based on alternative protease cleavage sites increasing the half-life of systemically applied hCFPs might offer suitable approaches to meeting this challenge. Further strategies include the induction of endosomal leakage and disruption by pH alteration or the use of membrane-destabilizing agents, and photochemical internalization (PCI) by light-induced activation of co-administered photosensitizers and have recently been reviewed [122].

The exact intracellular routing of ITs and hCFPs after endosome formation meaning the differentiation between the lysosomal pathway and the retrograde transport via the ER is important and often yet unknown. Direct visualization by confocal microscopy of the intracellular trafficking route of a targeted plant RIP (Gelonin) showed that it accumulated preferentially in the lysosome in resistant cells but not in sensitive cells [141]. These observations implicate the process of increased lysosomal degradation as the most likely basis for resistance and underline the importance of a potent endosomal escape.

6. Comparative Analysis of Anti-CD64 hCFPs and H22(scFv)-ETA'

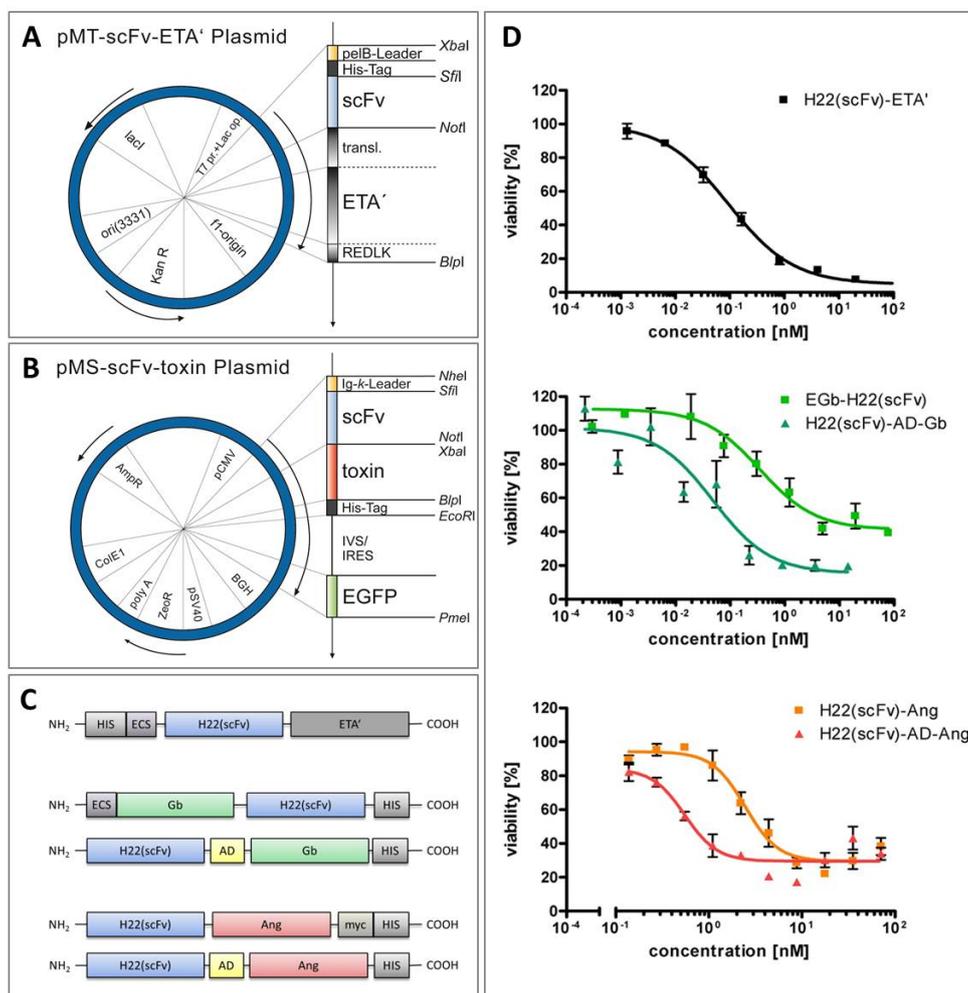
We successfully used an adapter derived from the components described above to increase the cytotoxic potency of the fusion construct H22(scFv)-Ang. The inclusion of the adapter led to a 20-fold increase in cytotoxic activity compared to the version lacking the adapter, with IC_{50} values in the nanomolar range [25]. These promising results, as well as previous studies concerning the insertion of

different fractions of the ETA translocation domain (PEAII) and different furin cleavage sites to optimize the efficacy of targeted granzyme B [142,143], motivated us to combine the modified adapter from our CD64-specific hCFP with granzyme B. Furthermore, a pH-sensitive fusogenic peptide has recently been fused to the C-terminus of a granzyme B-based hCFP targeting Her2/neu [101]. The authors used immunofluorescence and western blot analysis to show that the optimized variant accumulated to higher levels in the cytosol, and resulted in a 10-fold reduction in IC₅₀ values against different cancer cell lines, compared to constructs lacking the peptide. For example, IC₅₀ values against the breast cancer cell line BT474 M1 were 253.3 nM for the standard construct and 29.3 nM for the modified construct.

To confirm that multifunctional adapters can optimize the endosomal release of hCFPs based on granzyme B, we used the angiogenin constructs [25] and H22(scFv)-ETA' [79,81] as gold standard for comparison with the granzyme B variants. We used two expression systems for the heterologous production of the fusion proteins. As previously described [81,105], the immunotoxin H22(scFv)-ETA' was cloned in vector pMT, which is derived from the commercial plasmid pET 27b (Novagen, Germany), and expressed in *Escherichia coli* strain BL21 (DE3). In contrast, the hCFPs were expressed in the mammalian HEK293T cell line using the pMS plasmid system based on the commercial pSec-Tag2 vector series (Invitrogen, USA). The recombinant proteins were purified by immobilized metal ion affinity chromatography (IMAC) and, in the case of H22(scFv)-ETA', also by subsequent size exclusion chromatography (SEC) and endotoxin removal [81,105]. The expression vectors and protein constructs are illustrated in Figure 1.

Figure 1. (A) Plasmid map pMT-scFv-ETA': The bacterial expression vector contains the following functional elements: the lactose repressor gene (*lacI*), the T7 promoter (T7 pr.), the IPTG-inducible *lac* operon (*Lac op.*), two origins of replication (*f1* origin and *ori(3331)*), a kanamycin resistance gene (*KanR*), and the signal peptide of the pectate lyase gene (*pelB*). The His-tag epitope allows protein detection and purification. Relevant restriction sites are indicated. (B) Plasmid map pMS-scFv-toxin: The generic mammalian expression vector is available as multiple derivatives for various orientations of the hCFP and/or adapter. The constitutive human cytomegalovirus promoter (*pCMV*) initiates the expression of the bicistronic mRNA, which is terminated with a bovine growth hormone polyadenylation site (*BGH*). The ampicillin resistance gene (*AmpR*) and the bacterial origin of replication (*ColE1*) were used for plasmid propagation in *Escherichia coli*. The Zeocin-resistance gene (*ZeoR*) is controlled by the SV40 promoter (*pSV40*) and is used for the selection of transfected HEK293T-cells. The murine Ig- κ -leader promotes the secretion of the hCFPs into the cell culture supernatant. The C-terminal His-tag epitope allows protein detection and purification. Downstream of the coding sequence, the synthetic intron (*IVS*) is used to stabilize the mRNA, and an internal ribosomal entry site (*IRES*) enables the co-translation of the enhanced green fluorescent protein (*EGFP*) and the selection of transfected cells. Relevant restriction sites are indicated. (C) Schematic comparison of the fusion proteins: The fusion proteins H22(scFv)-ETA', EGb-H22(scFv), H22(scFv)-AD-Gb, H22(scFv)-Ang, and H22(scFv)-AD-Ang are shown as schematic maps. ECS = enterokinase cleavage site, ETA' = truncated version of *Pseudomonas* exotoxin A (PE40),

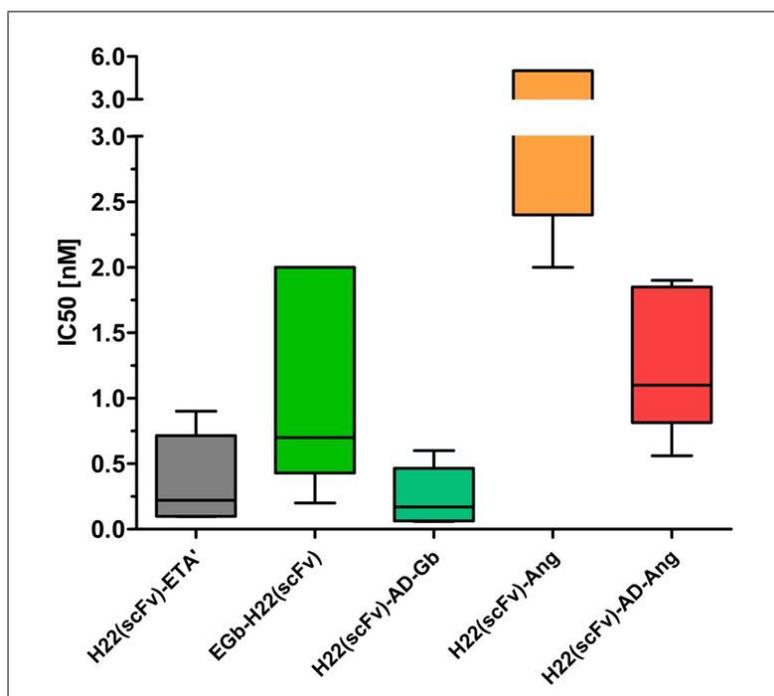
Gb = granzyme B, HIS = polyhistidine tag, AD = adapter with endosomal cleavable peptide, membrane transfer peptide and cytosolic cleavable peptide, Ang = angiogenin, myc = polypeptide tag derived from the c-myc gene. **(D)** XTT viability assay for hCFPs and ITs against IFN γ -stimulated HL-60 cells: We incubated 1.5×10^4 cells with different concentrations of purified proteins for 72 h. Cell viability was determined by measuring the conversion of XTT to its formazan derivative at 450–630 nm, using an ELISA reader (Epoch BioTek). Samples were analyzed in triplicate and the error bars indicate standard deviations (SD). The relative viability [%] was calculated based on a scale from 0% substrate consumption (incubation with Zeocin) to 100% substrate consumption (incubation with PBS) and was displayed with GraphPad Prism (La Jolla, CA, USA). The IC₅₀ values are 0.04 nM for H22(scFv)-ETA', 0.35 nM for EGb-H22(scFv), 0.05 nM for H22(scFv)-AD-Gb, 2.45 nM for H22(scFv)-Ang, and 0.55 nM for H22(scFv)-AD-Ang.



As discussed in previous reports [25,81,93], we confirmed specific binding of the fusion proteins to CD64 by flow cytometry, the induction of apoptosis by annexin V/PI staining (data not shown) and the impact on cell viability by XTT cytotoxicity assays (Figure 1). The concentration-dependent viability of promyelocytic leukemia HL-60 cells in the presence of each fusion protein is shown as an example. The data confirm the influence of the adapter because the values are shifted left compared to the constructs lacking the adapter. The constructs containing the adapter repetitively achieved lower IC₅₀

values than constructs lacking the adapter (Figure 2). The IC_{50} values for the constructs based on granzyme B were 0.06–0.33 nM with the adapter compared to 0.2–2 nM without the adapter. Similarly, the IC_{50} values for the constructs based on angiogenin were 0.56–1.9 nM with the adapter and 2.8–5 nM without, although angiogenin is generally less toxic towards HL-60 cells than granzyme B. We were thus able to validate that molecular adapters promoting the cytosolic release of human effector molecules increase the cytotoxic efficacy of hCFPs. Remarkably, the granzyme B construct with the adapter was not only more potent than its counterpart lacking an adapter, but it was also of similar potency to the bacterial gold standard, H22(scFv)-ETA' (Figure 2). This means that we have developed a hCFP, which possesses the same cytotoxic potency as the bacterial IT H22(scFv)-ETA' and is potentially safer because it is likely to be less immunogenic and therefore more suitable for repetitive administration and non-life-threatening diseases.

Figure 2. Box plot of IC_{50} values: Five IC_{50} values determined in the XTT viability assays are compared for H22(scFv)-ETA', EGb-H22(scFv), H22(scFv)-AD-Gb, H22(scFv)-Ang, and H22(scFv)-AD-Ang.



7. Conclusions

Immunotoxins (ITs) are a promising tool in targeted therapies for a variety of diseases including cancer, chronic inflammation and autoimmune disorders. Traditional ITs, based on monoclonal antibodies chemically conjugated to potent cytotoxic proteins originating from bacteria or plants, suffer from several disadvantages in the clinic, such as nonspecific toxicity, immunogenicity and the inability to manufacture a homogenous product. Stepwise optimization has led to the establishment of fourth generation ITs now called human cytolytic fusion proteins (hCFPs) which are genetic fusions of truncated antibody fragments and human effector molecules.

Although hCFPs are probably much less immunogenic and may therefore be safer than traditional ITs when used for long-term, repetitive treatment regimens, they tend to be trapped in the endosome following endocytosis and their cytotoxic potency is therefore reduced. ITs based on bacterial toxins possess intrinsic translocation mechanisms that allow endosomal escape, which are missing in human effector proteins. One of the most promising future trends in the optimization of hCFPs is therefore the addition of heterologous translocation domains included within sophisticated adapters comprising a membrane translocation domain flanked by endosomal and cytosolic cleavable peptides. This strategy has been shown to increase the *in vitro* cytotoxic potency of hCFPs containing granzyme B or angiogenin for the treatment of CD64+ diseases. Furthermore, hCFPs engineered to promote endosomal escape could potentially compete with traditional ITs, as illustrated by the comparison of the anti-CD64 hCFPs H22(scFv)-AD-Ang and H22(scFv)-AD-Gb and the analogous bacterial IT based on a truncated version of Pseudomonas exotoxin A (ETA').

A deeper understanding of the molecular mechanisms of cellular uptake and endosomal release will also facilitate the development of efficient delivery tools that ensure the cytosolic delivery of human effector proteins. Engineering hCFPs in such a way could increase the therapeutic value of these fusion proteins and advance them as a novel class of targeted therapeutic proteins not only for the treatment of life-threatening diseases, such as cancer, but also indications such as chronic inflammation and autoimmune disorders.

Acknowledgments

This work was funded in part by a grant from the German province NRW from EFRE “European Fund for Regional Development” under the theme “Europe - Investment in our Future” and by Fraunhofer internal projects. We thank Richard M. Twyman for critical reading of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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