Chapter 4

SPECIFIC, PRO-POPTOTIC CELL-SIGNALING: DESIGN OF NOVEL, RECOMBINANT TARGETED ANTITUMOR AGENTS OPERATING EXCLUSIVELY THROUGH MODULATION OF CELLULAR APOPTOTIC EVENTS

Novel, Targeted Pro-Apoptotic Therapeutic Agents

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Abstract: Normal cellular homeostasis employs a delicate balance between pro-apoptotic (cell death) and anti-apoptotic (growth-stimulatory) forces. Unrestricted/unregulated cell growth, which occurs in neoplasia frequently, accompanies an imbalance in these forces and frequently the apoptotic pathways are disrupted leading to increased metastatic spread, resistance to chemotherapy and radiotherapy. Pro-apoptotic enzymes such as the serine protease Granzyme B can activate the pro-apoptotic cascade within tumor cells through multiple, independent mechanisms. We have generated novel cell-targeting fusion constructs containing enzymatically active GrB and have demonstrated that these constructs are highly cytotoxic to target cells. Apoptotic cascades can be maximally activated within 4 hr of treatment and these agents are synergistic with chemotherapeutic agents. We propose that these types of signaling agents, which operate exclusively through activation of pro-apoptotic mechanisms, may describe a new class of targeted therapeutic agents with unique biological properties.

Key words: apoptosis, caspases, fusion protein, Granzyme B, targeted therapy

Cellular homeostasis requires a balance between cell proliferation and programmed cell death (apoptosis). Apoptosis is defined as genetically programmed autonomous cell death, and it occurs in normal healthy tissues

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such as breast cells at varying rates during the estrus cycle in response to changes in hormone levels [1]. Apoptosis is also regulated by non-hormonal signals. Changes in the genetics of apoptotic regulatory mechanisms may result in an increase in cell numbers, as well as the preservation of genetically altered cells, which begins the process of tumorigenesis [2]. Studies on apoptosis have led to the identification of a central tripartite death switch i.e. apoptosome consisting of Apaf-1, Apaf-2 and Apaf-3. The caspases, a family of cysteine-dependent aspartate directed-proteases, constitute the central executioners of apoptosis. Much of the attention on programmed cell death is focused on caspases, however, apoptosis can still occur even when the caspase cascade is blocked, revealing the existence of caspase-independent alternative pathway(s). The mitochondrial release of cytochrome c following a programmed cell death inducing stimulus in both plants and animals suggests the evolutionary conservation of death pathways [3]. The major apoptosis pathways are summarized in Figure 1. There are at least three recognized points at which apical proteases are activated to initiate apoptosis. The extrinsic pathways include delivery of Granzyme B (GrB) to the cell as well as receptor interaction with certain ligands. The mitochondrial pathway is an intrinsic pathway triggering apoptosis and is apparently subordinate to the dominant, direct pathway in most cell types.

In addition, many of the survival pathways including growth factors or their receptors such as ErbB-2 [4;5] and oncogenes such as Bcl-2 that drive tumor cells into unregulated growth and directly inhibit cellular apoptosis pathways in multiple ways. Bcl-2 is an anti-apoptotic and anti-proliferative protein over-expressed in several different human cancers including breast. Gain of Bcl-2 function in mammary epithelial cells was superimposed on the WAP-TAg transgenic mouse model of breast cancer progression to determine its effect on epithelial cell survival and proliferation at three key stages in oncogenesis: the initial proliferative process, hyperplasia, and cancer. During the initial proliferative process, Bcl-2 strongly inhibits both apoptosis and mitotic activity. However as tumorigenesis progresses to hyperplasia and adenocarcinoma, the inhibitory effects on mitotic activity were lost. In contrast, anti-apoptotic activity persisted in both hyperplasias and adenocarcinomas. These results demonstrate that the inhibitory effect of Bcl-2 on epithelial cell proliferation and apoptosis can separate during cancer progression. In this model, retention of anti-apoptotic activity with loss of anti-proliferative action resulted in earlier tumor presentation [6;7].

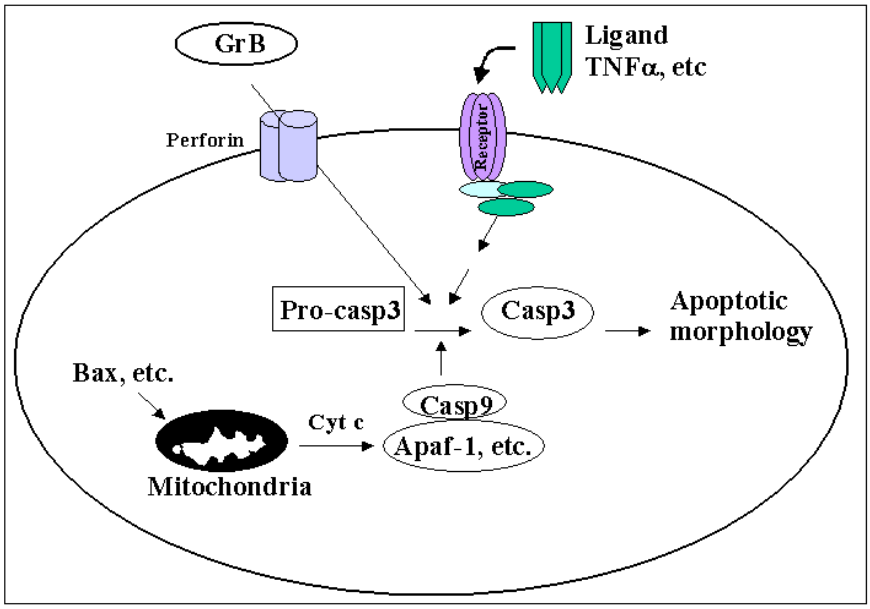


Figure 1. Major cellular apoptotic pathways: the extrinsic pathways include delivery of Granzyme B (GrB) to the cell as well as receptor interaction with certain ligands. The mitochondrial pathway is an intrinsic pathway triggering apoptosis.

Traditional chemotherapeutic approaches for treatment of neoplastic disease have generally relied upon the targeting of rapidly proliferating cells by inhibiting DNA replication or cell division. Although this strategy has been effective, its innate lack of selectivity for tumor cells has resulted in diminishing returns, approaching the limits of acceptable toxicity. Apoptosis also contributes to cell death in tumors treated with various anticancer agents [8]. Development of resistance of cancer cells to chemotherapeutic agents has been clearly shown to be correlated with a blockade of apoptotic signaling in resistant cells [9;10]. Metastatic spread of breast cancer also appears to directly involve the apoptotic pathway. Some studies have shown that high metastatic potential is strictly associated with increased resistance to apoptosis [11-14]. Suppression of drug-induced apoptosis by over-expression of oncogenes such as bcl-2, growth factors or their receptors [15-18] may be an important cause of both metastases and intrinsic chemo- and radiation- resistance.

Recent clinical studies [19] in patients with breast cancer suggest a direct link between tumor apoptosis, labeling index (Ki-67) and patient response to chemotherapeutic regimens. The studies assess the in vivo relationship of

apoptosis to proliferation and Bcl-2 protein in human breast tumors both prior to chemotherapy and in the residual resistant cell population at the completion of treatment by evaluation of apoptotic index (AI), Ki67 and Bcl-2 protein expression in the tissue of patients with operable breast cancer immediately before ECF [6 cycles of epirubicin (50 mg/m2, iv, Day 1), cisplatin (50 mg /m2, iv, Day 1) and continuous infusional 5 Fu (200 mg/m2 /24 hrs)] preoperative chemotherapy. There was a significant positive association between AI and Ki67 both before and after chemotherapy. In the residual specimens AI and Ki67 were significantly reduced compared with pre-treatment biopsies, while Bcl-2 expression showed a significant increase. These data suggest that apoptosis and proliferation are closely related in vivo. It is possible that the phenotype of reduced apoptosis and increased Bcl-2 may be associated with breast cancer cells resistant to cytotoxic chemotherapy [20]. Several studies have suggested a direct correlation between response to chemotherapy and activation of apoptotic pathways [21-24]. Chemo-resistance appears to frequently accompany clinical progression of breast cancers from a hormone-dependent, non-metastatic, antiestrogen-sensitive phenotype to a hormone-independent, invasive, metastatic, antiestrogen-resistant phenotype [25].

A growing understanding of the molecular events that mediate tumor growth and metastases has led to the development of rationally designed targeted therapeutics that offer the dual hope of maximizing efficacy and minimizing toxicity to normal tissue [26;27]. In recent years, a strategy in cancer therapy in general, and breast cancer in particular, has been the use of maximum tolerated doses of toxic non-specific agents as well as the investigation of a range of new agents that specifically target tumor-related molecules through a variety of biological pathways. Approaches to directly modulate of apoptosis pathways are of particular interest in terms of new drug development in breast cancer. The activation of apoptotic mechanisms in breast tumor cells has been directly implicated in the response of patient tumors to chemotherapy, response to radiotherapy and propensity to metastasize (Figure 2).

Therapeutic intervention strategies focusing on inactivation of anti-apoptotic pathways have employed strategies such as antisense molecules to down-regulate constitutively over-expressed Bcl-2 [28]. Treatment with antisense molecules has been shown to restore sensitivity to chemotherapeutic agents in breast tumors. In addition, dendritic cells [29-31] have been shown to initiate apoptosis in breast tumor cells (in vitro and in vivo) which are resistant to chemotherapeutic agents. As expected, dendritic cells appear to synergize with chemotherapeutic agents in generating cytotoxic effects on resistant breast tumor cells.

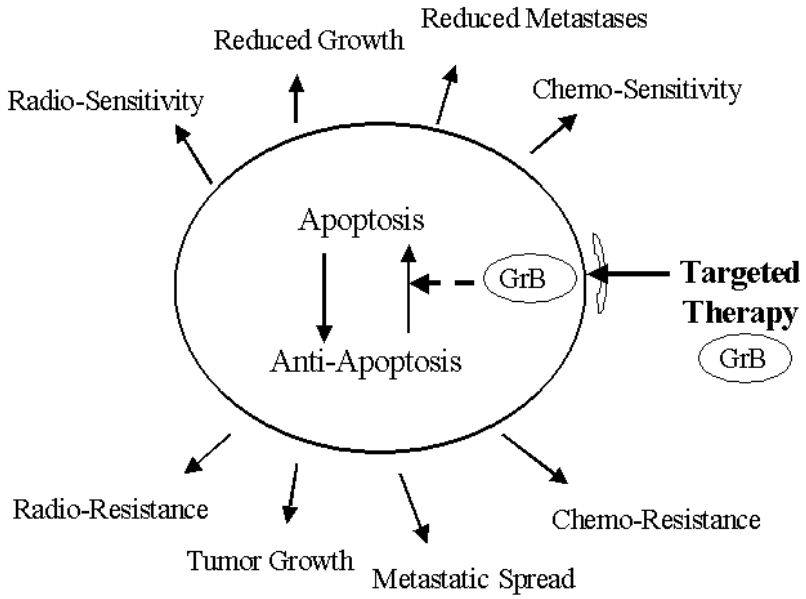


Figure 2. Apoptosis and anti-apoptotic events directly control ability of tumor cells to respond to external factors and control response to chemotherapeutic agents, radiation therapy and metastatic spread.

Cytotoxic T-lymphocytes (CTL) and Natural Killer (NK) cell types destroy tumor cells by direct physical delivery of the serine protease granzyme B (GrB) thereby inducing apoptosis and this is one of the major mechanisms in the cellular immune-response [32;33]. Cytotoxic T-lymphocyte granules contain perforin (PFN), a pore-forming protein, and a family of serine proteases termed granzymes. In CTL-mediated cytolysis, PFN is initially released and it inserts into the target cell membranes where it polymerizes to form transmembrane pores [34;35], which facilitates access of NK or CTL-released GrB to the target cell cytoplasm. Alternatively, other authors have suggested that after release from CTLs, GrB may be internalized into target cells by receptor-mediated endocytosis and that the role of PFN is to mediate release of GrB from endocytic vesicles. These studies suggest that PFN can be replaced by other vesicle-disrupting factors such as those produced by adenovirus [36-38].

Once delivered to the cytoplasm, GrB induces apoptosis (Figure 3) by directly activating caspases and inducing rapid DNA fragmentation [39;40].

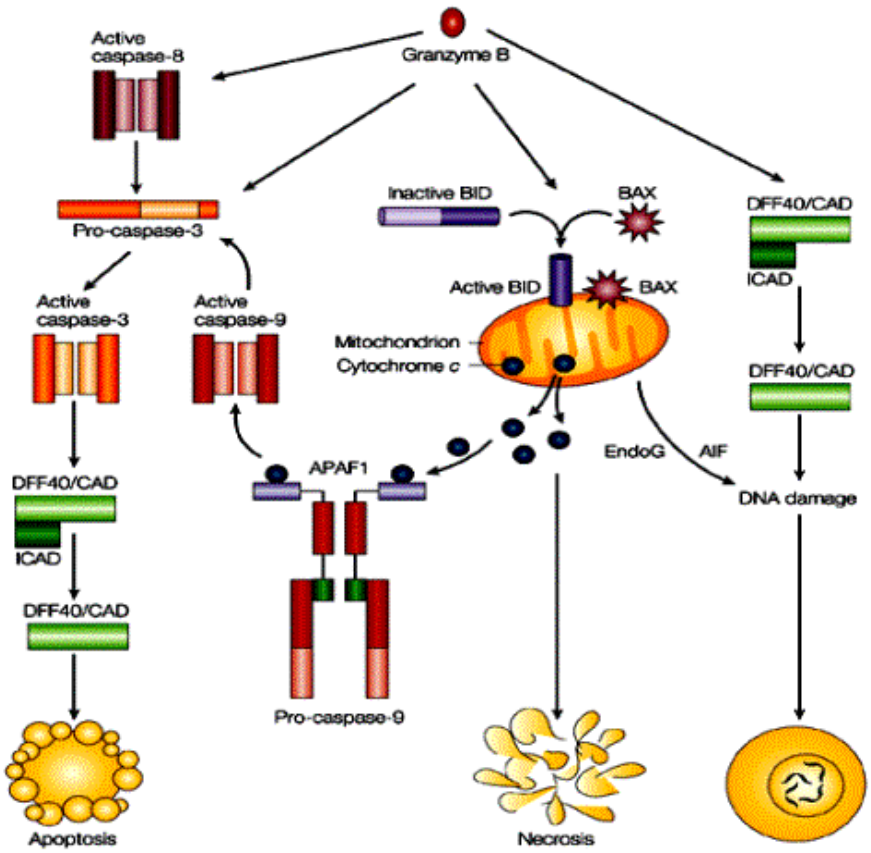


Figure 3. Pathways to apoptotic cell death initiated by GrB. Once released into the cytoplasm, GrB can initiate apoptotic cell death through both caspase-dependent and caspase-independent pathways. (From M. Barry and R. C. Bleackley. "Cytotoxic T Lymphocytes: All Roads Lead to Death". Nature Reviews-Immunology. 2002, 2: 401-409).

GrB can cleave many procaspases in vitro, and has been an important tool in analyzing the maturation of caspase-3 [41], caspase-7 [42;43], caspase-6 [44], caspase-8 [45], caspase-9 [46], and caspase-10a/b [47;48]. Although many procaspases are efficiently cleaved in vitro, GrB-induced caspase activation occurs in a hierarchical manner in intact cells, commencing at the level of "executioner caspases" such as caspase-3, followed by caspase-7 [49]. Some studies have shown that GrB activated cell death pathways through cleavage of Bid and activation of the mitochondrial death pathway in intact cells [50;51]. In addition to the caspase-mediated cytotoxic events, GrB can also rapidly translocate to the nucleus and cleave poly (ADP-ribose) polymerase and nuclear matrix

antigen, utilizing different cleavage sites than those preferred by caspases [52;53]. In addition, some studies have demonstrated that GrB can directly damage to non-nuclear structures such as mitochondria, subsequently induce cell death through caspase-independent pathway [54;55].

Since almost all cells contain mechanisms responsible for mediating cell death (apoptosis) we propose that targeted delivery of GrB protein to the interior of cells will result in cell death through apoptotic mechanisms assuming that sufficient quantities of active enzyme can be successfully delivered to the appropriate subcellular compartment.

Recombinant single-chain Fv antibody (scFv)-based agents have been used in pre-clinical studies for cell-targeted delivery of cytokines [56] and intracellular delivery of highly cytotoxic n-glycosidases such as recombinant gelonin (rGel) toxin [57-59]. The smaller size of these antibody fragments may allow better penetration into tumor tissue, improved pharmacokinetics, and a reduction in the immunogenicity observed with intravenously administered murine antibodies. Initially, to target melanoma cells, we chose a recombinant single-chain antibody designated scFvMEL which recognizes the high-molecular-weight glycoprotein gp240, found on a majority (80 %) of melanoma cell lines and fresh tumor samples [60]. Our group and others have demonstrated that this antibody possesses high specificity for melanoma and is minimally reactive with a variety of normal tissues, making it a promising candidate for further study [61-63].

Recently, studies have demonstrated that the gp240 antigen is present not only on melanoma but also on 66 % of lobular breast cancer [64]. Lobular breast cancer is an important subtype classification of breast cancer which defines a population of women with tumors which are relatively radiation resistant and resistant to chemotherapeutic regimens [65;66]. Data suggests that cellular apoptotic mechanisms in this population of tumors may be inhibited thus suppressing response to therapeutic agents although this is speculative.

Antibodies designated ZME-018 or 225.28 S targeting the gp240 antigen have been extensively studied in melanoma patients and have demonstrated an impressive ability to localize in metastatic tumors after systemic administration [64;67-69] (Figure 4).

The recombinant scFvMEL single-chain antibody has been used extensively in our laboratory to target gp240 bearing cells in vitro and using xenograft models [70-77]. This antibody binds to target cells and is efficiently internalized making this an excellent carrier to deliver toxins or other therapeutic payloads.

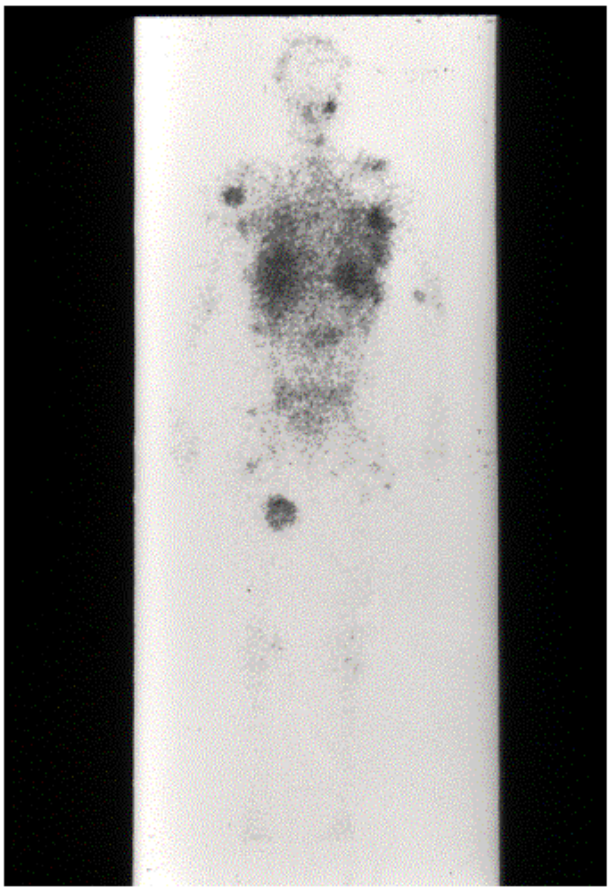


Figure 4. Tumor uptake of ^{111} In monoclonal antibody ZME-018 in a patient with metastatic melanoma after systemic administration.

More importantly, the gp240 antigen is not expressed on normal cells thus making this an interesting target for therapeutic intervention. In this study, we used scFvMEL as a tumor cell-targeting carrier and designed a novel recombinant fusion construct designated GrB/scFvMEL, containing human pro-apoptotic enzyme GrB. The purpose of these studies was to determine whether we could deliver sufficient quantities of active GrB enzyme to drive cellular apoptotic events specifically in breast cancer target cells. Moreover, we propose to examine if apoptotic modulation by delivery of GrB into breast tumor is able to re-establish sensitivity of breast cancer tumor cells resistant to chemotherapy or to specifically sensitize tumor cells to radiation therapy.

Delivery of human pro-apoptotic proteins to tumor cells is a novel concept. We have recently demonstrated that nascent cellular apoptotic cascades which are normally held in check can be specifically activated using a directed therapeutic approach in which activated signal transduction cascade proteins are delivered to cell cytoplasm using recombinant fusion constructs containing antibodies or growth factors capable of binding to a cell surface antigen and internalizing a payload.

The concept of delivering human pro-apoptotic proteins as functional components of targeted therapeutics we believe is a novel and previously untried approach. Our recent publications on constructs containing GrB are the first reported such constructs found in the literature [78;79]. The development of gp240-targeted therapeutic agents containing GrB offers an unprecedented opportunity to utilize such agents to probe the criticality of apoptotic processes during tumor growth, metastatic spread and response to therapeutic agents. In addition, information regarding these studies will also be used to further develop the GrB/scFvMEL fusion construct as a potential therapeutic agent.

1. CAN WE GENERATE A RECOMBINANT CONSTRUCT CONTAINING A TARGETING ANTIBODY AND ACTIVE GRB?

We successfully generated a recombinant construct GrB/scFvMEL containing active GrB and single-chain Fv fragment targeting the gp240 antigen present on 80% of melanoma and 66% of lobular breast cancer. First, we successfully obtained the human pre-mature GrB gene composed of mature GrB and a signal sequence from human cutaneous T-cell lymphoma (Hut-78) cell RNA utilizing reverse transcription-PCR (Figure 5).

In the premature GrB protein, the first 20 amino acids at as a signal sequence. In cytotoxic T cells, active GrB is nominally generated by dipeptidyl peptidase I (DPPI)-mediated proteolysis [80] which removes the two-residue (Gly Glu) propeptide and exposes a terminal Ile ^{21} residue. The NH2-terminal Ile-Ile-Gly-Gly sequence of GrB is necessary for enzymatically active GrB.

In our PCR engineering design and construction of the final molecule, this enzymatic requirement dictated that the GrB protein leads the molecule followed by a flexible linker and the targeting antibody. In addition, we insured that the EK cleavage site (DDDDK) for removal of the purification tag was immediately adjacent to Ile ^{21}.

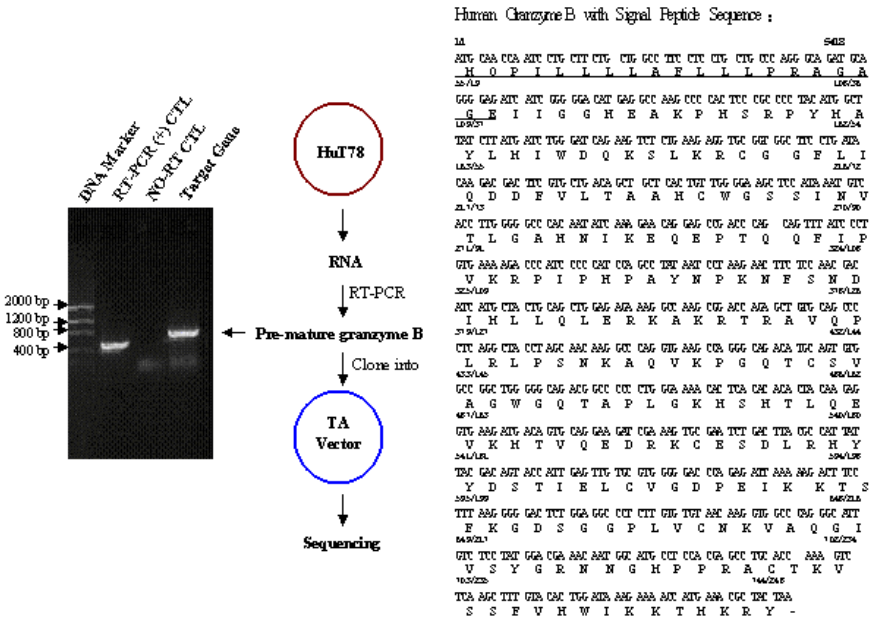


Figure 5. Cloning the human GrB gene from HuT-78 cells. RNA was isolated, and premature GrB cDNA (~ 800 bp) was amplified by reverse transcription –PCR and cloned into the PCR2.1 TA vector. The human GrB sequence with a 20- amino acid signal sequence was confirmed and designated as pre-mature GrB. Once the signal peptide was removed, the mature amino-terminal Ile-Ile-Gly-Gly sequence of active GrB was generated.

The fusion gene was then introduced into the pET32a (+) bacterial expression vector to form pET32GrB/scFvMEL (Figure 6). The recombinant protein GrB/scFvMEL was expressed in bacterial expression system (Figure 7) as a polyhistidine-tagged protein (A, lane 2) and then purified by nickel-NTA metal affinity chromatography (A, lane 3). The his-tag was cleaved by addition of recombinant enterokinase (rEK) to produce active form of GrB fusion and then Q-sepharose ion exchange resin was used for final purified GrB/scFvMEL (A, lane 4). Specificity of the cleaved fusion protein was confirmed by Western blot using either mouse anti-GrB or rabbit anti-scFvMEL antibody (B). To assess the biological activity of the GrB component of the fusion construct, the ability of the enzyme to cleave a BAADT substrate was assessed and compared to native GrB [81]. The fusion construct GrB/scFvMEL was shown to have intact GrB enzymatic activity with ΔmA/min = 68.6 and a Specific Activity (SA) = 2.6×10^5 units/ μmole. This activity was comparable to that of native GrB with ΔmA/min = 48.2 and a SA = 4.8×10^5 units/ μmole. As expected, the GrB/scFvMEL

construct containing the purification tag (before rEK digestion) was shown to be unable to cause hydrolysis of the BAADT substrate (ΔmA/min ≤ 5).

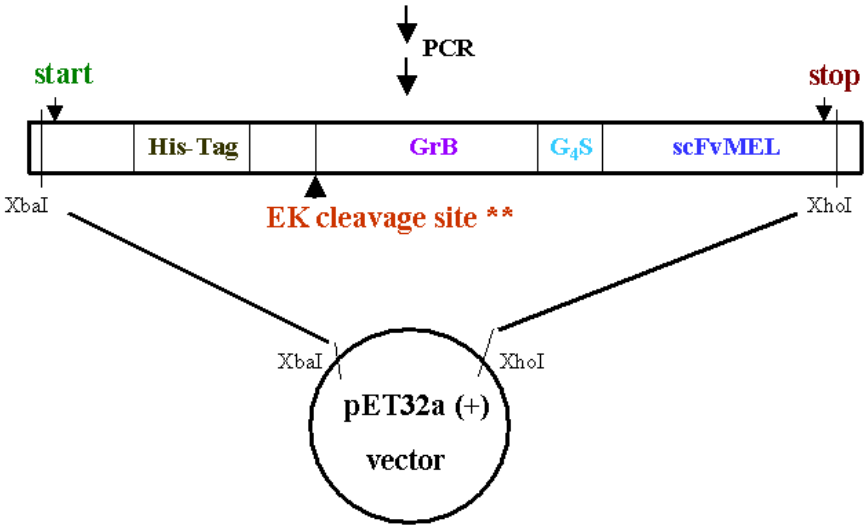
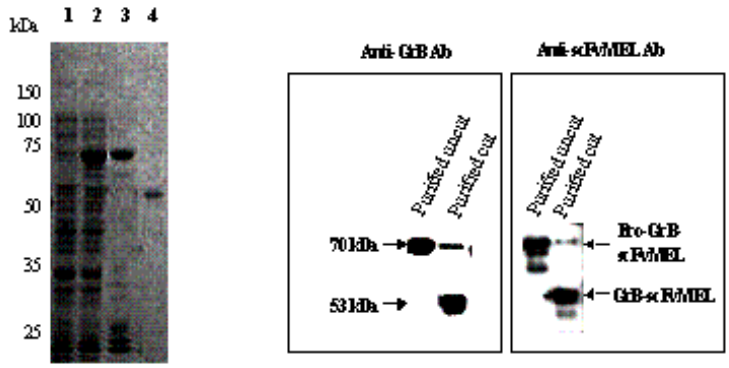


Figure 6. Construction of the GrB/scFvMEL fusion toxin by PCR and insertion into pET32a (+) vector. Mature GrB was attached to the recombinant antibody scFvMEL via a flexible tether (G_4S). A cleavage site for EK (DDDDK) was inserted upstream of the first amino acid (Ile) of mature GrB. The fused gene was then introduced into pET32a (+) vector to form the expression vector pET-32GrB/scFvMEL. Once the protein tag was removed by rEK digestion, the first reside (Ile) of mature GrB was exposed, thereby activating the GrB moiety of the fusion construct.



Enzymatic Activity of GrB Moiety of Fusion Protein Compared with Native GrB *

Samples	ΔmOD/min	Units (U)	U/μg	MW (kDa)	Specific Activity (U/μM)
Native GrB	48.2	1.0	19.2	25	4.8×10^5
GrB/scFvMEL (Un-rEK cut)	2.0**	-	-	70	-
GrB/scFvMEL (rEK-cut)	68.6	1.42	4.7	53	2.6×10^5

* BAADT Assay
** The rate of non-enzymatic hydrolysis of BAADT at 0.2 mM, in 0.3 mM Ellman's Buffer at 25 °C is ≤ 5 Δm OD/min

Figure 7. SDS-PAGE and Western analysis of GrB/scFvMEL expression in E. coli. A. 10 % SDS-PAGE gel with coomassie blue staining under reducing conditions showed that the GrB/scFvMEL construct was expressed as a 70- kDa molecule (53 + 17 kDa purification tag). The size of final purified GrB/scFvMEL was ~ 53 kDa. Lane1, non-induced bacterial cell lysate; lane 2, induced cell lysate; lane 3, pro-GrB/scFvMEL (+ tag) after purification by nickel-NTA metal affinity chromatography; lane 4, final purified GrB/scFvMEL. B. Western blotting confirmed that the fusion protein reacted with both mouse anti-GrB and rabbit anti-scFvMEL antibodies.

2. DOES THIS RECOMBINANT GRB/SCFVMEL INTERNALIZE EFFICIENTLY INTO GP240 POSITIVE CELLS?

The GrB moiety of the fusion construct was efficiently delivered into the cytosol of gp240 positive A375-M melanoma cells after treatment with GrB/scFvMEL for 1 h (D) or 6 h (E) as assessed by confocal microscope imaging detected using goat anti-GrB antibody (Figure 8). Pretreatment of cells with the original anti-gp240 antibody ZME-018 followed by incubation with the GrB/scFvMEL was shown to completely suppress internalization of this agent (B, C). This effectively demonstrates that binding of the construct to gp240 on the tumor cell surface is responsible for internalization of the construct.

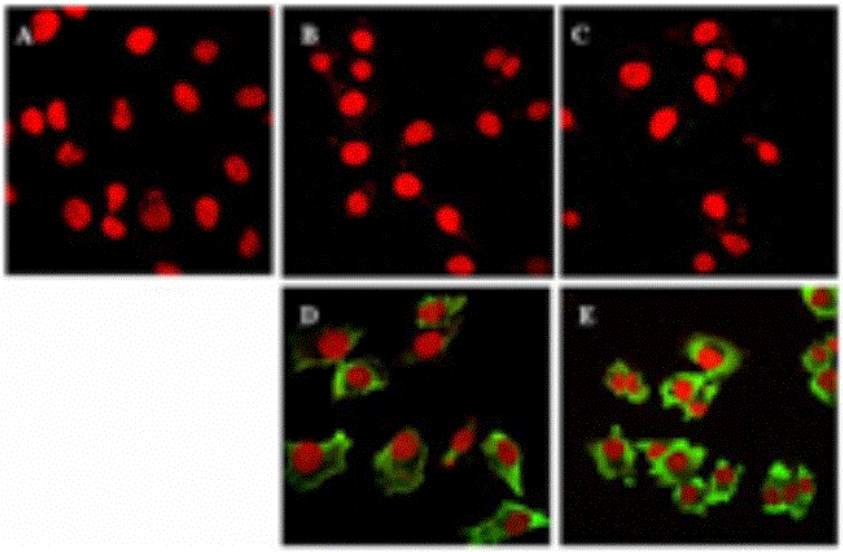


Figure 8. Internalization of GrB/scFvMEL into A375-M cells assessed by confocal microscopy. A375-M cells were treated with 40 nM GrB/scFvMEL for 1 or 6 h. As indicated, some cells were pre-treated with ZME018 to assess antigen-mediated internalization. Molecules bound to the cell surface were removed by brief treatment with glycine buffer (pH 2.5). Cells were then fixed, permeabilized and incubated with goat anti-GrB antibody, followed by FITC-coupled anti-goat IgG and propidium iodide (PI). The slides were mounted and analyzed by Zeiss LSM 510 confocal laser scanning microscopy. A, no treatment control. B, pretreatment with ZME-018, then GrB/scFvMEL treatment for 1 h. C, pretreatment with ZME-018, then GrB/scFvMEL treatment for 6 h. D, GrB/scFvMEL treatment for 1 h. E, GrB/scFvMEL treatment for 6 h.

3. DOES THE GRB/SCFVMEL CONSTRUCT GENERATE APOPTOSIS IN TARGET CELLS?

Both antigen-positive and antigen-negative cells were treated with an IC_{50} concentration of the GrB/scFvMEL fusion construct. Apoptotic cells were observed at 8 h after treatment when assessed by TUNEL assay. In contrast, there were no apoptotic cells in non-target cells treated with identical doses of the fusion construct, thereby demonstrating cell specificity of the construct (Figure 9).

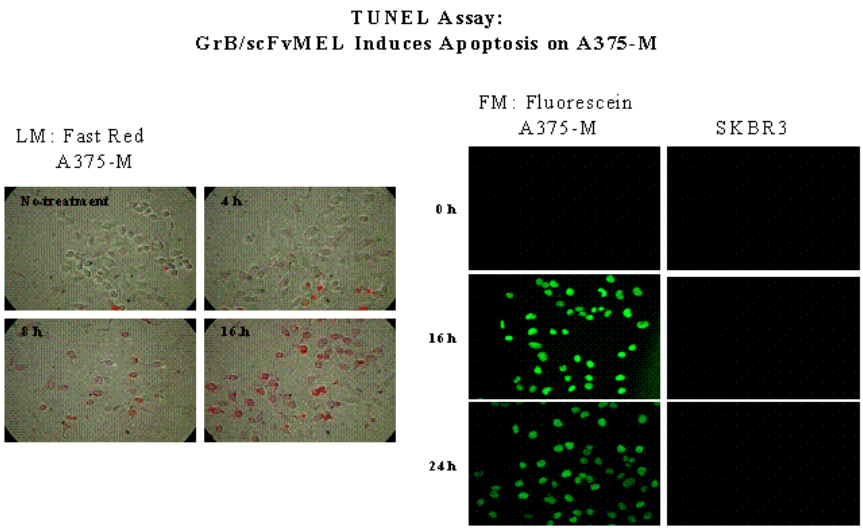


Figure 9. Treatment with GrB/scFvMEL induces apoptosis on antigen-positive A375-M cells but not on antigen-negative SKBR3 cells as assessed by TUNEL assay.

Procaspase-3 was cleaved into one fragment (~ 20 kDa) at 4 h, and further cleaved into smaller fragments after treatment for 8 h. We were not able to demonstrate caspase-3 cleavage on antigen-negative SKBR3 cells treated with GrB/scFvMEL (Figure 10). Treatment of GrB/scFvMEL was shown to result in cytochrome c translocation from the mitochondrial compartment into cytosol by 4 h after treatment of A375-M cells but not observed on SKBR3 cells (Figure 11). These studies showed that the cytotoxic /apoptotic events observed after treatment of target cells with the fusion construct are the result of both caspase-dependent and caspase-independent mechanisms.

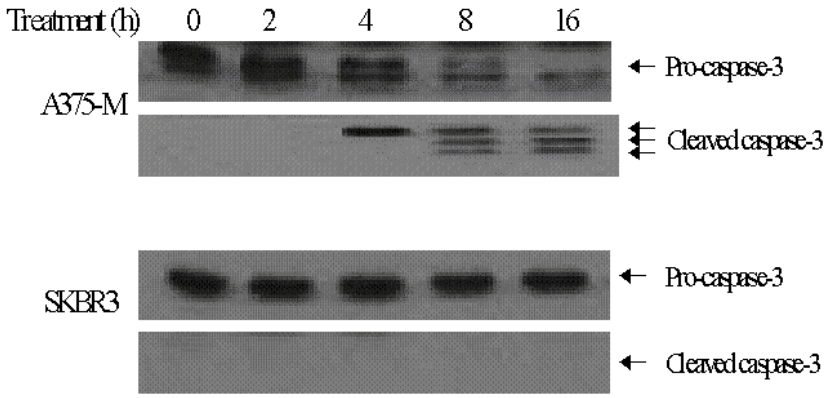


Figure 10. GrB/scFvMEL induced caspase-3 cleavage on antigen-positive A375-M cells. A375-M and SKBR3 cells were treated with GrB/scFvMEL at 50 nM for various times. Whole cell lysates (30 μg) were analyzed by 12 % SDS-PAGE and followed by immunoblotting to detect caspase-3 or cleaved caspase-3. Pro-caspase-3 was cleaved into an active fragment at 4 h and further cleaved into smaller fragments after treatment for 8 h by GrB/scFvMEL on A375-M cells. We found no caspase-3 cleavage on SKBR3 cells treated with GrB/scFvMEL.

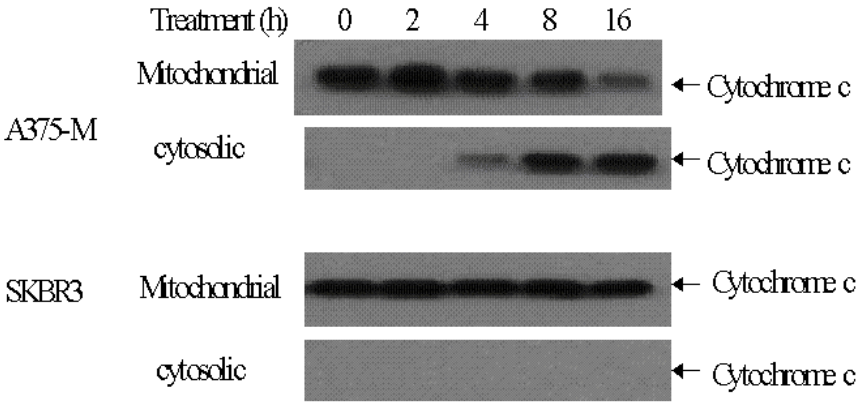


Figure 11. Cytochrome c was released from mitochondria fractions into cytosol by GrB/scFvMEL on A375-M cells. Cells were treated with GrB/scFvMEL at 50 nM for various times. Cells were collected and the cytosolic and mitochondrial fractions were isolated. Fractions (30 μg) from nontreated and treated cells were analyzed by 15 % SDS-PAGE followed by immunoblotting and detection with an anti-cytochrome c antibody. As shown, cytochrome c was found to be released from mitochondria into the cytosol on A375-M cells but not on SKBR3 cells within 4 h after treatment by GrB/scFvMEL.

4. IS THE GRB/SCFVMEL CONSTRUCT CYTOTOXIC TO TARGET CELLS?

The cytotoxicity of GrB/scFvMEL was assessed against log-phase gp240 positive A375-M, MDA-MB435 and negative SKBR3 cells in culture. A 50 % growth inhibitory effect was found at a concentration of ~ 20 nM on A375-M cells and MDA-MB435 cells.

However, no cytotoxic effects were found on antigen-negative SKBR3 cells at doses of up to 1 μM (Figure 12). By comparison, the cytotoxic effects of GrB/scFvMEL were approximately the same as that of another fusion toxin, MELsFv/rGel [82] on A375-M cells.

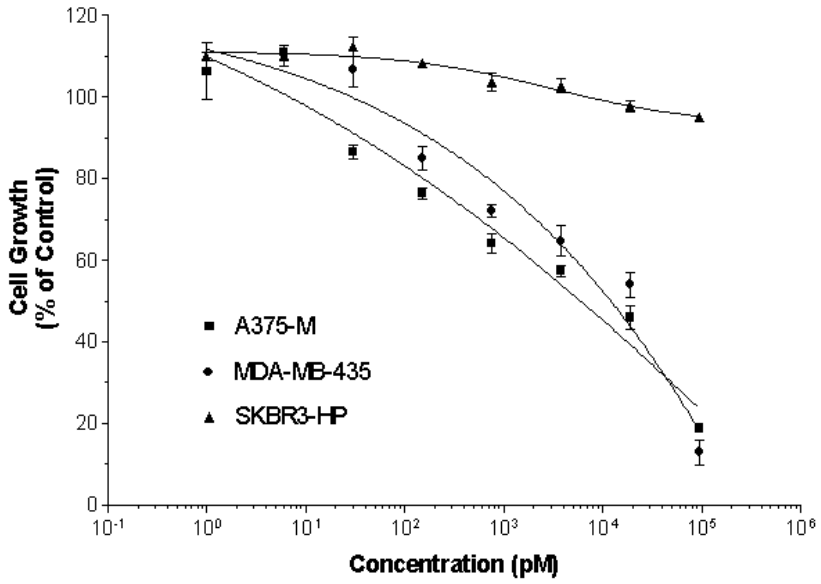


Figure 12. Cytotoxicity of the GrB/scFvMEL fusion toxin on A375-M, MDA-MB435 and SKBR3-HP cells. Log-phase cells were plated into 96-well plates at a density of 2.5 x 10^3 cells per well and allowed to attach for 24 h. The medium was replaced with medium containing various concentrations of GrB/scFvMEL. After 72 h, the effect of fusion toxin on the growth of cells in culture was determined using crystal violet staining. The IC_{50} of GrB/scFvMEL was ~ 20 nM on A375-M and MDA-MB435 cells. In contrast, no cytotoxicity was observed on SKBR3 cells.

When A375-M cells were pretreated with a full-length anti-gp240 antibody (ZME-018) for 6 h and then treated with GrB/scFvMEL for 72 h,

the cytotoxicity of GrB/scFvMEL was abolished, thereby demonstrating a requirement for antigen recognition in the cytotoxic effect of the GrB/scFvMEL fusion construct (Figure 13). These data confirm the confocal imaging data that demonstrate that pre-treatment of cells with native ZME-018 can abolish internalization of this agent. In addition, the uncut GrB/scFvMEL construct or rEK showed no cytotoxicity to target cells as expected (Figure 13).

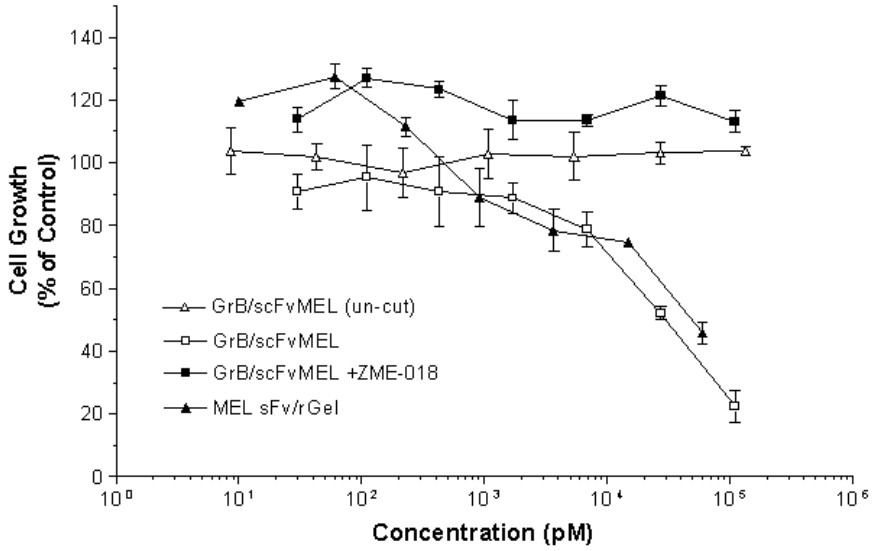


Figure 13. Comparative cytotoxicity of GrB/scFvMEL and MEL sFv/rGel and effect of addition of ZME-018 on cytotoxicity of GrB/scFvMEL against A375-M cells. Log-phase cells were plated into 96-well plates at a density of 2.5 x 10^3 cells per well and allowed to attach for 24 h. Cells were treated with different concentrations of GrB/scFvMEL or MEL sFv/rGel and were also pretreated with ZME-018 (40 mg/ml) for 6 h and then co-treated with various concentrations of GrB/scFvMEL. After 72 h, the cells were stained with crystal violet. The IC_{50} of GrB/scFvMEL was approximately identical to that of MEL sFv/rGel on A375-M. ZME-018 pretreatment inhibited the cytotoxicity of GrB/scFvMEL on A375-M cells.

5. CAN GRB/SCFVMEL FUSION PROTEIN SENSITIZE CELLS TO CONVENTIONAL CHEMOTHERAPEUTIC AGENTS?

Cells in exponential growth phase were plated into 96-well plates. After 24 hr, the cells were treated with drug-containing medium. At the end of the indicated incubation period, growth inhibition was assessed by crystal violet staining. In order to determine the effects of sequencing, cells were treated with the different sequences.

Sequence I (C1): cells were pretreated with chemotherapeutic agent for 6 h, and then co-administered with chemotherapeutic agent and GrB/scFvMEL for 72 h.

Sequence II (C2): cells were pretreated with GrB/scFvMEL for 6 h, and then co-administered with chemotherapeutic agent and GrB/scFvMEL for 72 h.

Sequence III: cells were pretreated with GrB/scFvMEL for 6 h, followed by treatment with chemotherapeutic agents for 72 h.

Sequence IV: cells were treated with various chemotherapeutic agents for 72 h without GrB/scFvMEL pretreatment.

Chemotherapeutic agents include doxorubicin (DOX), vincristine (VCR), etoposide (VP-16), cisplantin (CDDP), cytarabine (Ara C) and 5 –FU.

There were synergistically cytotoxic effects on A375-M cells when the GrB/scFvMEL fusion construct in combination with the chemotherapeutic agents such as DOX, VCR and CDDP. In addition, this new class of agents has additive interactions with VP-16, Ara-C and 5-Fu. The cytotoxicities significantly increased when A375-M cells were pretreated with GrB/scFvMEL for 6 h followed by co-treatment with chemotherapeutic agents (sequence II-C2) compared to that the cells were pretreated with chemotherapeutic agents for 6 h followed by combination treatment (sequence I-C1) (Figure 14). Our data also demonstrated that the cytotoxicities of chemotherapeutic agents significantly increased when cells were pretreated with the fusion construct for 6 h followed by various chemotherapeutic agents for 72 h (sequence III) compared to without GrB/scFvMEL pretreatment (sequence IV) ($p < 0.01$). The results indicated that the effects of conventional chemotherapeutic agents could be sensitized by pretreatment with GrB/scFvMEL fusion protein for 6 h on gp240 positive targeted cells.

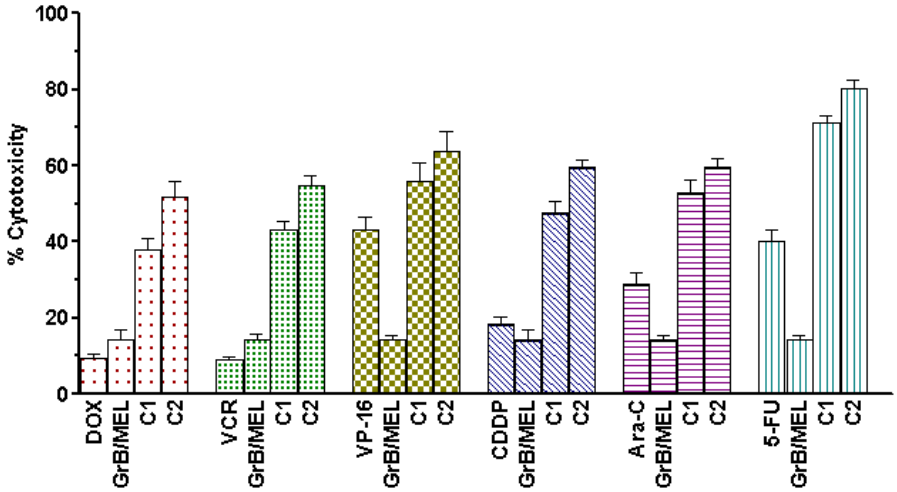


Figure 14. Studies of GrB/scFvMEL in combination with various chemotherapeutic agents. Antigen-positive(A375) cells were pretreated (at IC_{25} doses) with various chemotherapeutic agents for 6 h followed by addition of GrB/scFvMEL(IC_{25}). The cells were then incubated for a total of 72 h (sequence C1). Alternatively, cells were first treated with GrB/scFvMEL for 6 h, and then various chemotherapeutic agents were added for 72 h (sequence C2). Chemotherapeutic agents include doxorubicin (DOX), vincristine (VCR), etoposide (VP-16), cisplantin (CDDP), cytarabine (Ara C) and 5-Fu.

6. DOES GRB/SCFVMEL LOCALIZE IN TUMOR XENOGRAFT CELLS AND INDUCE TUMOR APOPTOSIS AFTER SYSTEMIC ADMINISTRATION?

Mice bearing A375-M xenograft tumors were administered GrB/scFvMEL (75 μg in 0.1 ml saline). Twenty-four hours later, animals were sacrificed and representative tissue sections were removed and formalin fixed and stained (H&E and TUNEL). Tumor tissue displayed apoptotic neuclei in treatment group (Figure 15).

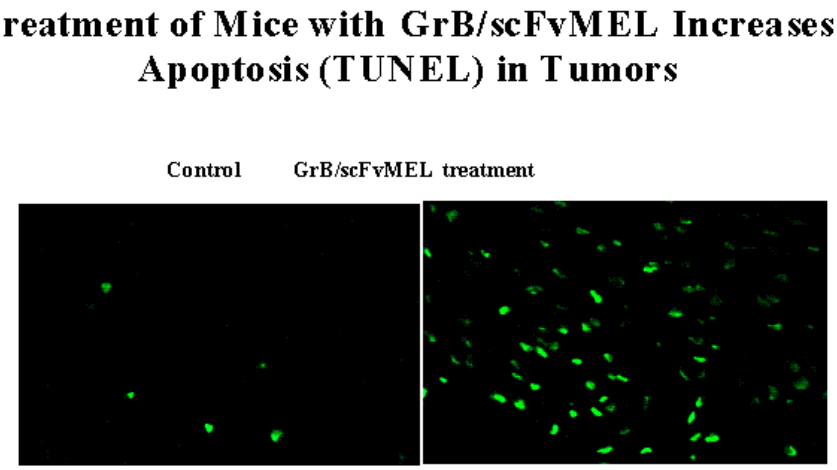


Figure 15. Mice bearing A375-M xenograft tumors were administered GrB/scFvMEL (37.5 mg/kg total dose, iv, tail vein in divided doses at the schedule indicated). Twenty-four hours after the last dose, a group of amimals were sacrificed and tumor tissues were removed and fixed. Tumor tissue sections were stained by TUNEL and analyzed under Nikon Eclipse TS 100 fluorescent microscope. Tumor tissue displayed highly apoptotic neuclei in the treatment group compared to the control group.

Immunohistochemical stains for GrB/scFvMEL detected by either anti-GrB or anti-scFvMEL antibody were performed. Localization or internalization of GrB/scFvMEL was observed in tumor tissue (Figure 16).

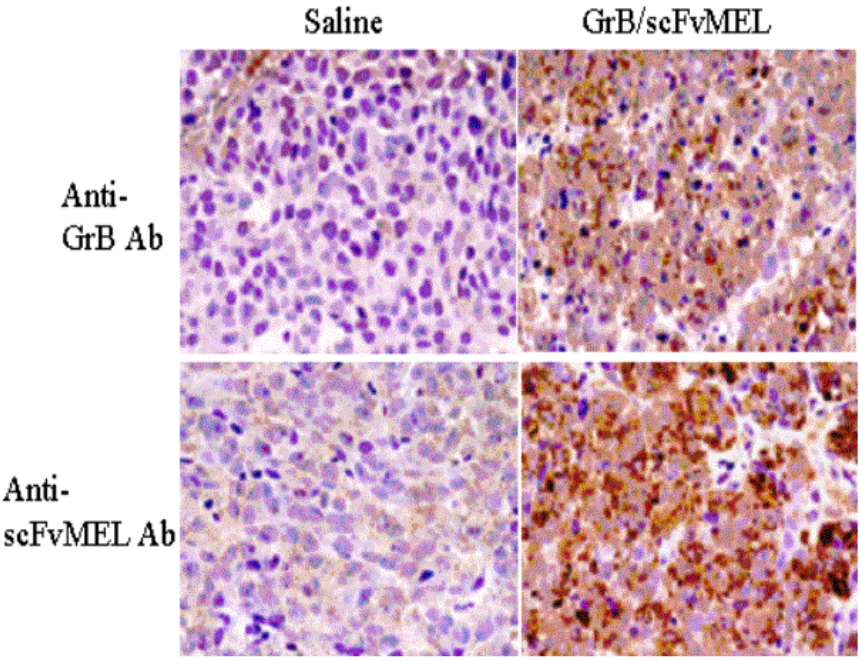


Figure 16. Mice bearing A375-M xenograft tumors were administered GrB/scFvMEL (37.5 mg/kg). Twenty-four hours after the last dose, animals were sacrificed and tumor tissues were removed, fixed and stained by immunohistochemical staining for GrB/scFvMEL detected by either anti-GrB or anti-scFvMEL antibody. Localization and internalization of GrB/scFvMEL was observed in tumor tissue in the treatment group but not in the control group.

7. DOES GRB/SCFVMEL HAVE IN VIVO ANTITUMOR EFFECTS?

Preliminary studies to examine the in vivo anti-tumor effects of GrB/scFvMEL were performed on A375-M human melanoma tumor xenografts. Mice bearing the tumors were treated (iv tail vein) 5 × every other day with either GrB/scFvMEL or saline. Tumor volumes were measured for 42 days. The saline-treated control tumors increased from 50 mm^3 to 1200 mm^3 over this period. Tumors treated with GrB/scFvMEL (37.5 mg/kg) increased from 50 mm^3 to 200 mm^3 (Figure 17).

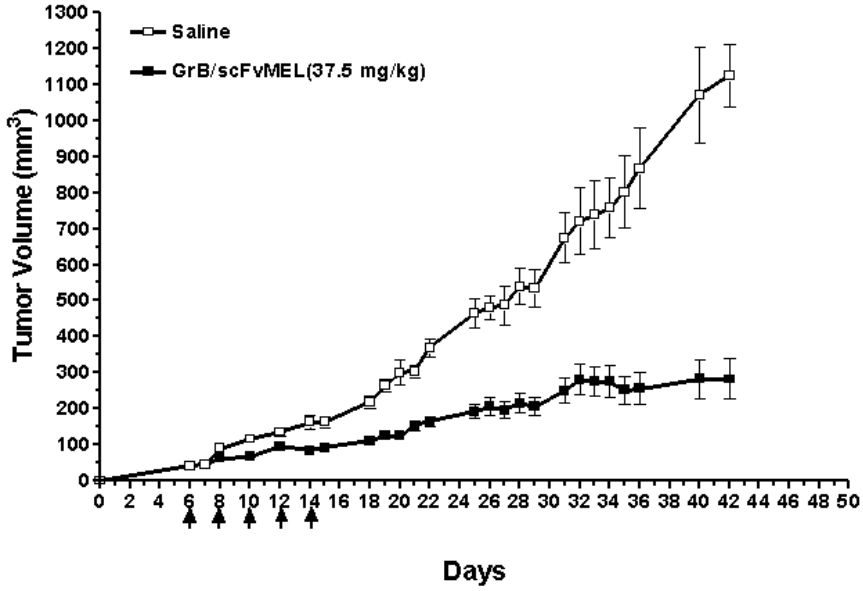


Figure 17. Athymic (nu/nu) mice, female, 6-8 weeks of age, were injected subcutaneously, right flank with 3 x 10^6 log-phase A375-M cells and tumors were allowed to establish. Once tumors reached measurable size (~ 30 – 50 mm^3), animals were treated via i. v. tail vein with either saline (control) or GrB/scFvMEL fusion construct (37.5 mg/kg total dose) for 5 times every other day. Animals were monitored and tumors were measured for an additional 28 days. The saline-treated control tumors increased from 50 mm^3 to 1200 mm^3 over this period. In contrast, tumors treated with GrB/scFvMEL (37.5 mg/kg) increased from 50 mm^3 to 200 mm^3.

This study clearly demonstrates the biological activity of a new class of tumor-targeted enzymes that are cytotoxic to target cells because they are capable of direct activation of the nascent cellular pro-apoptotic pathway. Although several groups have generated antibody-enzyme chemical conjugates and fusion constructs, the purpose of the majority of these targeted enzymes have been to locally convert inactive pro-drugs to active therapeutic agents [83]. The primary types of directly cytotoxic enzymes commonly delivered by cell- targeting proteins such as antibodies and growth factors usually fall into the class of ribosome-inhibiting proteins (RIPs). Toxins such as pseudomonas exotoxin (PE) and gelonin (rGel) have been successfully utilized because only a few molecules are needed to irretrievably intoxicate a target cell [84;85]. Recently, Newton et al. described a new class of immunoconjugates containing human RNase which has in vitro and in vivo cytotoxic activity against human tumor cell lines and

xenografts [86] primarily through degradation of RNA. The current construct, to our knowledge, is one of the first descriptions of a targeted enzymatic agent that operates primarily through activation of the pro-apoptotic cascade process. We were encouraged to note that the GrB/scFvMEL fusion construct demonstrated equivalent cytotoxic effect on target cells compared to a fusion toxin containing a highly potent plant n-glycosidase such as recombinant gelonin. Studies in our laboratory demonstrate that the scFvMEL/rGel fusion construct apparently cells through a necrotic rather than an apoptotic process. These comparative studies demonstrate that the robust cytotoxic effects of the rGel toxin can be matched by that of the GrB apoptotic effects.

On interaction of a cytotoxic T lymphocyte (CTL) with a target cell, there is a directed exocytosis of the CTL granules into the extracellular space between the two cells. The original view of entry for GrB into the target cell was that perforin polymerized to form a pore in the target cell membrane through which GrB could pass [87]. More recently, the discovery of a receptor for GrB that is the mannose-6-phosphate receptor has indicated that GrB might be taken up by receptor-mediated endocytosis [88;89] and that perforin may act to release endosomal GrB into the cytosol of the target cell [90].

The current study clearly demonstrates that an antibody delivery vehicle can provide the cellular entry access for the enzyme specifically on antigen-positive cells and is capable of delivering the enzyme to cytoplasm without the need for perforins. Moreover, the delivery vehicle can provide enzyme concentrations in the cytoplasm which are apparently sufficient to drive apoptosis. The anti-gp240 scFv antibody used has been well-studied in our laboratory and is capable of internalizing and delivering attached proteins such as toxins into the cytoplasm of target cells. The exact mechanism by which this particular antibody internalizes has not been fully elucidated. However, our studies suggest that there is no need for perforins or vesicle-disrupting factors in the antibody-mediated delivery of GrB to the cytoplasm. In addition, since the recombinant GrB protein is unglycosylated, it will not be taken up through the mannose-6-phosphate receptors, thereby reducing the likelihood of uptake by antigen-negative cells.

We have also described other constructs containing GrB as the cytotoxic moiety. Studies describing GrB fused to $VEGF_{121}$ for directed apoptosis targeting tumor vasculature indicated that the vasculature of many solid tumors were susceptible to the cytotoxic properties of the fusion construct [91]. In addition, as in the current study, the $I.C._{50}$ values for the GrB/$VEGF_{121}$ were similar to that of the $VEGF_{121}$/rGel construct.

The current study of GrB/scFvMEL extends these observations. These studies demonstrate that delivery of GrB to the cytosol of human tumor cells

in culture can induce apoptosis. Recently studies showed that apoptosis induced by GrB is characteristically a rapid process taking approximately 1 h to result in the hallmarks of apoptosis such as DNA fragmentation, caspase cleavage and cytochrome c release[92]. Our studies indicated that apoptotic event happened within 8 h. This is because the antibody internalization process requires several hours for optimal delivery and the current process requires delivery of GrB to the cytoplasm in quantities sufficient to overcome normal homeostasis and to drive cells into apoptosis. The apoptosis induced by antibody delivery of GrB suggests that tumor cells, like normal endothelial cells, contain sufficient pro-apoptotic substrates capable of activation by GrB and that there appear to be no apparent downstream control-point mechanisms nominally in place to prevent pharmacologically directed apoptosis using this particular mechanistic approach. Mechanistic studies demonstrate release of cytochrome c from the mitochondria to the cytosol induced by the fusion protein GrB/scFvMEL as well as clear activation of caspase-3 specifically in target cells. These studies suggest that the cytotoxic/apoptotic events observed after treatment of target cells with the fusion construct are the result of both caspase-dependent and caspase-independent mechanisms. It remains to be determined whether there are cellular resistance mechanisms capable of protecting cells against GrB-containing targeted therapeutic agents. In addition, still to be determined is whether there are specific cell types inherently or iatrogenically resistant to this cytotoxic approach.

This appears to be the first description of the use of the proapoptotic, serine protease GrB in targeted therapeutic constructs. This agent is the culmination of our search for a human therapeutic protein that is both highly active and potentially universal. In addition, as a critical consideration, we demonstrated that the GrB enzyme was effective even while it remained covalently attached to an assembled targeting vehicle. This advance avoids problems with linker technology that can result in premature release of the active component prior to target internalization or loss of biological activity of the active component due to permanent attachment to the targeting molecule. Importantly, the demonstration of biological activity with a GrB-containing fusion protein does not appear to be confined to the current approach using $VEGF_{121}$ as a vehicle to target tumor endothelium. Recent studies in our laboratory have demonstrated impressive cytotoxic activity of GrB-containing fusion constructs composed of single-chain antibodies targeting numerous human tumor cell types. The unique mechanism of action of GrB- based fusion targeted therapeutic agents may also provide for novel interactions with other types of therapeutic agents or with other modalities such as hyperthermia or radiation therapy. Further in vitro and animal studies with this agent are clearly warranted.

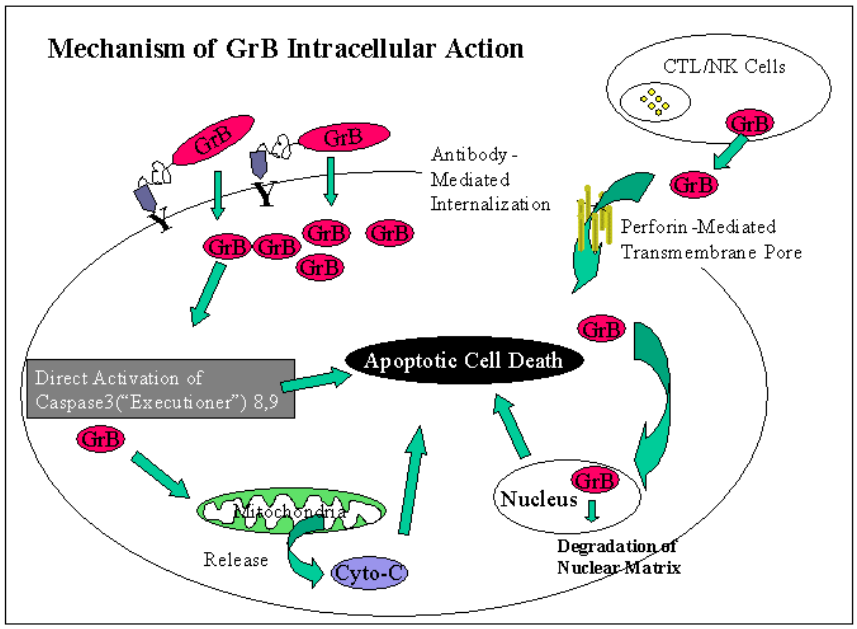


Figure 18. GrB intracellular activity can be produced by both perforin- and antibody-mediated internalization.

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