Review

Selective Induction of Cancer Cell Death by Targeted Granzyme B

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Abstract: The potential utility of immunotoxins for cancer therapy has convincingly been demonstrated in clinical studies. Nevertheless, the high immunogenicity of their bacterial toxin domain represents a critical limitation, and has prompted the evaluation of cell-death inducing proteins of human origin as a basis for less immunogenic immunotoxin-like molecules. In this review, we focus on the current status and future prospects of targeted fusion proteins for cancer therapy that employ granzyme B (GrB) from cytotoxic lymphocytes as a cytotoxic moiety. Naturally, this serine protease plays a critical role in the immune defense by inducing apoptotic target cell death upon cleavage of intracellular substrates. Advances in understanding of the structure and function of GrB enabled the generation of chimeric fusion proteins that carry a heterologous cell binding domain for recognition of tumor-associated cell surface antigens. These hybrid molecules display high selectivity for cancer cells, with cell killing activities similar to that of corresponding recombinant toxins. Recent findings have helped to understand and circumvent intrinsic cell binding of GrB and susceptibility of the enzyme to inhibition by serpins. This now allows the rational design of optimized GrB derivatives that avoid sequestration by binding to non-target tissues, limit off-target effects, and overcome resistance mechanisms in tumor cells.

Keywords: granzyme B; cancer therapy; epidermal growth factor receptor; ErbB2; HER2; transforming growth factor α; single-chain Fv antibody; recombinant fusion protein

1. Introduction

Monoclonal antibodies are well established as targeted therapeutics for the treatment of cancer, and an increasing number of such reagents are in clinical use. Prominent examples include the anti-CD20 antibody rituximab (Rituxan/MabThera) [1], the epidermal growth factor receptor (EGFR)-specific antibody cetuximab (Erbitux), and the ErbB2 (HER2)-specific antibody trastuzumab (Herceptin) [2,3]. Nevertheless, responses could not be achieved in all patients with cancers expressing high levels of the respective target antigens, and in a significant proportion of patients, initial responses are followed by the development of resistance despite continued antigen expression [4,5]. This suggests that patient- and tumor-specific factors such as limited recruitment of endogenous immune effector mechanisms and activation of alternative signaling pathways can influence treatment outcome. In contrast to regular antibodies, protein conjugates and recombinant fusion proteins that link antibody- or ligand-mediated recognition of cancer cells with a potent cytotoxic effector function can achieve their antitumoral activity independent of the signaling capabilities of the target antigen, and do not require endogenous immune effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) or complement fixation. Hence, such targeted therapeutics may constitute a valuable treatment option, in particular in cases where current antibody therapies are ineffective.

Antibody-toxins, also termed immunotoxins, were initially derived by chemically coupling bacterial or plant toxins to monoclonal antibodies specific for molecules on the surface of tumor cells. The elucidation of the molecular structure of bacterial toxins such as *Pseudomonas* exotoxin A (ETA, PE), and the development of recombinant antibody formats have allowed to miniaturize these molecules through recombinant DNA techniques, and to produce them as single polypeptides in large quantities and of consistent quality in bacteria [6]. Recombinant ETA-based toxins are derived by replacing the toxin's N-terminal cell binding domain with a heterologous function for cell recognition, such as a natural peptide ligand or a single-chain Fv (scFv) antibody fragment [7,8]. This basic principle has been applied successfully for antibody-toxins targeted to many different tumor-associated surface antigens including EGFR, ErbB2, mesothelin, and differentiation antigens like CD22, some of which have entered clinical development [9–11]. Nevertheless, while clinical trials with antibody-toxins for the treatment of hematologic malignancies have yielded impressive response rates, reports on successful application of such molecules in patients suffering from cancers of epithelial origin are still rare [9,12,13]. This is at least in part due to the type of target antigens available. Normal expression of target receptors such as CD22 is restricted to a defined population of differentiated cells, thereby limiting potential adverse effects, whereas epithelial antigens targeted for therapy usually display significantly enhanced expression in tumors, but might also be present at varying levels on different normal tissues. Consequently, for recombinant toxins targeted to epithelial tumor antigens the therapeutic index, *i.e.*, the difference between the minimum effective dose and the maximum tolerated dose might be smaller.

In principle, repeated treatment cycles or continuous therapy for a prolonged time period may overcome this problem. This, however, is hampered by the high immunogenicity of current antibody-toxins, resulting in rapid development of neutralizing antibodies against their toxin portion [8,9]. Different approaches have been proposed to reduce immunogenicity, including combined treatment with immunosuppressive reagents, chemical modification of the toxin moiety with polyethylene glycol

(PEGylation), and elimination of dominant B and T cell epitopes [6,8]. Alternatively, employing a cytotoxic protein of human origin as an effector function in immunotoxin-like molecules may be considered a straightforward way to circumvent the problem of high immunogenicity. Target cell killing by bacterial toxins such as ETA is mediated by the inhibition of protein synthesis, followed by the induction of apoptosis *via* indirect mechanisms [14,15]. Consequently, human molecules that transmit strong pro-apoptotic signals are prime candidates for the development of targeted fusion proteins for cancer therapy [16]. Different strategies have been proposed to exploit the process of cellular self-destruction for the ordered elimination of tumor cells, including the fusion of cell targeting domains to cell death inducing cytokines of the tumor necrosis factor family [17] or pro-apoptotic members of the Bcl-2 protein family [18,19]. These molecules function upstream in the apoptosis cascade, and their activity may be affected by reduced sensitivity of cancer cells to pro-apoptotic signals [20]. Hence, also human pro-apoptotic effectors have been employed to develop immunotoxin-like molecules that act at late stages of the apoptotic signaling cascade and can affect multiple pathways simultaneously. These include apoptosis inducing factor (AIF) and granzyme B (GrB) [21–25]. Thereby the serine protease GrB, similar to protein toxins, modifies its substrates enzymatically, which allows amplification of its effects and can result in cytotoxicity even at low enzyme concentrations in the target cell cytosol. In this review, we focus on the current status and future prospects in the development of targeted GrB fusion proteins, with special emphasis on molecules directed to cancer cells overexpressing the growth factor receptors EGFR or ErbB2.

2. Granzyme B Fusion Proteins for Targeted Cancer Therapy

2.1. Induction of Programmed Cell Death by the Serine Protease Granzyme B

Granzyme B (GrB) is naturally expressed by cytotoxic T-lymphocytes (CTL) and natural killer (NK) cells. The serine protease plays a crucial role in the immune defense against virus-infected and malignant cells by inducing apoptotic target cell death upon cleavage of intracellular substrates [26]. Initially, GrB is produced as an inactive precursor protein. This pre-pro-GrB carries an N-terminal signal peptide directing packaging of the protein into secretory granules. Subsequent removal of the activation dipeptide Gly-Glu by the cysteine protease cathepsin C generates the enzymatically active form of GrB of approximately 32 kDa [27], which is stored together with other granzymes and perforin in the dense core of lytic granules [28]. Following target recognition and effector cell activation, the lytic granules are polarized towards the immunological synapse, where they fuse with the plasma membrane and release their contents into the synaptic cleft between effector and target cell [29,30]. After its release, GrB enters target cells with the help of the pore-forming protein perforin, and rapidly induces apoptosis *via* caspase-dependent and caspase-independent mechanisms [31]. The exact mechanisms of perforin pore formation and perforin-mediated GrB entry are still not fully understood. Recent studies indicate that perforin monomers released into the synaptic cleft bind to the target cell membrane, oligomerize, and undergo a major conformational rearrangement to form transmembrane pores [32,33]. Initially, it was thought that perforin pores may allow direct diffusion of GrB into the target cell cytosol. More recently, it has been suggested that pore-formation triggers membrane repair by an endocytic mechanism that facilitates co-internalization of perforin and GrB into vesicular compartments, followed by perforin-mediated

endosomolysis and release of GrB into the cytosol [34,35] (Figure 1a). For recombinant human GrB expressed in mammalian cells also uptake independent of perforin has been described, which required binding to cell surface-bound heat shock protein 70 (Hsp70) and Hsp70-mediated internalization [36,37].

Figure 1. Cellular uptake of granzyme B and targeted granzyme B fusion proteins. (**a**) Perforin monomers released into the synaptic cleft bind to the target cell membrane, oligomerize, and undergo conformational rearrangement to form transmembrane pores. These may allow direct diffusion of GrB, or trigger co-internalization of perforin and GrB into vesicular compartments, followed by perforin-mediated endosomolysis and release of GrB into the cytosol [34,35]. (**b**) Targeted GrB derivatives specifically interact with tumor-associated cell surface antigens such as EGFR or ErbB2 *via* their heterologous cell binding domain. Receptor-mediated endocytosis then results in uptake into endosomes. Efficient endosome release and translocation to the cytosol can be achieved by addition of an endosomolytic activity such as chloroquine [23,38].
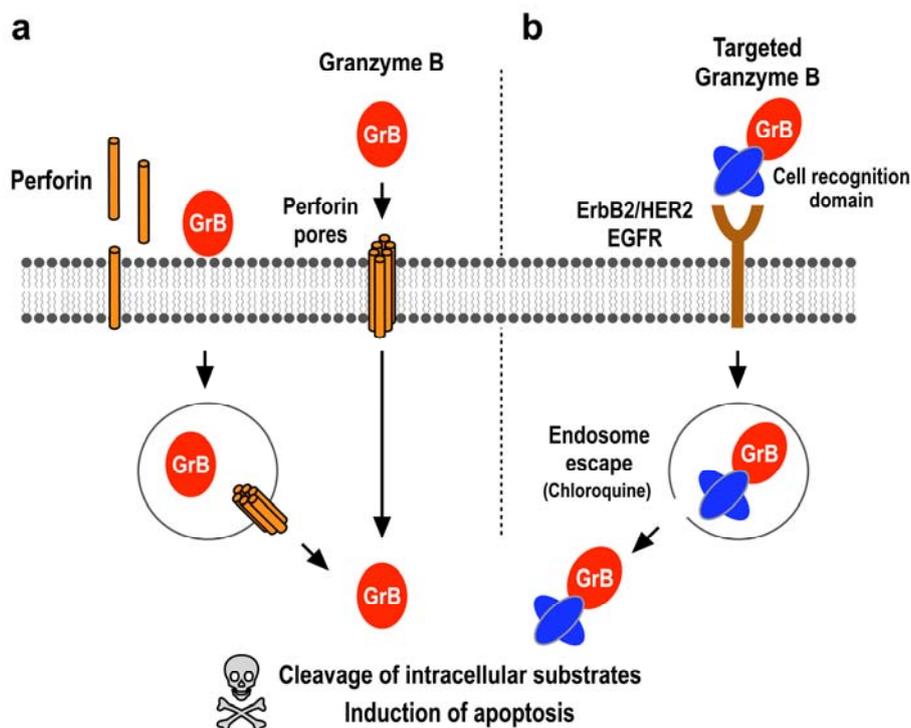
While cell death-inducing cytokines of the tumor necrosis factor family such as FASL and TRAIL require intact receptor systems and downstream signaling pathways to induce activation of initiator and effector caspases, cytosolic GrB can activate the apoptosis machinery directly and at different levels. This ensures induction of cell death even if one pathway is blocked [39]. GrB shares the substrate specificity of caspases, and cleaves its target proteins C-terminal of specific aspartate residues [40]. Important GrB substrates include caspase-3 and other initiator and effector caspases [41], as well as central caspase substrates such as the BH3-only protein Bid [42,43], and the inhibitor of caspase-activated DNase (ICAD) [44,45]. In addition, GrB directly cleaves components of the cytoskeleton [46], lamin B [47], PARP [48], and proteins involved in cellular homeostasis and stress response [39].

2.2. Expression Systems for Production of Granzyme B in Recombinant Form

The availability of GrB in recombinant form is an important prerequisite for functional analysis of the protein, and essential for application of GrB as a therapeutic effector molecule. Thereby the production of enzymatically active protein is complicated by the requirement to eliminate the pre-pro domains from GrB in order to generate the free N-terminus of the mature molecule [27,49]. Large amounts of GrB and targeted GrB derivatives could be produced in *E. coli* as inactive precursors fused to heterologous N-terminal protein domains such as glutathione-S-transferase (GST), requiring refolding and additional *in vitro* cleavage with proteolytic enzymes of purified recombinant proteins [50–52]. Similarly, GrB and targeted GrB were expressed as inactive precursor proteins in mammalian cells for *in vitro* activation *via* cleavage of a synthetic enterokinase site within the molecule [53,54]. Recently, an interesting approach for the generation of self-activating GrB in *E. coli* was also reported [52]. Thereby the GrB-specific cleavage site IEPD was introduced between GrB and a heterologous N-terminal prodomain, but so far, this approach has only been demonstrated to function for wildtype GrB not fused to a cell targeting ligand.

As a basis for subsequent studies on immunotoxin-like fusion proteins harboring GrB as an effector domain, we established a eukaryotic expression system utilizing the methylotrophic yeast *Pichia pastoris* for the production of human GrB in secreted, enzymatically active form [49]. *Pichia pastoris* is well established for the expression of secreted proteins [55], and had previously been employed to generate recombinant GrB from different mammalian species [56,57]. We fused amino acid residues 21 to 247 of GrB to the yeast α-factor signal peptide, which is removed in the secretory pathway by the *Pichia* protease kexin, resulting in recombinant protein with the free N-terminus of mature human GrB released into the culture supernatant. In contrast to production of GrB in bacterial expression systems, the final product was glycosylated and did not require further refolding and/or proteolytic activation *in vitro*. Single-step purification by immobilized metal affinity chromatography (IMAC) utilizing a C-terminal polyhistidine tag attached to the GrB sequence yielded 1 to 2 mg of purified protein per liter of culture supernatant. Recombinant GrB from yeast cleaved natural and synthetic GrB substrates with kinetic constants similar to those of human GrB isolated from IL-2-activated lymphocytes [49]. Direct cytosolic delivery of GrB with a cationic lipid-based transduction reagent resulted in rapid induction of apoptotic cell death, demonstrating the preserved cell-death inducing capacity of the recombinant protein [49]. Alternatively, GrB was generated in *Pichia pastoris* as a fusion with an N-terminal maltose binding protein (MBP) domain as a chaperone, resulting in enhanced levels of free GrB upon *in vivo* processing of a kexin-sensitive cleavage site [58].

2.3. Tumor Cell-Specific Granzyme B Fusion Proteins

GrB requires a correctly processed, free N-terminus for enzymatic activity [49]. Hence, attachment of a heterologous cell binding domain to the C-terminus of GrB allows to redirect the resulting fusion molecule to a tumor-associated cell surface antigen while retaining functionality of the serine protease (Figure 1b). Successful generation of a targeted GrB protein was first reported by the group of Rosenblum, who fused vascular endothelial growth factor (VEGF) 121 to GrB [22]. Upon bacterial expression and proteolytic activation *in vitro*, the fusion protein specifically eliminated endothelial

cells expressing the FLK-1/KDR receptor, which may be employed to target the tumor vasculature. Likewise, GrB was fused to a scFv antibody fragment targeting the melanoma antigen gp240, resulting in rapid killing of antigen-positive target cells *in vitro* with an IC_{50} of 20 nM [50], and growth delay of melanoma xenografts in a murine *in vivo* model [59]. Interestingly, in the latter case treatment with the GrB-scFv protein sensitized tumors to subsequent chemotherapy or ionizing irradiation. Targeted cytotoxicity in the absence of perforin was also achieved with an immunoconjugate of bacterially expressed and *in vitro* activated GrB linked *via* a disulfide bond to a dsFv antibody fragment specific for the Lewis Y carbohydrate antigen [51]. Depending on caspase-3 and target antigen expression by tumor cells, half-maximal killing was achieved in *in vitro* cytotoxicity assays after 48 h at concentrations of 35 to 98 nM, which compares to concentrations of 1.8 to 42 nM for a corresponding *Pseudomonas* exotoxin A immunoconjugate under the same experimental conditions. For an *in vitro* activated GrB-scFv fusion protein targeting CD64-positive acute myeloid leukemia (AML) cells, also IC_{50} values in the nanomolar range were reported [53]. After 3 days of treatment, half-maximal killing of established AML cells was achieved at concentrations of 1.7 to 17 nM.

Following a strategy similar to the one outlined above for expression of recombinant wildtype GrB in *Pichia pastoris*, we generated chimeric GrB fusion proteins harboring at their C-terminus the EGFR ligand transforming growth factor (TGF) α or the ErbB2-specific scFv antibody fragment scFv(FRP5) for selective targeting to tumor cells [23]. Overexpression of EGFR and the closely related ErbB2 protein have been described for many tumors of epithelial origin, and have been shown to contribute to cellular transformation [3]. Importantly, these growth factor receptors are accessible from the extracellular space, making them attractive targets for monoclonal antibodies as well as antibody-toxins or recombinant toxins that employ natural peptide ligands for targeting [9,13,60–62]. Yeast-expressed GrB-TGFα (GrB-T) and GrB-scFv(FRP5) (GrB-5) proteins were bifunctional, cleaving synthetic and natural GrB substrates, and displaying strongly enhanced binding to cells carrying the respective EGFR or ErbB2 target receptors [23]. Following cell binding and receptor-mediated uptake, the chimeric molecules were rapidly internalized, but at the concentrations applied did not induce target cell death. Instead, GrB-T and GrB-5 remained trapped in intracellular vesicles, unable to gain access to cytosolic GrB substrates. Nevertheless, this problem was resolved by addition of an endosomolytic reagent such as chloroquine, now resulting in efficient release of the fusion proteins from endosomal vesicles and targeted cytotoxicity (Figure 2). Chloroquine accumulates in acidic compartments such as late endosomes and lysosomes, where it interferes with the pH equilibrium, finally leading to osmotic rupture of the vesicles [63]. Hence, retargeting of GrB to ErbB2 or EGFR must have resulted in routing to an acidic environment sensitive to chloroquine, as expected upon uptake of the fusion proteins *via* classical receptor-mediated endocytosis, but not typical for wildtype GrB. This was confirmed by the inability of chloroquine to release unfused GrB from intracellular vesicles upon uptake *via* natural GrB internalization mechanisms [49]. In the presence of chloroquine concentrations of 50 to 100 μM, GrB-5 and GrB-T were able to specifically kill target cells with IC_{50} values measured after 14 hours of treatment in the picomolar to nanomolar range, whereas non-target cells were not affected at considerably higher concentrations [23,38] (Table 1). Cytotoxic activity was accompanied by clear signs of apoptosis such as chromatin condensation, membrane blebbing, formation of apoptotic bodies and activation of endogenous initiator and effector caspases.

Figure 2. Cell killing activity and selectivity of an ErbB2-specific GrB - antibody fusion protein. ErbB2-expressing Renca-lacZ/ErbB2 (left panel) and ErbB2-negative Renca-lacZ renal carcinoma cells (right panel) were incubated for 14 h with the indicated concentrations of recombinant GrB-scFv(FRP5) (GrB-5) fusion protein in the presence of 50 μM chloroquine. The relative number of viable cells in comparison to controls only treated with chloroquine was determined in cell viability assays as described [23]. For Renca-lacZ/ErbB2 target cells an IC_{50} value of 0.29 nM (20 ng/mL) was determined.
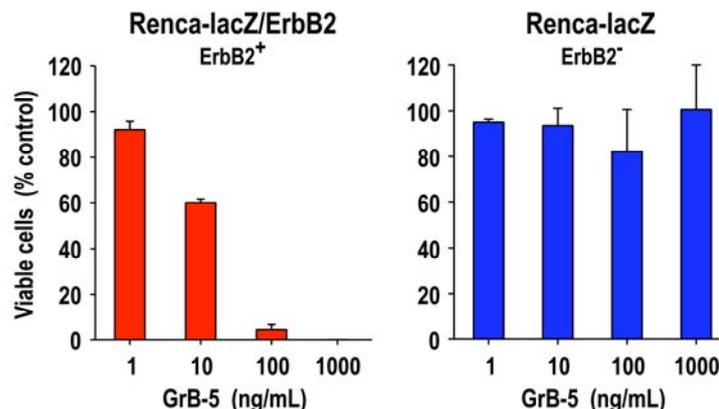
Table 1. Specificity and cytotoxicity of GrB fusion proteins and recombinant toxins.

Reagent [a]	Specificity	Tumor cell line		
		MDA-MB468 EGFR$^+$, ErbB2$^-$	A431 [b] EGFR$^+$, ErbB2$^+$	Renca-lacZ/ErbB2 EGFR$^-$, ErbB2$^+$
GrB-5	ErbB2/HER2	no killing at 14.5 nM	5.8 nM	0.29 nM
5-ETA	ErbB2/HER2	no killing at 15 nM	0.5 nM	0.09 nM
GrB-T	EGFR	0.25 nM	3.5 nM	no killing at 25 nM
T-ETA	EGFR	0.06 nM	0.02 nM	n.d.[c]

[a] IC_{50} values for granzyme B fusion proteins GrB-scFv(FRP5) (GrB-5) and GrB-TGFα (GrB-T) were determined in cell viability assays upon treatment of cells with recombinant proteins for 14 hours in the presence of chloroquine as described [23,38]. IC_{50} values for *Pseudomonas* exotoxin A fusion proteins scFv(FRP5)-ETA (5-ETA) and TGFα-ETA (T-ETA) were determined in cell viability assays upon treatment of cells with recombinant proteins for 40 hours as described [61,62,64]. MDA-MB468 and MDA-MB453 are established human breast carcinoma cells. Renca-lacZ/ErbB2 cells are murine renal carcinoma cells stably expressing human ErbB2 [64]. [b] Reduced sensitivity of human A431 squamous cell carcinoma cells to GrB fusion proteins was attributed to endogenous expression of GrB-specific serine protease inhibitor serpin P9 (PI-9) [23]. [c] n.d., not determined.

Similar to a Lewis Y-specific GrB immunoconjugate [51], cell killing by GrB-T and GrB-5 fusion proteins was 3–4 times less effective than that by *Pseudomonas* exotoxin A fusion proteins which employ the same cell targeting domains (Table 1). However, while the bacterial toxins required incubation times of at least 40 h for maximum *in vitro* cell killing, high cytotoxic activity of the GrB fusion proteins was already found after 14 h of treatment, and apoptotic morphology of target cells was observed as early as 2 hours after addition of chimeric GrB molecules, following kinetics similar to the GrB/perforin system [23]. Furthermore, target cells were killed even in the presence of the pan-caspase inhibitor zVAD-fmk, albeit to a lesser extent. This ability of GrB fusion proteins to also activate caspase-independent cell death pathways, possibly through cleavage of Bid or ICAD [43,44], can be relevant for elimination of tumor cells with a block in caspase-dependent apoptosis.

For GrB fusion proteins targeting surface antigens other than growth factor receptors, selective cytotoxicity was obtained without the need for an endosome escape activity [22,50,51,53]. Nevertheless, in such cases 10 to 300 times higher protein concentrations and/or extended treatment times were required for half-maximal killing activity *in vitro*. Interestingly, also GrB-T displayed cell killing in the absence of chloroquine. However, to achieve a measurable effect, high protein concentrations ($\geq$12.5 nM) and incubation for 48 h were required [38]. These findings suggest that the type of target receptor determines uptake and intracellular routing of chimeric GrB molecules, and kinetics and efficiency of their access to the cytosol.

3. Opportunities and Challenges for Further Development of Targeted Granzyme B

3.1. Target Cell Specificity of Granzyme B Fusion Proteins

So far experience with chimeric GrB fusion proteins in *in vivo* models is limited. While remaining difficulties to scale up protein production to levels required for treatment of larger cohorts of experimental animals may be overcome by utilizing optimized expression systems in yeast, insect and mammalian cells [24,58,65], also intrinsic features of GrB need to be addressed that may adversely affect availability of GrB fusions at the tumor site. With a calculated pI around 10, GrB is a highly basic protein with a positively charged surface. This enables binding to glucosaminoglycans and other negatively charged structures on the surface of different cell types [66–68]. While natural GrB is released in complex with the chondroitin sulfate proteoglycan serglycin that shields its positively charged surface [69,70], the therapeutic applicability of recombinant GrB derivatives may be limited by promiscuous binding of uncomplexed GrB to cell surface proteoglycans *via* electrostatic interactions [66,67,71]. This, in turn, could limit the amount of protein available for specific tumor cell killing. Bird *et al.* identified two cationic sequence loops RKAKRTR (residues 116 to 122) and KKTMKR (residues 241 to 246) within GrB that electrostatically interact with heparan-sulfate-containing molecules [66] (Figure 3a,b). Mutation of these sequences resulted in diminished cell binding and suppression of subsequent endocytosis, but disturbed perforin-assisted cytotoxicity. More recently, also residues K133 and K137 were implicated in non-selective cell binding of GrB [68].

Following an approach similar to that of Bird *et al.*, we mutated the two cationic heparin-binding motifs responsible for non-selective electrostatic interactions of GrB with cell surface structures to generate a surface charge-modified GrB variant termed GrBcs. Yeast-expressed GrBcs retained the enzymatic activity of wildtype GrB, but displayed markedly reduced intrinsic cell binding [38]. When fused to TGFα for tumor targeting, the resulting GrBcs-T molecule showed enzymatic activity in cell-free assays that was indistinguishable from that of unmodified GrB-T. However, binding of GrBcs-T to EGFR-negative cells was abolished (Figure 3c), while binding to EGFR-positive cells and target-specific cell killing were retained (Figure 3d). When tested in mixed cultures of EGFR-negative and EGFR-positive cells, GrBcs-T in contrast to GrB-T was not sequestrated by binding to cells devoid of target antigen. This greatly increased the availability of the modified GrBcs-T molecule for specific target cell killing [38]. Hence, systemically applied chimeric molecules that employ surface charge-modified GrB will be less likely than similar proteins based on wildtype GrB to be trapped by binding to non-target tissues before reaching the tumor site.

Figure 3. (**a**) Electrostatic surface potential of granzyme B. Positively charged areas are represented in blue. Residues mutated in the charge-modified derivative GrBcs are indicated. The model is based on the crystal structure of human GrB (1FQ3) [72] (generated with DeepView Swiss-PdbViewer; spdbv.vital-it.ch). (**b**) Positively charged residues within GrB (indicated in blue) were replaced by alanine residues (indicated in red) to obtain the charge-modified derivative GrBcs [38,66]. (**c**) Differential cell binding of targeted GrB proteins consisting of TGFα fused to the C-terminus of wildtype GrB (GrB-T) or charge-modified GrBcs (GrBcs-T). Unlabeled EGFR-negative MDA-MB453 breast carcinoma cells (blue) were mixed with fluorescently labeled EGFR-positive MDA-MB468 breast carcinoma cells (red) at a ratio of 1:1 or 10:1 prior to incubation with GrB-T (left panels) or GrBcs-T fusion proteins (right panels). Cell binding was analyzed by flow cytometry with Alexa Fluor 647-conjugated GrB-specific antibody as described [38]. (**d**) Cytotoxic activity of surface charge-modified GrBcs-T. EGFR-positive MDA-MB468 (upper panel) and EGFR-negative MDA-MB453 cells (lower panel) were treated with the indicated concentrations of purified GrB-T (blue circles) or GrBcs-T protein (red circles) for 14 hours in the presence of 50 µM chloroquine. The relative number of viable cells in comparison to controls treated only with chloroquine was determined in cell viability assays as described [38].
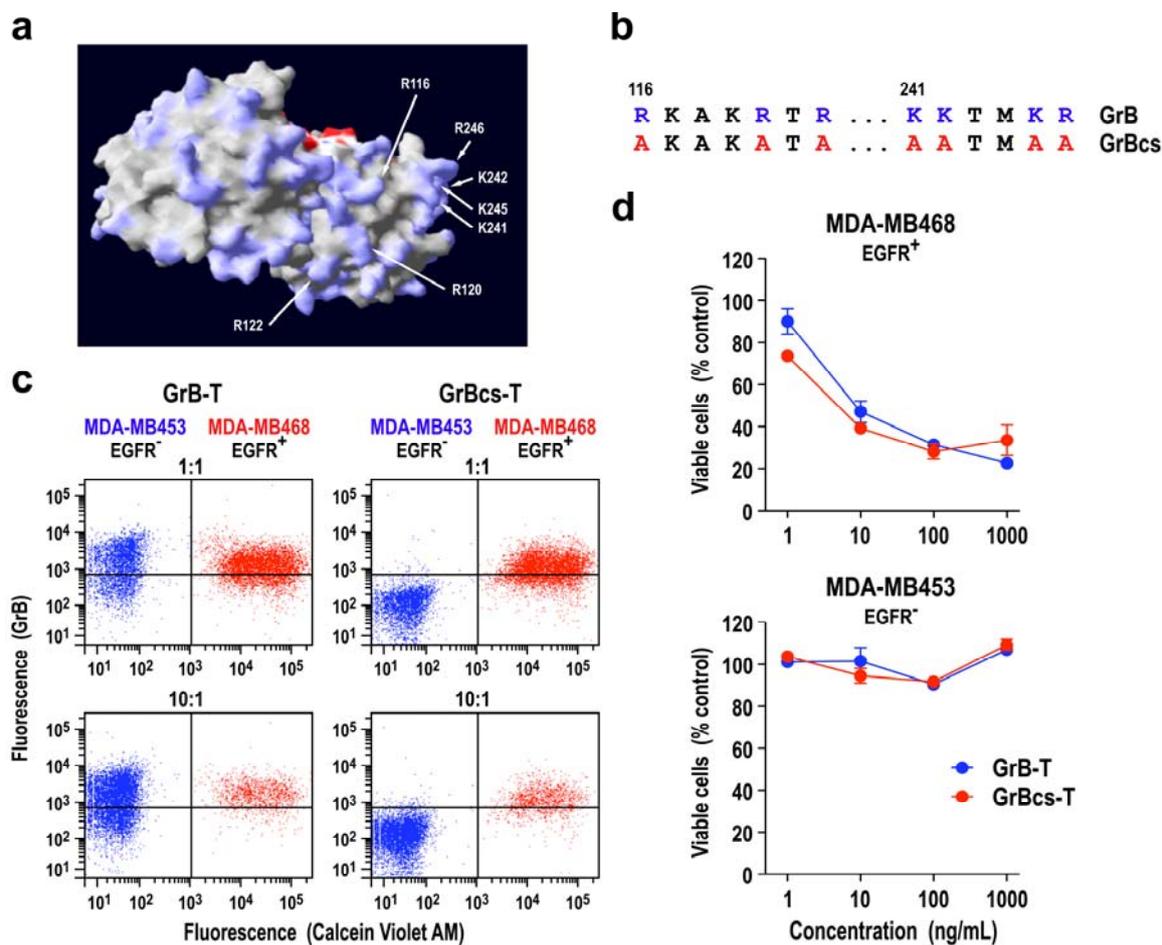
3.2. Extracellular Activity of Granzyme B

In addition to its apoptosis-inducing activity within target cells, the serine protease GrB can also process components of the extracellular matrix [39,73], which may be important for tissue remodeling in the course of an ongoing immune reaction. However, excessive extracellular activity of GrB has been linked to pathophysiological conditions such as rheumatoid arthritis [74,75], cardiovascular [76–78] and neurodegenerative diseases [79], and may complicate application of large doses of recombinant GrB proteins for therapeutic purposes. Like intrinsic cell binding, interaction of GrB with extracellular substrates has been linked to the high positive surface charge of the molecule [80]. To investigate potential differences in the extracellular activities of wildtype GrB and GrBcs derivatives, we employed human HeLa cervix carcinoma cells as a model. These cells undergo morphological changes upon degradation of their extracellular matrix by GrB [49], but are not sensitive to the apoptosis-inducing effects of EGFR-specific GrB-T or GrBcs-T proteins in the absence of chloroquine. While exposure to GrB-T or untargeted GrB resulted in a concentration-dependent loss of adherent cells, GrBcs-T and GrBcs induced only minimal cell detachment [38]. This confirms that electrostatic interactions play an important role for the extracellular proteolytic activity of GrB and GrB fusion proteins, which can be controlled by surface charge-modification as in the case of unspecific cell binding.

3.3. Granzyme B Resistance of Tumor Cells

The cellular serine protease inhibitor (serpin) PI-9 is an effective and highly specific physiological inhibitor of GrB [81,82]. PI-9 is abundantly expressed in CTL and NK cells to protect them from misdirected endogenous GrB. In addition, significant PI-9 levels have been found in B cells [81], monocytes [83], dendritic cells [84,85], and other bystander cell types that need to be shielded from GrB-mediated killing during an ongoing immune response. PI-9 is also present in normal human plasma, but at the given concentrations, it does not efficiently inhibit GrB activity [51]. This suggests that systemic application of targeted GrB fusion proteins may not be drastically affected by PI-9 circulating in the blood. More importantly, PI-9 expression has been found in tumor cells, where it constitutes a potential resistance mechanism to escape elimination by cytotoxic lymphocytes [86,87]. While PI-9 does not provide complete protection of tumor cells, it reduces their sensitivity for GrB-mediated cell death. Consistent with this, a strong correlation between PI-9 expression in the tumor and disease progression has been shown for different cancer types [88–90]. It is conceivable that this variable expression of PI-9 in tumor cells could significantly affect susceptibility to targeted GrB fusion proteins, as already evident from *in vitro* assays, where sensitivity for EGFR- and ErbB2-specific GrB-T and GrB-5 proteins was approximately 20 times lower for cancer cells with PI-9 expression despite high levels of the target antigens (see Table 1). Using a computational approach, Losasso *et al.* recently identified residues of human GrB important for interaction with PI-9 [91]. Employing molecular dynamic simulations, the mutations R28K, R201A and R201K within GrB were found to significantly destabilize GrB-PI-9 interaction, and the modified GrB variants retained enzymatic activity in the presence of PI-9. From this study, in particular the GrB mutant R201K emerged as a promising candidate suitable for the generation of novel, PI-9-resistant GrB fusion proteins. Also combination of GrB fusion proteins with reagents counteracting anti-apoptotic mechanisms in tumor

cells may enhance targeted cell killing, as recently observed for GrB combined with the Bcl-2 inhibitor ABT-737 [92].

3.4. Intracellular Routing and Cytosolic Delivery of Granzyme B

Perhaps the biggest hurdle for targeted cytotoxic proteins that act inside cells is effective cytosolic delivery of the toxic payload. In general, for such chimeric proteins to have the desired antitumoral activity, they must bind specifically to a tumor-associated cell surface antigen, followed by internalization into target cells, and translocation of the whole molecule or an enzymatically active fragment to the cytosol for induction of cell death. Bacterial toxins such as *Pseudomonas* exotoxin A or diphtheria toxin (DT) have endogenous endosome escape activity, which can be readily employed for cytosolic delivery of recombinant ETA- and DT-based toxins [7]. While certain GrB fusion proteins are obviously able to reach the cytosol to some degree on their own [24], this does not appear to be as efficient as desirable, and GrB fusion proteins targeted to EGFR or ErbB2 were shown to be trapped in endosomal vesicles after receptor-mediated uptake. They require an exogenously provided endosomolytic activity like chloroquine to induce cell death at low concentrations [23,38]. Chloroquine has already been employed in animals models as an endosome release agent in conjunction with other therapeutic molecules [93], and is being used since many years for the treatment of malaria and other diseases in humans [94]. Nevertheless, high doses and long term use of chloroquine can be associated with toxicity. Furthermore, the development of combined treatment regimens may be complicated by the different pharmacokinetics of a low molecular weight drug such as chloroquine and much larger chimeric GrB fusion proteins. In principle, integration of the translocation domains of ETA or DT into GrB fusion proteins could be useful to enhance cytosolic delivery [95–97]. Nevertheless, this compromises the aim to develop fully humanized immunotoxin-like molecules. Alternatively, functional domains from members of the Bcl-2 protein family may be employed, some of which exhibit a high degree of structural similarity with the DT translocation domain and have membrane-inserting capabilities [98]. In addition, perforin-derived peptides or full-length perforin may be able to cooperate with targeted GrB, if the size restriction of perforin pores is not being exceeded [50,68].

3.5. Activity of Granzyme Fusion Proteins against Resting Cancer Cells

While the majority of malignant cells within a tumor may grow rapidly, some of the cells including cancer stem or cancer initiating cells can be quiescent and in a resting state (G0 phase of the cell cycle). Cancer stem cells are characterized by self-renewal and multi-lineage differentiation capacity, but also by an intrinsic resistance to chemotherapeutics [99]. In contrast to most cytotoxic drugs, targeted protein toxins and apoptosis inducing molecules such as GrB do not rely on interference with DNA replication and cell division for their effects. Nevertheless, also apoptosis sensitivity is reduced in resting tumor cells, which may impact on the efficacy of immunotoxin-like molecules. So far, this aspect has not been addressed in the context of targeted GrB fusion proteins. In pilot experiments, we investigated sensitivity of human breast carcinoma cells for EGFR-specific GrBcs-T and ErbB2-specific GrB-5 fusion proteins depending on whether or not they were actively dividing. Resting EGFR-expressing MDA-MB468 cells were still killed by GrBcs-T, albeit requiring significantly

higher protein concentrations than dividing cells (Figure 4a, left panel; see Figure 3d for comparison). In contrast, GrB-5 had only minimal activity against resting ErbB2-expressing MDA-MB453 cells, but cytotoxicity was rapidly restored upon addition of serum to induce proliferation (Figure 4b, left panels). These results suggest that activity of GrB fusion proteins against resting cells may be affected by the type of the cell binding domain, and the activation state and internalization rate of the target receptor. While GrBcs-T contains functional TGFα and can activate EGFR for signaling and internalization in the absence of growth factors from serum, this is different for monovalent GrB-5, which does not induce ErbB2 dimerization, activation and internalization on its own (Figure 4, right panels). Further work and in-depth analysis will be required to elucidate killing of resting cells by GrB fusion proteins in detail, and extend these findings to molecules targeting other surface antigens.

Figure 4. Cytotoxic activity of targeted GrB proteins against resting cells. (**a**) EGFR-positive MDA-MB468 breast carcinoma cells were starved for 4 h by incubation in low serum (0.5% FCS), before treatment for 24 h with 1 µg/mL (25 nM) of EGFR-specific GrBcs-T protein in the presence of 50 µM chloroquine in medium also containing 0.5% FCS. (**b**) ErbB2-positive MDA-MB453 breast carcinoma cells were starved for 4 h by incubation in low serum (0.5% FCS), before treatment for 24 h with 1 µg/mL of ErbB2-specific GrB-5 protein in the presence of 50 µM chloroquine in medium also containing 0.5% FCS (left panel). Alternatively, cells were treated for 4 h with 1 µg/mL of GrB-5 protein in medium containing 0.5% FCS in the absence of chloroquine, washed, and incubated for another 20 h with complete medium (10% FCS) (middle panel). In (**a**) and (**b**) control cells were treated with untargeted GrBcs protein. The relative number of viable cells in comparison to controls treated without GrB proteins was determined in cell viability assays as described [38]. The activation state of EGFR and ErbB2 upon binding of GrB-T and GrB-5 proteins is schematically shown on the right.
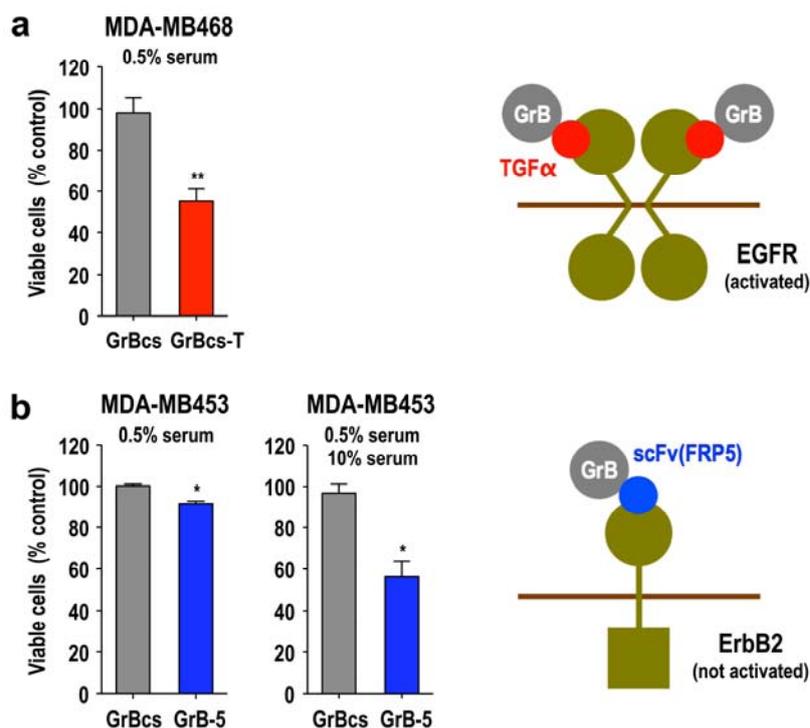
3.6. Local Delivery of Targeted Granzyme B by Genetically Modified Lymphocytes

T-lymphocytes and NK cells have the intrinsic potential to extravasate and to reach their targets in almost all body tissues. These cells are therefore ideally suited to invade tumors *in vivo*. Utilizing genetically modified immune cells for *in vivo* production of targeted GrB upon adoptive transfer could bypass the necessity for large-scale production of recombinant GrB fusion proteins, and may enhance availability of the therapeutic proteins in the tumor vicinity. Previously, Zhao *et al.* designed an ErbB2-specific GrB fusion protein for expression in T cells based on the structure of *Pseudomonas* exotoxin A. This molecule carried an N-terminal scFv antibody domain for cell recognition, fused *via* the ETA translocation domain to GrB as a C-terminal domain [95]. Established Jurkat T cells that expressed the targeted GrB displayed activity against ErbB2-expressing tumor cells *in vitro* and *in vivo*, which was attributed to the activity of the fusion protein. Unfortunately, the study did not address the question how a correctly processed and enzymatically active GrB fragment could be generated based on the chosen protein design, and whether a fusion protein carrying GrB at the N-terminus may have been more effective. Possibly, activation of GrB occurred by partial proteolytic degradation upon uptake into target cells. To investigate feasibility and consequences of expression of chimeric GrB fusion proteins reflecting the structure of current targeted GrB molecules, we genetically modified human NK cells by transduction with lentiviral vectors (Figure 5). NK cells possess all pathways required for processing, packaging, and triggered release of endogenous wildtype GrB, and may be readily employed for ectopic expression of retargeted GrB.

Figure 5. Intracellular localization of a GrB fusion protein expressed in natural killer cells. Established human NKL cells [100] were transduced with a lentiviral vector encoding human pre-pro-GrB genetically fused to enhanced green fluorescent protein (EGFP) (left), or a control vector encoding unfused EGFP (right). Intracellular localization of GrB-EGFP and EGFP proteins was analyzed by confocal laser scanning microscopy (upper panels). Bright field microscopic images of the same cells are shown in bottom panels.
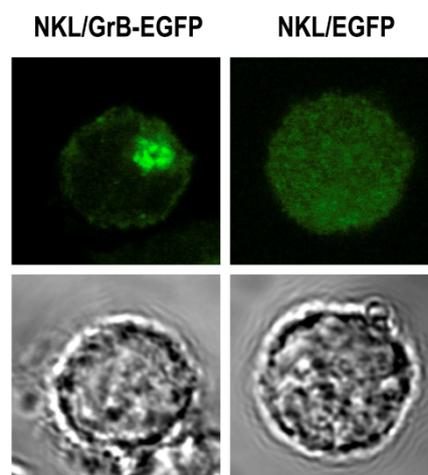
For initial analysis, we chose a model protein that carried full-length human GrB at the N-terminus, fused to a C-terminal enhanced green fluorescent protein (EGFP) domain as a marker. The GrB-EGFP protein was readily expressed by gene-modified NK cells, and routed to vesicular structures consistent

with cytotoxic granules. Likewise, GrB-5 and GrB-T molecules were successfully expressed, and shown to be released in correctly processed and enzymatically active form together with endogenous granzymes and perforin upon triggered activation of the respective NK cells [101]. Combined expression of targeted GrB and tumor-specific chimeric antigen receptors in NK cells may now allow selective enrichment of such cells within a tumor [7,102], and increased antitumoral activity through cooperation of GrB fusion proteins with natural cytotoxicity mechanisms.

4. Conclusions

Targeted GrB fusion proteins hold promise as tools for directed cancer therapy. They structurally and functionally reflect recombinant toxins, but employ an effector domain of human origin expected to result in low or no immunogenicity. GrB is an enzyme, enabling amplification of its cell-death inducing activity through cleavage and activation of cellular caspases, and induction of caspase-independent apoptosis pathways. Targeted GrB fusion proteins are relatively novel molecules, investigated for less than a decade. During this time, suitable protein designs have been developed based on the structure and activation mechanism of the parental molecule, which allows successful combination of GrB with heterologous cell binding domains. Importantly, the prototypic fusion proteins described so far fulfill the basic requirement of specificity with respect to cytotoxic activity against tumor cells *in vitro* and in animal models. Significant progress has been made towards the development of optimized GrB derivatives with enhanced bioavailability and antitumoral activity. Recent advances in understanding and circumventing intrinsic cell binding of GrB and susceptibility of the enzyme to inhibition by serpins will now allow the rational design of next-generation GrB derivatives that avoid sequestration by binding to non-target tissues, limit off-target effects, and overcome resistance mechanisms in tumor cells. While endosomal entrapment of targeted GrB remains a critical issue, protein delivery across the plasma membrane is a very general problem and under active investigation from many sides. Ongoing approaches to convert GrB into molecules of true therapeutic value will also continue to benefit from advances in the field of apoptosis research, providing details of this enzyme's mode of action, and its multiple functions in normal physiology and various disease states.

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