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 showed 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 cytotoxicity, induction of apoptosis, and efficient downregulation of PI3K/Akt and Ras/ERK pathways. The endogenous presence of the GrB proteinase inhibitor 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 showed no cross-resistance to the GrB-based fusion proteins. Administration (intravenous, 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 showed excellent delivery of GrB to tumors and a dramatic induction of apoptosis compared with saline treatment. This study clearly showed 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.

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Introduction

Bacterial and plant toxin-based immunotoxins have shown 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; refs. 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). Our group first showed that various fusion constructs targeting tumor cells and tumor endothelium and containing GrB have impressive proapoptotic and cytotoxic activity (14–18). Several other groups have since confirmed these findings using other GrB-containing constructs (19, 20). Because endogenous GrB is present in plasma in both normal and pathologic states, it is unlikely that this molecule would engender an immune response.

Dalken and colleagues have described a GrB/FRP5 fusion construct targeting Her2/neu, which displayed selective and rapid tumor cell killing in vitro (21). However, studies have shown that the fusion construct required the presence of the endosome-disrupting agent chloroquine for biologic activity and suggested that an endosomal release process may be necessary for Her2/neu-targeted agents. Studies by Wang and colleagues 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 seemed 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 showed that scFvs with intermediate affinity ($K_d \approx 10^{-11} \text{ mol/L}$) as opposed to high affinity ($K_d \approx 10^{-12} \text{ mol/L}$) were optimal carriers of protein toxins (23, 24). Therefore, we used 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 showed the in vivo antitumor efficacy of the functionalized GrB chimeric protein against a human breast xenograft model.

Materials and Methods

Cell lines

The cell lines BT474 M1, NCI-N87, Calu3, MDA MB435, and Me180 were all obtained from American Type Culture Collection (ATCC). The human breast cancer cell lines MDA MB453 and eB-1 were generously supplied by Drs. Zhen Fan and Dihua Yu (MD Anderson Cancer Center, Houston, TX). The BT474 M1 Herceptin- and lapatinib-resistant cells were derived from BT474 M1 cells after a 12-month selection in the continuous presence of 1 μmol/L Herceptin or 1.5 μmol/L lapatinib. BT474 M1 MDR-1 cells were generated by the transfection of plasmid pHaMDR1 to parental BT474 M1 cells. The HEK 293T cell line was supplied by Dr. Bryant G. Darnay (MD Anderson Cancer Center). All cell lines were maintained in Dulbecco's Modified Eagle Medium or RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mmol/L L-glutamine, and 1 mmol/L antibiotics.

Cell lines were validated by short tandem repeat (STR) DNA fingerprinting using the AmpF/STR Identifier Kit according to the manufacturer's instructions (Applied Biosystems). The STR profiles were compared with known ATCC fingerprints (ATCC.org), to the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808 (http://bioinformatics.istge.it/clima/; Nucleic Acids Research 37:D925-D932 PMCID: PMC2686526) and to the MD Anderson fingerprint database. The STR profiles matched known DNA fingerprints or were unique.

Plasmid construction

The GrB/4DS/26, GrB/4DS, GrB/26, and GrB DNA constructs were generated by an overlapping PCR method. Illustrations of the constructs are shown in Fig. 1A. We designed a universal 218 linker (GSTSGSGKPGSGEGSTKG) incorporated between the individual components of GrB, 4DS, or peptide 26. Peptide 26 (AALAEALAEALEALAEALAEAA) was generated from the 29-residue amphipathic peptide without the 3 C-terminal amino acids, which are responsible for dimerization (25). All construct genes were cloned into the mammalian cell expression vector pSecTag (Life Technologies).

Expression, purification, and activation of GrB-based proteins

The GrB-based proteins were expressed in HEK 293T host cells and purified by immobilized metal affinity chromatography as described in Supplementary 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 extracellular domain (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 N-$\alpha$-$\beta$-butoxycarbonyl-$\alpha$-alanin-$\alpha$-$\beta$-alanin-$\alpha$-$\beta$-aspartyl-thienobenzylester (BAADT) as a specific substrate (18). Assays consisted of commercial human GrB (Enzyme Systems...
Assays for caspase activation and apoptosis

Biotechnology). Bodies recognizing cytochrome c and Bax (Santa Cruz Biotechnology). Aliquots of each cytosolic and mitochondrial fraction were analyzed by Western blotting with anti-cytosolic extraction buffer mix (BioVision) and then homogenized 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 (AAALEALAEALEALAEALAEAAAA) 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

Internalization analysis

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

In vitro cytotoxicity assays

Log-phase cells were seeded (~5 × 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 hours. Cell viability was determined using the crystal violet staining method 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/well) and incubated with 100 nmol/L GrB/4D5/26 at 37°C for 24 or 48 hours. Aliquots of cells were washed with PBS 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) 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).

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) as well as p-Her2/neu (Tyr877), p-Her2/neu (Tyr1165/1166), EGF receptor (ER), progestrone receptor (PR), Akt, p-Akt, extracellular signal–regulated kinase (ERK), p-ERK (Thr177/Thr180), IGF1 receptor, p-IGF1 receptor (Tyr1165/1166), estrogen receptor (ER), progesterone receptor (PR), Akt, p-Akt, extracellular signal–regulated kinase (ERK), p-ERK (Thr 177/Thr 180), PTEN, proteinase inhibitor 9 (PI-9), 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 before the injection of 1 × 10^5 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) 5 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, 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 dUTP 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 conducted 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 cytol (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 (AAALEALAEALEALAEALAEAAAA) 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

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293T). Following purification, the final products migrated at the expected molecular weights, with a purity of more than 95% (Fig. 1B).

**Analysis of binding affinity**

The binding affinities (Kₘ values) of GrB/4D5/26 and GrB/4D5 were assessed by ELISA using purified Her2/neu 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 Kₘ values were determined by calculating the concentration of fusion constructs that produced half-maximal specific binding. GrB/4D5 and GrB/4D5/26 showed apparent Kₘ values of 0.329 and 0.469 nmol/L, respectively, to Her2/neu ECD and 0.383 and 0.655 nmol/L, respectively, to BT474 M1 cells. These results are in general agreement with the published Kₘ value for native Herceptin to the Her2/neu receptor (0.15 nmol/L; ref. 27).

**Enzymatic assay of GrB-based fusions**

To assess the biologic activity of the GrB component of the fusions, we compared the ability of the constructs to cleave the substrate BAADT with that of native, authentic GrB (Fig. 2B). GrB/4D5 and GrB/4D5/26 had intact GrB enzymatic activity (1.54 × 10⁵ and 1.57 × 10⁵ U/μmol, respectively). These activities were comparable with that of the native GrB standard (1.19 × 10⁵ U/μmol). 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 conducted 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), showing that both constructs were efficient in cell binding and internalization after exposure to Her2/neu–positive cells. The internalization efficiency of the

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**Figure 2.** Characterization and comparison of GrB-based fusion proteins. A, Kₘ 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 hours of treatment with 25 nmol/L 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 nmol/L GrB fusion constructs in BT474 M1 cells.
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 30 minutes. 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 hours of treatment, whereas GrB delivery by GrB/4D5/26 was observed starting at approximately 4 hours of treatment and presented a tremendously high level of free GrB up to 48 hours (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 hours exposure, GrB/4D5/26 showed specific cytotoxicity to Her2/neu–positive cells, with IC50 values of less than 100 nmol/L (Table 1), and GrB/4D5 showed cytotoxic effects at somewhat higher doses (>200 nmol/L). In addition, GrB/26 showed minimal cytotoxicity at doses more than 600 nmol/L, but no significant activity of GrB itself was observed at doses up to 1.5 μmol/L. When Her2/neu–positive MDA MB453 cells were pretreated with Herceptin (5 μmol/L) for 6 hours and then treated with GrB/4D5/26 for 72 hours, the cytotoxicity of GrB/4D5/26 was reduced (Supplementary Fig. S1), thereby showing a requirement for antigen binding of the GrB/4D5/26 construct.

We further investigated the expression levels of the endogenous PI-9 in different tumor cells (Supplementary Fig. S2 and 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 ER pathway (30). In this study, we developed a model of Herceptin- and lapatinib-resistant variants of BT474 M1 cells. Parental BT474 M1 cells were readily sensitive to both Herceptin (IC50, 52.5 nmol/L) and lapatinib (IC50, 34.7 nmol/L; Table 2). Herceptin-resistant cells showed resistance to Herceptin [IC50, 10.1 μmol/L; fold resistance (FR), 192] but remained sensitive to lapatinib (IC50, 34.7 nmol/L). Lapatinib-resistant cells showed resistance to high micromolar concentrations of both Herceptin (IC50, 74.1 μmol/L; FR, 1411) and lapatinib (IC50, 8.2 μmol/L; FR, 237). As shown in Table 2, cells

Table 1. Comparative IC50 values of GrB-based fusion constructs against various types of tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type</th>
<th>Her2/neu level</th>
<th>PI-9 level</th>
<th>IC50 (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GrB/4D5/26</td>
</tr>
<tr>
<td>BT474 M1</td>
<td>Breast</td>
<td>++++</td>
<td>+</td>
<td>29.3</td>
</tr>
<tr>
<td>Calu3</td>
<td>Lung</td>
<td>++++</td>
<td>+++++++++</td>
<td>40.5</td>
</tr>
<tr>
<td>NCI-N87</td>
<td>Gastric</td>
<td>++++</td>
<td>+</td>
<td>90.4</td>
</tr>
<tr>
<td>MDA MB453</td>
<td>Breast</td>
<td>++++</td>
<td>+</td>
<td>56.8</td>
</tr>
<tr>
<td>eB-1</td>
<td>Breast</td>
<td>++</td>
<td>–</td>
<td>93.1</td>
</tr>
<tr>
<td>MDA MB435</td>
<td>Breast</td>
<td>+</td>
<td>–</td>
<td>&gt;500.0</td>
</tr>
<tr>
<td>Me180</td>
<td>Cervical</td>
<td>+</td>
<td>+</td>
<td>&gt;500.0</td>
</tr>
</tbody>
</table>

NOTE: +, indicates the Her2/neu expression level in different cancer cells.

Table 2. Cytotoxic effects of Her2/neu–targeted therapeutic agents on IC50 values in BT474 M1 cells and resistant variants

<table>
<thead>
<tr>
<th>Agent</th>
<th>BT474 M1 Herceptin-resistant</th>
<th>BT474 M1 lapatinib-resistant</th>
<th>BT474 M1 + EGFb</th>
<th>BT474 M1 + NRG-1c</th>
<th>BT474 M1 + β-estradiold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herceptin</td>
<td>52.5 (1)</td>
<td>10,100.5 (192)</td>
<td>74,100.0 (1,411)</td>
<td>26,305.0 (501)</td>
<td>23,033.0 (439)</td>
</tr>
<tr>
<td>Lapatinib</td>
<td>34.7 (1)</td>
<td>32.4 (1)</td>
<td>8,225.0 (237)</td>
<td>543.0 (16)</td>
<td>547.1 (16)</td>
</tr>
<tr>
<td>GrB/4D5/26</td>
<td>32.9 (1)</td>
<td>26.8 (1)</td>
<td>66.1 (2)</td>
<td>21.7 (1)</td>
<td>18.1 (1)</td>
</tr>
</tbody>
</table>

aFR represents IC50 of agent on BT474 M1–resistant variants/that on BT474 M1 parental cells.

b,c,dCells were pretreated with b20 ng/mL EGF, c50 ng/mL NRG-1, or d10 ng/mL β-estradiol for 72 hours before drug treatment.
resistant to Herceptin showed equivalent sensitivity to the GrB/4D5/26 construct (IC50 ~30 nmol/L for both Herceptin-resistant and parental BT474 M1 cells, respectively). For lapatinib-resistant cells, the IC50 was marginally increased (2-fold) compared with parental cells (66.1 vs. 32.9 nmol/L).

We also showed that addition of 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- to 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 showed 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, Herceptin-, and lapatinib-resistant cells.

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

![Figure 3](image-url)

Figure 3. Effects of GrB-based fusions on apoptotic pathways of BT474 M1 parental, Herceptin-, and lapatinib-resistant 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 analysis of apoptosis kinetics and specificity of GrB/4D5/26. Cells were treated with GrB/4D5/26 for up to 24 hours with or without 100 μmol/L zVAD-fmk for 24 hours in parental or Herceptin-resistant cells and for up to 48 hours in lapatinib-resistant cells.
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, -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 Herceptin-resistant cells, the activations of caspase-9, -3, and PARP were delayed in lapatinib-resistant 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, Herceptin- and lapatinib-resistant cells, and found that cleavage occurred after 2 hours of drug exposure for parental and Herceptin-resistant cells but at 24 hours for lapatinib-resistant 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, Herceptin- and lapatinib-resistant cells, GrB/4D5/26 treatment activated BID and downregulated the antiapoptotic 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 hours with GrB/4D5/26 was
shown to activate the mitochondrial pathway in both BT474 M1 parental and Herceptin-resistant cells, but this activation was delayed in lapatinib-resistant 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 PR and PI-9. In contrast, in lapatinib-resistant 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 showed 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 shown 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 seem to be able to inactivate all the signaling pathways in these cells. We also observed the delaying signaling effects of GrB/4D5/26 on lapatinib-resistant cells compared with parental or Herceptin-resistant cells, which was in agreement with the apoptotic cell death results observed for the lapatinib-resistant cells. Notably, there was an increased mRNA and protein level of PI-9 in this resistant line but not in the parental or Herceptin-resistant cells (Supplementary Figs. 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 with 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 shown in Table 3, 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 show 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 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 show significant tumor growth-suppressive effects in the absence of observable toxicity. Staining of tumor tissue nuclei with TUNEL (Fig. 6C) clearly showed that the tumor tissues displayed apoptotic nuclei in the GrB/4D5/26 treatment group. In addition, the intratumoral distribution of GrB/4D5/26 seemed 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 (ADC) and immunotoxins. There are now a number of clinically effective ADCs showing 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 ADCs, 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 deimmunized 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 seems to be an ideal payload for targeted therapeutic applications in part because this serine protease exerts a multimodal 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, proapoptotic pathways activated by this molecule and suggesting that emergence of resistance to this class of agents may be difficult from a biologic 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).

**Table 3. Cytotoxicity of chemical agents and GrB-based fusions on MDR-1-expressing cells**

<table>
<thead>
<tr>
<th>Agent</th>
<th>IC50 (nmol/L) BT474 M1</th>
<th>IC50 (nmol/L) BT474 M1 MDR-1</th>
<th>FRa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taxol</td>
<td>5.2</td>
<td>1,047.3</td>
<td>209</td>
</tr>
<tr>
<td>Vinblastin</td>
<td>1.3</td>
<td>105.1</td>
<td>89</td>
</tr>
<tr>
<td>GrB/4D5</td>
<td>311.8</td>
<td>318.9</td>
<td>1</td>
</tr>
<tr>
<td>GrB/4D5/26</td>
<td>34.1</td>
<td>35.5</td>
<td>1</td>
</tr>
</tbody>
</table>

aFR represents IC50 of agent on BT474 M1 MDR-1 cells/that on BT474 M1 parental cells.
the fusogenic peptide dramatically augments intracellular delivery and biologic 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 biologic activity compared with 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 and colleagues described the construction and biologic 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- to 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 seemed 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 seem to impact

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 intravenous injection of saline and GrB/4D5/26. Twenty-four hours 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.
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 seem 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 intravenous administration in our xenograft studies.

The antitumor efficacy studies showed 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 approximately 140 mg/m². In comparison, studies of the ADC T–DM1 conjugate in the BT474 tumor model used doses of 3 to 15 mg/kg (~10–50 mg/m²; ref. 44), which is lower than the doses for the GrB construct. However, our study showed 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 Herceptin- or lapatinib-resistant 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 MDR 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 and colleagues (50) reported hydrophilic linkers showed higher retention in MDR-1–expressing cells than similar conjugates made with the cyclohexane-1-carboxylate (SMCC), which is found in T–DM1. Therefore, the emergence of MDR may provide cross-resistance to GrB/4D5/26, this agent had an IC₅₀ value that was only 2-fold higher than parental cells, despite the fact that 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 showed that a novel Her2/neu–targeted functionalized GrB fusion constructs using 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 show 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 lapatinib-resistant 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Study supervision: L.H. Cheung, M.G. Rosenblum

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References


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

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