Published in final edited form as:

Mol Cancer Ther. 2013 June ; 12(6): 979–991. doi:10.1158/1535-7163.MCT-13-0002.

Construction and Characterization of Novel, Completely Human Serine Protease Therapeutics Targeting Her2/neu

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Abstract

Immunotoxins containing bacterial or plant toxins have shown promise in cancer-targeted therapy, but their long-term clinical use may be hampered by vascular leak syndrome and immunogenicity of the toxin. We incorporated human granzyme B (GrB) as an effector and generated completely human chimeric fusion proteins containing the humanized anti-Her2/neu single-chain antibody 4D5 (designated GrB/4D5). Introduction of a pH-sensitive fusogenic peptide (designated GrB/4D5/26) resulted in comparatively greater specific cytotoxicity although both constructs showed similar affinity to Her2/neu-positive tumor cells. Compared with GrB/4D5, GrB/4D5/26 demonstrated enhanced and long-lasting cellular uptake and improved delivery of GrB to the cytosol of target cells. Treatment with nanomolar concentrations of GrB/4D5/26 resulted in specific cell killing accompanied by induction of apoptosis mechanisms and efficient downregulation of the PI3K/Akt and Ras/ERK pathways. The endogenous presence of the GrB proteinase inhibitor 9 (PI-9) did not impact the response of cells to the fusion construct. Surprisingly, tumor cells resistant to Lapatinib or Herceptin, and cells expressing MDR-1 resistant to chemotherapeutic agents demonstrated no cross-resistance to the GrB-based fusion proteins. Administration (i.v., tail vein) of GrB/4D5/26 to mice bearing BT474 M1 breast tumors resulted in significant tumor suppression. In addition, tumor tissue excised from GrB/4D5/26-treated mice demonstrated excellent delivery of GrB to tumors and a dramatic induction of apoptosis compared with saline treatment. This study clearly demonstrated that the completely human, functionalized GrB construct can effectively target Her2/neu expressing cells and displays impressive *in vitro* and *in vivo* activity. This construct should be evaluated further for clinical use.

Keywords

granzyme B; Her2/neu; fusogenic peptide; Herceptin resistance; Lapatinib resistance; Multidrug resistance

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Research conducted, in part, by the Clayton Foundation for Research.

Conflict of interest

The authors declare no conflicts of interest.

Introduction

Bacterial and plant toxin-based immunotoxins have demonstrated remarkable potency and specificity, but a number of obstacles limit their clinical application (1, 2). The toxin component of these fusion proteins can elicit vascular damage leading to loss of vascular integrity (vascular leak syndrome, VLS) (3, 4). Immune responses to the toxins in patients also result in rapid clearance of subsequent courses of therapy (5, 6). Toxin immunogenicity is being addressed by engineering b-cell epitopes on the structure (7, 8) but these molecules may be difficult to humanize completely (9).

A new class of immunotoxins have recently been developed containing cytotoxic human proteins (10, 11). Granzyme B (GrB) is a well-known serine protease generated by cytotoxic lymphocytes to induce apoptotic cell death in target cells (12, 13). Studies by our group first demonstrated that various fusion constructs targeting tumor cells and tumor endothelium and containing GrB have impressive pro-apoptotic and cytotoxic activity (14–18). Several other groups since have confirmed these findings using other GrB-containing constructs (19, 20). Because endogenous GrB is present in plasma in both normal and pathological states, it is unlikely that this molecule would engender an immune response.

Dalken et al described a GrB/FRP5 fusion construct targeting Her2/neu and which displayed selective and rapid tumor cell killing *in vitro* (21). However, studies demonstrated that the fusion construct required the presence of the endosome-disrupting agent chloroquine for biological activity and suggested that an endosomal release process was necessary for Her2/neu targeted agents. Studies by Yang et al suggested that incorporation of a furin-sensitive linker into GrB-based fusion constructs may promote effective cytoplasmic delivery of an active GrB fragment into target cells (22). However, the recombinant molecule appears to be stable only when generated *in situ* by protein-expressing transfected cells.

We previously examined a series of anti-Her2/neu single-chain antibodies (scFv) fused to the recombinant gelonin (rGel) toxin, and clearly demonstrated that scFvs with intermediate affinity (K_d 10^{-11} M) as opposed to high affinity (K_d 10^{-12} M) were optimal carriers of protein toxins (23, 24). Therefore, we employed an intermediate-affinity, humanized anti-Her2/neu scFv designated 4D5 for the construction of our GrB-containing fusion constructs. In this study, we provided data on the cytotoxicity of Her2/neu targeted GrB fusions against a panel of human tumor cell lines and explored the mechanism of *in vitro* activity of these fusion constructs. Finally, we demonstrated the *in vivo* antitumor efficacy of the functionalized GrB chimeric protein against a human breast xenograft model.

Materials and Methods

Plasmid construction

The GrB/4D5/26, GrB/4D5, GrB/26, and GrB DNA constructs were generated by an overlapping polymerase chain reaction method. Illustrations of the constructs are shown in Fig. 1A. We designed a universal 218 linker (GSTSGSGKPGSGEGSTKG) incorporated between the individual components of GrB, 4D5, or peptide 26. Peptide 26

(AALEALAEALAEALAEALAEAAAA) was generated from the 29-residue amphipathic peptide without the three C-terminal amino acids, which was responsible for dimerization (25). All construct genes were cloned into the mammalian cell expression vector pSecTag (Life Technologies, Carlsbad, CA).

Expression, purification, and activation of GrB-based proteins

The GrB-based proteins were expressed in HEK 293T cells and purified by immobilized metal affinity chromatography essentially described in Supplemental Methods.

Determination of K_d by ELISA

The K_d value and specificity of GrB-based protein samples were evaluated by ELISA on Her2/neu ECD, Her2/neu-positive BT474 M1 cells, and Her2/neu-negative Me180 cells. Rabbit anti-c-myc antibody and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G were used as tracers in this assay, as described previously (24).

GrB activity assays

The enzymatic activity of the GrB component was determined in a continuous colorimetric assay using BAADT as a specific substrate (18). Assays consisted of commercial human GrB (Enzyme Systems Products, Livermore, CA) or GrB-based fusion proteins in BAADT at 25°C. The change in absorbance at 405 nm was measured on a Thermomax plate reader (Columbia, MD). Increases in sample absorbance were converted to enzymatic rates by using an extinction coefficient of $13,100 \text{ cm}^{-1} \text{ M}^{-1}$ at 405 nm. The specific activity of GrB-based fusion proteins was calculated using native GrB as the standard.

Internalization analysis

Immunofluorescence-based internalization studies were performed using BT474 M1 and Me180 cells. Cells were treated with 25 nM GrB/4D5/26 for 4 h and subjected to immunofluorescent staining with anti-GrB antibody (fluorescein isothiocyanate [FITC]-conjugated secondary antibody). Nuclei were counterstained with PI. Visualization of immunofluorescence was performed with a Zeiss LSM510 confocal laser scanning microscope Zeiss LSM510 (Carl Zeiss, Thornwood, NY).

***In vitro* cytotoxicity assays**

Log-phase cells were seeded ($\sim 5 \times 10^3$ /well) in 96-well plates and allowed to attach overnight. Cells were further incubated with various concentrations of GrB-based fusion proteins, GrB, or medium at 37°C for 72 h. Cell viability was determined using the crystal violet staining method followed by solubilization of the dye in Sorenson's buffer as described previously (23).

Annexin V/PI staining

The Annexin V/PI staining assay was used to quantitatively determine the percentage of cells undergoing apoptosis after exposure to GrB/4D5/26. Cells were seeded onto 6-well plates (5×10^5 cells per well) and incubated with 100 nM GrB/4D5/26 at 37°C for 24 or 48 h. Aliquots of cells were washed with phosphate-buffered saline and then incubated with

Annexin V-FITC antibody. PI solution was added at the end of the incubation, and the cells were analyzed immediately by flow cytometry.

Cytochrome c release assay and Bax translocation

After treatment with GrB/4D5 or GrB/4D5/26, cells were collected and resuspended with 0.5 mL of 1× cytosol extraction buffer mix (BioVision, Milpitas, CA) and then homogenized in an ice-cold glass homogenizer. The homogenate was centrifuged, and the supernatant was collected and labeled as the cytosolic fraction. The pellet was resuspended in 0.1 mL of mitochondrial extraction buffer and saved as the mitochondrial fraction. Aliquots of each cytosolic and mitochondrial fraction were analyzed by western blotting with antibodies recognizing cytochrome c and Bax (Santa Cruz Biotechnology, Santa Cruz, CA).

Assays for caspase activation and apoptosis

Western blot analysis was used to identify activation of caspases-3, and -9 as well as PARP cleavage. In addition, apoptosis was analyzed using antibodies recognizing Bcl-2 and BID (Santa Cruz Biotechnology).

Impact on cell signaling pathways

After treatment, cell lysates were analyzed by western blotting with antibodies recognizing Her2/neu and phosphorylated (p)-mTOR (S2448) (Cell Signaling Technology, Danvers, MA) as well as p-Her2/neu (Tyr877), p-Her2/neu (Tyr 1221/1222), EGF receptor, p-EGF receptor (Thr845), Her3, p-Her3 (Tyr1328), IGF1 receptor, p-IGF1 receptor (Tyr 1165/1166), ER, PR, Akt, p-Akt, ERK, p-ERK (Thr 177/Thr 160), PTEN, PI-3, and β -actin (all from Santa Cruz Biotechnology). Immunoreactive proteins were visualized by enhanced chemiluminescence.

In vivo efficacy studies

We used Balb/c nude mice to evaluate the *in vivo* effect of GrB/4D5/26 against aggressive breast cancer. Each mouse received a weekly subcutaneous injection of 3 mg/kg estradiol cypionate (26) starting 2 weeks prior to the injection of 1×10^7 BT474 M1 cells into the right flank. On the third day after cell inoculation, mice were injected intravenously (tail vein) either with saline or GrB/4D5/26 (44 mg/kg) five times per week for 2 weeks. Animals were monitored, and tumors were measured (calipers) for an additional 50 days.

Immunofluorescence analysis

Twenty-four hours after the final injection of saline or GrB/4D5/26, the mice were sacrificed and tumor samples were frozen immediately in preparation for section slides. The sample slides were incubated with either anti-GrB antibody (FITC-conjugated secondary antibody) or a terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) reaction mixture, as well as with an anti-mouse CD31 antibody (phycoerythrin-conjugated secondary antibody), and were further subjected to nuclear counterstaining with Hoechst 33342. Immunofluorescence observation was performed under a Zeiss Axioplan 2 imaging microscope (Carl Zeiss).

Results

Construction, expression, and purification of GrB-based fusions

The sequence of the humanized anti-Her2/neu scFv 4D5 was derived from the published Herceptin light- and heavy-chain variable domain sequences (27). Previous observations suggested that use of fusogenic peptides facilitates endosomal escape and delivery of large molecules into the cytosol (28, 29). We therefore incorporated the fusogenic peptide 26 (Fig. 1A). GrB-based fusions were generated by fusing GrB to 4D5 with (designated GrB/4D5/26) or without (designated GrB/4D5) the addition of pH-sensitive fusogenic peptide 26 (AALEALAEALAEALAEALAEAAAA) to the C-terminal of the construct.

Furthermore, GrB and GrB/26 were used as controls. All fusion proteins were expressed in human embryonic kidney cells (HEK 293T). Following purification, the final products migrated at the expected molecular weights, with a purity of >95% (Fig. 1B).

Analysis of binding affinity

The binding affinities (K_d values) of GrB/4D5/26 and GrB/4D5 were assessed by ELISA using purified Her2/neu extracellular domain (ECD), Her2/neu-positive BT474 M1 human breast cancer cells, and Her2/neu-negative Me180 human cervical cancer cells. Both fusions specifically bound to Her2/neu ECD and BT474 M1 cells but not to Me180 cells (Fig. 2A). The apparent K_d values were determined by calculating the concentration of fusion constructs that produced half-maximal specific binding. GrB/4D5 and GrB/4D5/26 demonstrated apparent K_d values of 0.329 nM and 0.469 nM, respectively, to Her2/neu ECD and 0.383 nM and 0.655 nM, respectively, to BT474 M1 cells. These results are in general agreement with the published K_d value for native Herceptin to the Her2/neu receptor (0.15 nM) (27).

Enzymatic assay of GrB-based fusions

To assess the biological activity of the GrB component of the fusions, we compared the ability of the constructs to cleave the substrate *N*- α -*t*-butoxycarbonyl-L-alanyl-L-alanyl-L-aspartyl-thiobenzylester (BAADT) with that of native, authentic GrB (Fig. 2B). GrB/4D5 and GrB/4D5/26 had intact GrB enzymatic activity (1.54×10^5 U/ μ mol and 1.57×10^5 U/ μ mol, respectively). These activities were comparable to that of the native GrB standard (1.19×10^5 U/ μ mol). Because the pro-GrB fusion constructs contain purification tags on the N-terminal end of GrB and render the molecule enzymatically inactive, these proteins were unable to cause hydrolysis of BAADT.

Cellular uptake and GrB delivery of fusion constructs

Immunofluorescence staining was performed with BT474 M1 and Me180 cells. The GrB moiety of both fusions was observed primarily in the cytosol after treatment with a fusion protein in BT474 M1 cells but not in Me180 cells (Fig. 2C), demonstrating that both constructs were efficient in cell binding and internalization after exposure to Her2/neu-positive cells. The internalization efficiency of the fusions was further examined by time-dependent western blot analysis of the GrB signal (full-length GrB fusion + free GrB) (Fig. 2D). Both constructs internalized rapidly into BT474 M1 cells within 30min. Compared

with GrB/4D5, GrB/4D5/26 displayed enhanced and long-lasting cell internalization. The intracellular delivery of GrB after endocytosis of GrB/4D5 or GrB/4D5/26 also was assessed by time-dependent western blotting (free GrB). We observed no GrB delivery by GrB/4D5 up to 48 h of treatment, whereas GrB delivery by GrB/4D5/26 was observed starting at approximately 4 h of treatment and presented a tremendously high level of free GrB up to 48 h (Fig. 2D).

***In vitro* cytotoxic effects of GrB-based fusions**

GrB-based fusions were then tested against a number of tumor cell lines. After 72 h exposure, GrB/4D5/26 demonstrated specific cytotoxicity to Her2/neu-positive cells, with IC₅₀ values of less than 100 nM (Table. 1), and GrB/4D5 demonstrated cytotoxic effects at somewhat higher doses (>200 nM). In addition, GrB/26 showed minimal cytotoxicity at doses >600 nM, but no significant activity of GrB itself was observed at doses up to 1.5 μM. When Her2/neu-positive MDA MB453 cells were pretreated with Herceptin (5 μM) for 6 h and then treated with GrB/4D5/26 for 72 h, the cytotoxicity of GrB/4D5/26 was reduced (Supplementary Fig. S1), thereby demonstrating a requirement for antigen binding of the GrB/4D5/26 construct.

We further investigated the expression levels of the endogenous proteinase inhibitor 9 (PI-9) in different tumor cells (Supplementary Fig. S2, Table 1). These studies failed to find an association between the response of cells to the cytotoxicity of the GrB constructs and the endogenous expression of PI-9. This may suggest that factors other than PI-9 may account for the observed differences in GrB/4D5/26 cytotoxicity to Her2/neu expressing target cells.

Cytotoxic effects of GrB/4D5/26 against cells resistant to Herceptin or Lapatinib

Acquired resistance to Herceptin or Lapatinib, can be mediated by concomitant upregulation of Her2/neu downstream signaling pathways or activation of signaling through the estrogen receptor (ER) pathway (30). In this study, we developed a model of Herceptin-resistant (HR) Lapatinib-resistant (LR) variants of BT474 M1 cells. Parental BT474 M1 cells were readily sensitive to both Herceptin (IC₅₀: 52.5 nM) and Lapatinib (IC₅₀: 34.7 nM) (Table. 2). HR cells demonstrated resistance to Herceptin (IC₅₀ 10.1 μM, F.R.: 192) but remained sensitive to Lapatinib (IC₅₀: 32.4 nM). LR cells showed resistance to high micromolar concentrations of both Herceptin (IC₅₀: 74.1 μM, F.R.: 1411) and Lapatinib (IC₅₀: 8.2 μM, F.R.: 237). As shown in Table 2, cells resistant to Herceptin demonstrated equivalent sensitivity to the GrB/4D5/26 construct (IC₅₀ ~30 nM for both HR and parental BT474 M1 cells respectively). For LR cells, the IC₅₀ was marginally increased (2-fold) compared to parental cells (66.1 nM vs. 32.9 nM).

We also demonstrated that addition of epidermal growth factor (EGF) or neuregulin-1 (NRG-1) growth factor, but not β-estradiol, to BT474 M1 parental cells can circumvent the cellular cytotoxic responses to Herceptin and Lapatinib. Seventy-two hours of pretreatment of BT474 M1 cells with 20 ng/mL EGF or 50 ng/mL NRG-1 resulted in a 400~500 fold increase in resistance to Herceptin and a 16-fold increase in resistance to Lapatinib (Table. 2). However, treatment of these resistant cells resulted in no cross-resistance to GrB/4D5/26 fusions compared with parental BT474 M1 cells.

A significant observation was that incubation of cells with GrB/4D5/26 in the presence of chloroquine did not improve cytotoxicity toward these cells (Supplementary Fig. S3). This finding demonstrated that the fusogenic peptide 26 efficiently releases GrB fusion proteins from intracellular vesicles, thereby allowing access to cytosolic GrB substrates and induction of apoptosis.

Mechanistic Studies of GrB/4D5/26 Cytotoxicity

We conducted a panel of experiments to assess the potential of GrB-based fusions to initiate the proteolytic cascade culminating in apoptosis of BT474 M1 parental, HR and LR cells.

Annexin V/Propidium Iodide (PI) Staining—Cells were incubated for 24 h or 48 h with 100 nM GrB/4D5/26, and apoptosis was detected via Annexin V/PI staining. GrB/4D5/26 induced apoptosis in BT474 M1 parental, HR and LR cells, as indicated by the reduced viable population combined with greater populations of early apoptosis (Fig. 3A). No apoptosis was induced by 100 nM GrB/4D5 in any of these cells (Supplementary Fig. S4). Her2/neu-negative Me180 cells were not affected by either construct.

Activation of Caspases—Caspase activation in BT474 M1 cells was detected by western blot analysis. Treatment with GrB/4D5/26 resulted to the cleavage of caspase 3, caspase 9 and PARP in all cells, but no activation occurred when cells were treated with GrB/4D5 (Fig. 3B). Compared with BT474 M1 parental and HR cells, the activations of caspase-9, caspase-3, and PARP were delayed in LR cells, which coincided with the observed decreased cytotoxic effects.

We further assessed the kinetics of PARP cleavage induced by GrB/4D5/26 on BT474 M1 parental, HR and LR cells, and found that cleavage occurred after 2 h of drug exposure for parental and HR cells but at 24 h for LR cells (Fig. 3C). In addition, in the presence of the pan-caspase inhibitor zVAD-fmk, PARP cleavage of GrB/4D5/26 was partially inhibited in all cells. This finding is in agreement with a mechanism relying on GrB activity for caspase-3 cleavage followed by PARP cleavage.

Impact on Mitochondrial Pathways—We detected cell death induced by GrB/4D5/26 via several mitochondrial-related pathways. In BT474 M1 parental, HR and LR cells, GrB/4D5/26 treatment activated BID and downregulated the anti-apoptotic Bcl-2 protein (Fig. 4A), and it triggered the release of cytochrome c from the mitochondria into the cytosol (Fig. 4B). Bax was normally present in both the cytosol and mitochondria of untreated cells. However, when the cells were treated with GrB/4D5/26, Bax was decreased in cytosol and increased in mitochondria (Fig. 4B). As previously described, treatment for 24 h with GrB/4D5/26 was shown to activate the mitochondrial pathway in both BT474 M1 parental and HR cells, but this activation was delayed in LR cells.

Effects of GrB fusions on Her- and ER-associated signaling pathways

We next examined the mechanistic effects of the constructs on Her- and ER-related signaling events in BT474 M1 parental cells and the resistant variants. As shown in Supplementary Fig. S5, cells resistant to Herceptin had enhanced Her family receptor

activity but reduced levels of progesterone receptor (PR) and PI-9. In contrast, in LR cells there was total downregulation of Her family receptor activity but higher levels of ER, PR, and PI-9.

Cells treated with GrB/4D5 or GrB/4D5/26 demonstrated the effects on these signaling pathways, corresponding to the cytotoxic results we observed (Fig. 5). Treatment with GrB/4D5/26 remarkably inhibited phosphorylation of Her2/neu and its downstream molecules Akt, mTOR and ERK, which are critical events in Her2/neu signaling cascade. In contrast, GrB/4D5 showed a comparatively reduced effect on these pathways. We observed a reduced ER level among all cells. Evidence from other researchers has demonstrated that upregulation of the ER pathway in ER- and Her2/neu-positive cell lines with Lapatinib creates an escape/survival pathway (30, 31), but GrB/4D5/26 appear to be able to inactivate all the signaling pathways in these cells. We also observed the delaying signaling effects of GrB/4D5/26 on LR cells compared with parental or HR cells, which was in agreement with the apoptotic cell death results observed for the LR cells. Notably, there was an increased mRNA and protein level of PI-9 in this resistant line but not in the parental or HR cells (Supplementary Fig. S5 and S6). Taken together, these results suggest that activation of the ER pathway upregulates the expression of PI-9 and this results in a slight inhibition of GrB/4D5/26 activity and a delay in apoptotic cell death compared to parental cells.

Our investigation suggests that the GrB/4D5/26 fusion is more cytotoxic than GrB/4D5 construct to Her2/neu-positive cells, even those that have acquired resistance to the traditional Her2/neu therapeutic agents Herceptin and Lapatinib. The cytotoxicity results coincide with the observed effects on signal transduction and monitoring these pathways may be useful as a monitor of drug efficacy.

Effects of GrB/4D5/26 on the MDR-1 expressing cells

Multidrug resistance (MDR) is a phenomenon, which results from various reasons. The most-characterized cause of MDR is the overexpression of a 170-kDa membrane glycoprotein known as P-glycoprotein (Pgp). To verify the effects of GrB-based fusions on the Her2/neu positive cells with MDR-1 expression, we generated the BT474 M1 MDR-1 cells by the transfection of plasmid pHaMDR1 to parental BT474 M1 cells. As Table 3 shown, compared with parental cells, BT474 M1 MDR-1 showed 209-fold resistance to Taxol, and 89-fold resistance to Vinblastin. However, we could not observe the cross-resistance of MDR-1 cells to GrB/4D5 and GrB/4D5/26 constructs. Therefore, GrB-based fusion constructs demonstrate a wide range cytotoxicity to target cells even those with acquired resistance to chemotherapeutic agents.

Antitumor activity of GrB/4D5/26 fusions in xenograft models

We evaluated the ability of the GrB/4D5/26 fusion construct to inhibit the growth of established BT474 M1 tumor xenografts in nude mice after systemic administration. Tumors were subcutaneously inoculated with BT474 M1 cells on day 0, and treatment was initiated on day 3. Treatment consisted of an intravenous injection for a total of 10 days with saline or 44 mg/kg GrB/4D5/26. Compared with saline, GrB/4D5/26 greatly slowed tumor progression over 50 days of observation (Fig. 6A). There were no obvious toxic effects of

GrB/4D5/26 on mice at this dose suggesting that the maximum tolerated dose at this schedule had not been reached.

Finally, we determined the localization of GrB/4D5/26 after administration to mice bearing BT474 M1 tumors. Immunofluorescence staining confirmed that GrB/4D5/26 localized quickly and specifically in tumor tissue (Fig. 6B). This observation further suggested that GrB/4D5/26 can effectively target tumor cells overexpressing Her2/neu *in vivo* and can demonstrate significant tumor growth-suppressive effects in the absence of observable toxicity. Staining of tumor tissue nuclei with TUNEL (Fig. 6C) clearly demonstrated that the tumor tissues displayed apoptotic nuclei in the GrB/4D5/26 treatment group. In addition, the intratumoral distribution of GrB/4D5/26 appeared to concentrate primarily in areas with extensive apoptotic response (compare Grb/4D5/26 distribution in Fig. 6B, with TUNEL staining in Fig. 6C).

Discussion

Antibody-based therapeutic agents are one of the fastest growing areas in the cancer therapeutic field. Two of the most promising strategies to enhance the antitumor activity of antibodies are antibody-drug conjugates (ADCs) and immunotoxins. There are now a number of clinically-effective ADCs demonstrating remarkable activity (32, 33) and many of these constructs were driven by the impressive success of the Trastuzumab-DM1 (T-DM1) conjugate. On the other hand, there are limitations with antibody-drug conjugates, such as facile aggregation, off-target toxicity and potential resistance from MDR-positive cells (34, 35).

The use of immunotoxins has always been a promising alternative strategy for cancer targeted therapy, but the potential for antigenicity precluding prolonged treatment cycles (36, 37) and the unresolved issue of VLS have limited interest in the overall development of this area. Currently, a number of groups have focused on the development of either de-immunized or fully human toxin type molecules as payloads (1, 10, 38, 39).

In this study, we constructed novel human anti-Her2/neu immunotoxins containing human GrB as an apoptosis-inducing effector. GrB appears to be an ideal payload for targeted therapeutic applications in part because this serine protease exerts a multi-modal and well-known mechanism of cytotoxic action (40, 41). Of interest, this study found that inhibitors of caspase activation had little impact on the overall cytotoxicity of the construct attesting to the presence of multiple, redundant, pro-apoptotic pathways activated by this molecule and suggesting that emergence of resistance to this class of agents may be difficult from a biological perspective.

In a nominal cytotoxic process, GrB penetrates directly into target cells through the action of perforin-mediated transmembrane pores. This process bypasses the lysosomal compartment allowing GrB accessibility directly to cytosolic substrates (42). Internalization of GrB through antibody-mediated events provides tumor cell specificity but in the case of Her2/neu, internalization likely proceeds through the lysosomal compartment. For our optimal construct, we included a 26-residue, fusogenic peptide. At neutral pH, this peptide has a

random configuration, but under acidic lysosomal conditions, this peptide assumes an amphipathic helix thereby disrupting the lysosomal membrane allowing improved delivery of the fusion construct into the cytosol (25). Studies on target cells demonstrate that the incorporation of the fusogenic peptide dramatically augments intracellular delivery and biological activity of the GrB-containing construct.

Previous studies determined that bacterially-expressed GrB fusion proteins targeting cell surface antigens other than Her2/neu have excellent cytotoxic activity (17, 43). The folding of the protein and the absence of carbohydrates on the final product could impact their biological activity compared to proteins produced in mammalian cells. We expressed the GrB/4D5 fusion in bacterial cells and the purified protein showed similar cytotoxicity to that produced in mammalian cells (data not shown). This suggests that intracellular routing of chimeric GrB molecules depends on the type of target receptor and was not affected by the presence of carbohydrates on the chimeric molecule.

Dalken et al described the construction and biological activity of Her2/neu targeted fusion construct GrB/FRP5 (21). This agent was shown to be specifically cytotoxic to target cells with IC₅₀ values in the subnanomolar range but the cytotoxic activity was dependent on the addition of the lysomotropic agent chloroquine. In the absence of chloroquine, the cytotoxicity of the agent was reduced 10–300 fold thus suggesting that the construct may have been primarily sequestered into the lysosomal compartment and not available to activate apoptotic cascade mechanisms. The incorporation of the fusogenic, pH-sensitive peptide 26 in our construct appeared to circumvent the need for a lysomotropic agent to augment the activity of GrB fusion and it provided a greater concentration of target protein in the cell. The use of this peptide did not appear to impact the enzymatic activity of the GrB component nor did it influence the binding activity of the 4D5 to Her2/neu receptor. Finally, the presence of the 26 component did not appear to augment the nonspecific toxicity of the construct against antigen-negative cells *in vitro* nor did it increase the apparent toxicity of the construct during i.v. administration in our xenograft studies.

The antitumor efficacy studies demonstrated that administration of GrB/4D5/26 to mice bearing well-developed BT474 M1 tumors was effective at total doses of 44 mg/kg. This dose translates to a total dose of ~140 mg/m². In comparison, studies of the ADC T-DM1 conjugate in the BT474 tumor model employed doses of 3 to 15 mg/kg (~ 10 to 50 mg/m²) (44), which is lower than the doses for the GrB construct. However, our study demonstrated that there were no deaths or weight loss during the treatment schedule suggesting the safety and tolerability of GrB-based agents. Although we did not observe complete regression of tumor xenografts, alternative schedules or higher doses need to be examined.

The Her2/neu-targeted therapeutic agents Herceptin and Lapatinib have significantly improved outcomes in cancer treatment, but their use is limited by resistance and tolerability issues (45, 46). Evaluating the cytotoxicity of functionalized GrB fusions to HR or LR cells represents an important step. Our results suggested that GrB/4D5/26 inhibits the proliferation and survival of resistant cells as a result of caspase-dependent and independent apoptotic effects. In addition, our investigation into cellular signaling indicated that GrB/

4D5/26 could efficiently downregulate the phosphorylation of Her2/neu and ER family members, resulting in inhibition of both PI3K/Akt and Ras/ERK pathways.

The development of multidrug resistance mechanisms affecting groups of therapeutic agents has been shown to be a central problem resulting in reduced response in cancer treatment (47). The emergence of MDR phenotypes could also be a serious problem for the application of ADCs (48, 49). Studies by Kovtun et al (50) reported that ADCs utilizing PEG-based hydrophilic linkers showed higher retention in MDR-1 expressing cells than similar conjugates made with the nonpolar linker SMCC which is found in T-DM1. Therefore, the emergence of MDR may provide cross-resistance to T-DM1, due to the efflux of free drug upon intracellular release from the antibody. In contrast, the current studies demonstrate that expression of MDR does not provide cross-resistance to GrB-based fusion constructs and this appears to be a significant advantage over the conventional ADC approach.

The only intracellular inhibitor of human GrB is the nucleocytoplasmic serpin, PI-9. PI-9 has been found to be endogenously expressed in lymphocytes, dendritic cells and mast cells, for self-protection against GrB-mediated apoptosis (40, 41). This may suggest that the endogenous PI-9 level in cancer cells could inhibit the GrB activity of our target molecules. However, our studies did not show any relationship between PI-9 levels and cell sensitivity to GrB/4D5/26 in Her2/neu positive cells.

We examined GrB sensitivity against Lapatinib-resistant cells and found these cells showed a slight (2-fold) increase in the GrB/4D5/26 IC₅₀. This coincided with an upregulation of PI-9 leading to a delay in apoptosis. This upregulation may be the indirect result of ER pathway changes induced by Lapatinib resistance. Therefore, in the cell lines that are both ER- and Her2- positive, for which upregulation of the ER pathway may occur as an escape pathway, the endogenous GrB inhibitor PI-9 could be upregulated to inhibit GrB activity.

In conclusion, we demonstrated that a novel Her2/neu targeted functionalized GrB fusion constructs employing the pH-sensitive fusogenic peptide 26 as an endosomolytic domain efficiently promotes the release of GrB into the cytoplasm, resulting in apoptotic cell death in Her2/neu-positive cancer cells. This fusogenic peptide could be useful for studying GrB-induced apoptosis without the requirement of perforin or chloroquine. In addition, our studies demonstrate that tumor cells highly resistant to either Lapatinib or Herceptin and the cells with MDR-1 expression resistant to chemotherapeutic agents were not cross-resistant to the GrB-based fusion protein. Although the induction of PI-9 expression in LR cells delayed the apoptotic cytotoxicity of GrB/4D5/26, this agent had an IC₅₀ value that was only 2-fold higher than parental cells, despite the fact that resistant cells were more than 200-fold resistant to Lapatinib.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research work was conducted, in part, by the Clayton Foundation for Research.

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Figure 1. Construction and preparation of GrB-based fusion constructs. A. Schematic diagram of GrB fusion constructs containing scFv 4D5 and GrB without or with fusogenic peptide 26. B. Purified GrB-based chimeric proteins were analyzed by SDS-PAGE under non-reducing conditions.

Figure 2.

Characterization and comparison of GrB-based fusion proteins. A. K_d of the fusion constructs to Her2/neu ECD, Her2/neu-positive BT474 M1 cells, and Her2/neu-negative Me180 cells by ELISA. B. Enzymatic activity of GrB moiety of fusion proteins compared with native GrB. C. Internalization analysis of BT474 M1 cells and Me180 cells after 4 h of treatment with 25 nM functionalized GrB fusions. Cells were subjected to immunofluorescent staining with anti-GrB antibody (FITC-conjugated secondary), with PI nuclear counterstaining. D. Western blot analysis of intracellular behavior of 25 nM GrB fusion constructs in BT474 M1 cells.

Figure 3.

Effects of GrB-based fusions on apoptotic pathways of BT474 M1 parental, HR and LR cells. A. Detection of apoptosis of GrB/4D5/26 by Annexin V/PI staining assay. Me180 cells served as a Her2/neu-negative control group. B. Western blot analysis of cleavage and activation of caspases-3 and -9 as well as PARP by GrB-based fusion constructs. C. Western blot investigation of apoptosis kinetics and specificity of GrB/4D5/26. Cells were treated with GrB/4D5/26 for up to 24 h with or without 100 μ M zVAD-fmk for 24 h in parental or HR cells and for up to 48 h in LR cells.

Figure 4.

Effects of GrB fusion constructs on mitochondrial pathway in BT474 M1 parental, HR and LR cells. A. Effects of GrB-based fusion proteins on the upstream components Bcl-2 and BID in mitochondrial pathway. B. Effects of the fusion constructs on cytochrome c release and Bax translocation.

Figure 5.

Western blot analyses of the effects of GrB/4D5 and GrB/4D5/26 in BT474 M1 parental, HR and LR cells on Her2/neu and ER signaling pathways. Cells were treated with 100 nM functionalized GrB constructs for 24 or 48 h, and total cell lysates were quantified and further measured by western blot analysis of pHer2/neu, pAkt, pmTOR, pERK, ER, PR and PI-9 level.

Figure 6.

Tumor apoptotic activity of GrB/4D5/26 in BT474 M1 tumor xenografts. A. Mice with BT474 M1 flank tumors were intravenously injected with saline or 44 mg/kg GrB/4D5/26 at the indicated times (arrows). Mean tumor volume was calculated as $W \times L \times H$ as measured with digital calipers. B. Immunofluorescence staining of tumor samples after i.v. injection of saline and GrB/4D5/26. 24h after injection, animal was sacrificed and frozen tumor sections were prepared and detected by anti-GrB antibody (green) and anti-mouse CD31 antibody (red). Hoechst 33342 (blue) was used for DNA staining. C. Apoptosis detection in tumor tissue by TUNEL assay.

Table 1

Comparative IC₅₀ values of GrB-baed fusion constructs against various types of tumor cell lines

Cell line	Type	Her2/neu level	PI-9 level	IC ₅₀ (nM)			
				GrB/4D5/26	GrB/4D5	GrB/26	GrB
BT474 M1	Breast	****	*	29.3	253.3	905.5	> 1500.0
Calu3	Breast	****	*****	40.5	242.4	863.0	> 1500.0
NCI-N87	Gastric	****	*	90.4	629.0	1106.0	> 1500.0
MDA MB453	Lung	***	*	56.8	436.0	694.2	> 1500.0
eB-1	Breast	**	-	93.1	551.3	1134.5	> 1500.0
MDA MB435	Breast	*	-	> 500.0	> 750.0	1031	> 1500.0
Me180	Cervical	*	*	> 500.0	> 750.0	> 1500.0	> 1500.0

Table 2

Cytotoxic effects of Her2/neu-targeted therapeutic agents on IC₅₀ values in BT474 M1 cells and resistant variants

Agent	IC ₅₀ (nM) with (Fold Resistance) *					
	BT474 M1	BT474 M1 HR	BT474 M1 LR	BT474 M1 + EGF**	BT474 M1 + NRG-1***	BT474 M1 + β -estradiol****
Herceptin	52.5 (1)	10100.5 (192)	74100.0 (1411)	26305.0 (501)	23033.0 (439)	74.1 (1)
Lapatinib	34.7 (1)	32.4 (1)	8225.0 (237)	543.0 (16)	547.1 (16)	33.9 (1)
GrB/4D5/26	32.9 (1)	26.8 (1)	66.1 (2)	21.7 (1)	18.1 (1)	31.3 (1)

* Fold Resistance (F.R.) represents IC₅₀ of agent on BT474 M1 resistant variants/that on BT474 M1 parental cells.
Cells were pretreated with **20 ng/mL EGF, ***50 ng/mL NRG-1, or ****10 ng/mL beta-estradiol for 72 h before drug treatment.

Table 3

Cytotoxicity of Chemical agents and GrB-based fusions on MDR-1 expressing cells.

	BT474 M1	BT474 M1 MDR-1	Fold Resistance [*]
	IC50 (nM)		
Taxol	5.2	1047.3	209
Vinblastin	1.3	105.1	89
GrB/4D5	311.8	318.9	1
GrB/4D5/26	34.1	35.5	1

* Fold Resistance (F.R.) represents IC₅₀ of agent on BT474 M1 MDR-1 cells/that on BT474 M1 parental cells.