Selective Cytotoxicity to HER2-Positive Tumor Cells by a Recombinant e23sFv-TD-tBID Protein Containing a Furin Cleavage Sequence

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Abstract

Purpose: The HER2 antigen is a recognized target on breast cancer cells for immunotherapy. To overcome the immunogenicity and systemic toxicity of traditional immunotoxins, a novel human immunoproapoptotic molecule was developed and its antitumor activity was investigated.

Experimental Design: Recombinant e23sFv-TD-tBID, consisting of a single-chain anti-HER2 antibody fragment linked to a human active truncated Bid by a 10-amino acid residue furin cleavage sequence, was bacterially expressed. Purified e23sFv-TD-tBID was tested for binding, internalization, and cytotoxic activity in cell and for tumor localization and antitumor activity in athymic nude mice bearing established human tumor xenografts.

Results: e23sFv-TD-tBID selectively binds to HER2-positive cells and induces apoptotic cell death in vitro and in vivo. An investigation of its mechanism of action has revealed that e23sFv-TD-tBID was internalized on binding to the surface of HER2-positive tumor cells, proteolytically cleaved and transported directly to cytosol. The antitumor activity of e23sFv-TD-tBID was shown in a dose-dependent manner when injected i.p. into immunodeficient mice bearing human breast carcinomas. Moreover, this immunoproapoptotic protein, either given as a single dose or in combination with chemotherapy agents, significantly inhibited tumor growth without any observed toxic side effects on mice. Magnetic resonance imaging further showed the specific targeting and good penetration of tumors by e23sFv-TD-tBID in vivo. The therapeutic value of e23sFv-TD-tBID to human was shown by its cytotoxic effects on primary patient-derived breast tumor cells but not on endothelial cells.

Conclusion: These data suggest that recombinant e23sFv-TD-tBID has therapeutic potential for HER2-positive tumors and warrant further testing for clinical applications. Clin Cancer Res; 16(8): 2284–94. ©2010 AACR.

The HER2/neu proto-oncogene encodes a 185-kDa transmembrane glycoprotein (p185HER2) belonging to a subfamily of growth factor receptors with intrinsic tyrosine kinase activity (1, 2). Overexpression of the HER2/neu gene is found in 25% to 30% of primary human breast cancers and is associated with a poor clinical outcome (3). Increased HER2 expression is also observed in colon, prostate, lung, and gastric cancers (4–7). Because of preferential expression in tumor cells and extracellular accessibility, HER2 was recognized as an attractive target for antibody-directed therapy. The humanized HER2 antibody trastuzumab (Herceptin) was the first HER2-targeted therapeutics approved by the Food and Drug Administration for the treatment of metastatic breast cancer. Immunotoxins are targeted fusion toxins designed for cancer therapy, which are composed of the cytotoxic domain of natural toxins chemically or genetically linked to target cell-selective peptide ligands such as monoclonal antibodies (mAb), antibody fragments, growth factors, or cytokines. Immunotoxins combine the potency of the toxin with the specificity of the attached ligand to kill cancer cells. The most potent immunotoxins are made from bacterial or plant toxins. To date, recombinant immunotoxins have been successfully used in clinical trials for hematologic malignancies (8–11). However, the clinical use of immunotoxins for treatment of solid tumors is severely limited...
by the poor penetration (12), nonspecific cytotoxicity (13), and immunogenicity (14) of the toxin component. Strategies to overcome these limitations are being pursued, including the use of human or mammalian toxins such as RNases (15) or proapoptotic proteins (16). Based on the previously constructed immunotoxin e23sFv-PEA40 (17), we developed a set of recombinant immunoproteasome molecules containing anti-HER2 single-chain variable antibody fragment (scFv), the translocation domain (domain II) of Pseudomonas exotoxin A, and different proapoptotic molecules such as active caspase-3, caspase-6, and granzyme B of human origin for targeted gene therapy of HER2-positive tumors and warrants further testing for clinical applications.

Materials and Methods

Construction of the recombinant cDNA encoding e23sFv-TD-tBID. A cDNA encoding e23sFv-TD-tBID was engineered by two successive PCRs. The PCR product of BIDΔ1-60 with a furin cleavage sequence generated by using primers A (5′-CATGACATGGCGATCCAGCTGACCCAGTCT-3′) and B (5′-TTTGGCGCCGGAAGCTGAAATGATGAGGACATCTGGGCCGCAACCGAGGC-3′) was cloned downstream of e23sFv sequence in pCMV (27). Primers A and C (5′-TTTTCTAGATGCACITATGTCATCCCATTTCTGGC-3′) were then used to amplify the whole coding sequence. The assembled gene was cloned in pQE30 (Qiagen) and confirmed by sequencing.

Expression, purification, and refolding of recombinant proteins. e23sFv-TD-tBID protein was expressed in E. coli M15 cells by 1 mmol/L isopropyl-1-thio-β-galactopyranoside (IPTG) induction 4 h at 37°C. The isolated inclusion bodies were resuspended in solubilizing buffer B with 8 mol/L urea and incubated at 4°C for 1 h. After centrifugation and filtration, the supernatant was applied to the Ni²⁺ chelate affinity column at a flow rate of 0.5 ml/min. Nonspecifically bound proteins were removed by extensive washing with buffer B, 25 and 100 mmol/L imidazole. Bound proteins were eluted with 250 mmol/L imidazole in buffer B and refolded by dialyzing against buffers with decreasing denaturant in 0.1 mol/L NaCl and 20 mmol/L Tris-HCl (pH 7.5): first 6 mol/L urea, then 4 mol/L urea, then 2 mol/L urea and 1 mol/L EDTA, and finally PBS (pH 7.4). Dialysis was done at 4°C for 16 h with three buffer changes. After each step, samples were centrifuged and supernatants were subjected to the next round of dialysis. The refolded proteins were quantitated by bicinechonic acid assays (Pierce).

Western blot analysis. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham Biosciences). Membranes were incubated with primary antibodies that recognize His (1:5,000; Qiagen), Bid (1:1,000; Cell Signaling Technology), and HER2 (1:500; Lab Vision and NeoMarkers) overnight at 4°C in PBS-Tween 20. After incubation with horseradish peroxidase–conjugated secondary antibody (1:2,000; ZhongShanJinQiao), Western blots were visualized using an enhanced chemiluminescence kit (Pierce).

Cell culture. The human breast cancer cell lines SK-BR-3, MDA-MB-453, and MCF7 and ovarian cancer cell SKOV-3
Isolation of primary tumor cells from pleural effusion.

After obtaining informed consent, primary breast cancer cells were collected from pleural effusion of a patient diagnosed with medullary carcinoma with liver, bone, and pleura metastases, HER2(+++), ER(−), PR(−). Cells in pleural fluid diluted with RPMI 1640 were centrifuged, and the pellet was washed twice with PBS. Cells were then collected from the interface after centrifuging over a Ficoll density gradient (28, 29). The freshly isolated primary breast cancer cells were resuspended in RPMI 1640 with 10% fetal bovine serum, 50 μg/mL penicillin, and 100 μg/mL streptomycin and identified as HER2 positive.

Cellular ELISA.

HER2-positive SK-BR-3 cells and HER2-negative human umbilical vascular endothelial cells (HUVEC) were seeded in a 96-well plate (1 × 10^5 per well). After blocking with PBS/6% bovine serum albumin, the plate was incubated with 1 μg/mL purified e23sFv-TD-tBID in ELISA buffer (PBS/3% bovine serum albumin) for 90 min. The cells were then washed twice and incubated with anti-His mAb and horseradish peroxidase anti-mouse IgG for 1 h each. After applying 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich), binding was determined from 450-nm absorbance values and reported as the mean of at least three determinations.

Flow cytometric analyses.

Suspensions of 1 × 10^5 cells were washed with PBS buffer containing 0.2% bovine serum albumin and 0.05% sodium azide and incubated in 100 μL of 1 μg/mL purified e23sFv-TD-tBID for 45 min on ice. After washing, cells were incubated with 1:3,000 anti-His mAb for 25 min on ice and 1:100 FITC-linked secondary antibody for 20 min at 4 °C. The cells were analyzed by flow cytometry (BD Bioscience).

Internalization of e23sFv-TD-tBID.

Cells grown on coverslips to 60% confluence were incubated with e23sFv-TD-tBID (100 μg/mL) for 2 h at 37 °C or 4 °C. Cells then were washed, fixed, and permeabilized. Intracellular e23sFv-TD-tBID was detected with anti-His mAb, followed by Cy3-anti-mouse IgG. Cells were imaged by confocal microscopy (FluoView FV1000, Olympus).

In vitro cytotoxicity of e23sFv-TD-tBID.

Cells were seeded in 96-well plates at 5,000 per well. After 24 h, serial dilutions of purified protein in 0.2% human serum albumin were added to the cells and incubated at 37 °C for another 48 h. The effect of e23sFv-TD-tBID on cell growth was determined by using MTT assays. Cell survival was expressed as percentage of viable cells in the presence of the protein compared with control cells.

Assessment of apoptosis by monitoring exposure of phosphatidylserine.

An early event in apoptosis is exposure of phosphatidylserine on the outer cellular membrane. This feature was analyzed by flow cytometry using an Annexin V-FITC/propidium iodide (PI) kit (BD Pharmingen).

Assessment of apoptosis by monitoring loss of mitochondrial membrane potential.

Mitochondrial membrane potential was assessed by flow cytometry using a TMRM kit (Molecular Probes) and a marker for the outer mitochondrial membrane (CASPASE-3, BD Biosciences).
(ΔΨ) was analyzed by flow cytometry using the cell-permeant green fluorescent lipophilic dye DiOC₆ (Molecular Probes), which is actively taken up by intact mitochondria of living cells only. After treatment, cells were harvested by centrifugation (1,000 × g, 5 min), incubated for 30 min at 37°C with fresh medium containing 0.1 μmol/L DiOC₆, washed once with PBS, and analyzed by flow cytometry.

**In vivo antitumor activity.** Female BALB/c athymic mice (4-6 wk old) were inoculated s.c. with 5 × 10⁶ human breast cancer SK-BR-3 cells in the right mammary fat pad. Eight days later, when tumors reached ~50 mm³, six animals were randomly assigned to each treatment group and treated i.p. with therapeutic agents. The dosage of these agents tested was as follows: e23sFv-TD-tBID (5 mg/kg, days 0, 2, 4, 6, and 8), Herceptin (10 mg/kg, days 0, 2, 4, 6, and 8), the taxane drug paclitaxel (15 mg/kg, days 1-3), the anthracycline antibiotic doxorubicin (5 mg/kg, day 1), and PBS as control. During the treatment, tumor volumes (V) were measured and calculated using the formula of rational ellipsoid: $V = \frac{A \times B^2}{2}$, where A is the axial diameter and B is the rotational diameter. The mice were weighed, and tumor sizes were measured two to three times per week. Survival curve plots and Kaplan-Meier analysis were done using Prism 5 (GraphPad Software).

**Contrast-enhanced magnetic resonance imaging of tumor-bearing mice.** The contrast agent Gold Magnetic nanoparticle conjugated with e23sFv-TD-tBID was prepared by LifeGen Co. Magnetic resonance imaging (MRI) was done on a 3.0T superconducting unit (Siemens magnetom trio#tim3.0T) using loop coils. T2-weighted SE coronal images were obtained with the following parameters: repetition time, 4,000 ms; echo time, 90 ms; slice thickness, 3 mm; acquisition matrix, 448 × 448; field of view, 150 mm; and number of signal averaging, 6.

**Results**

Recombinant e23sFv-TD-tBID protein was expressed and purified from E. coli. The chimeric protein (Fig. 1A) is
composed of anti-HER2 e23sFv and active tBID separated by a 10-residue sequence containing a furin cleavage site (A187GNRVRSSVG196) from DT translocation domain. The recombinant cDNA was cloned in a T5 promoter-based E. coli expression vector (pQE30) equipped with a hexahistidine affinity tag for purification. The recombinant plasmid was transformed into E. coli, and the IPTG-induced target proteins were detected by SDS-PAGE. As shown in Fig. 1B, the observed molecular weight of e23sFv-TD-tBID was ∼43 kDa, consistent with the expected size. These fusion proteins, mainly existing in inclusion bodies (Fig. 1B, lane 2), were solubilized with 8 mol/L urea and purified by Ni²⁺ chelate affinity chromatography under denaturing conditions (Fig. 1C). The proteins were then refolded by dialysis against gradually decreased urea concentrations (from 4 to 2 mol/L) and finally in PBS (pH 7.4). The purity of the target protein reached >90%, and it was further analyzed by Western blot with anti-His and anti-Bid antibodies (Fig. 1D).

**Purified e23sFv-TD-tBID protein could bind and subsequently be internalized into HER2-positive tumor cells.** Binding of purified e23sFv-TD-tBID protein to HER2-positive cells was investigated by cellular ELISA and flow cytometry. As shown in Fig. 2A, purified e23sFv-TD-tBID protein bound to HER2-overexpressing breast cancer cell SK-BR-3 but not to HER2-negative HUVECs. To further analyze the binding specificity of e23sFv-TD-tBID proteins, flow cytometric and competitive flow cytometric analyses were done. The data showed that e23sFv-TD-tBID protein could bind to the surfaces of all five HER2-positive cells (SK-BR-3, MDA-MB-453, MCF7, SKOV-3, and SGC7901). Although we detected various levels of e23sFv-TD-tBID binding to the different cells due to the differences in HER2 expression, no binding to HER2-negative HUVECs was detected (Fig. 2B), confirming the specificity of the recombinant e23sFv-TD-tBID for HER2. Furthermore, binding of the e23sFv-TD-tBID protein was highest with the HER2-overexpressing SK-BR-3 cell line. This interaction was completely blocked by addition of 10 μg/mL anti-HER2 mAb (Fig. 2C), indicating that the attachment of tBID to anti-HER2 scFv did not alter the binding specificity of e23sFv.

To exert its cytotoxicity, internalization of e23sFv-TD-tBID is required. When SK-BR-3 cells were incubated with e23sFv-TD-tBID at 4°C for 2 hours, the protein was largely found on the cell surface; however, at the permissive temperature of 37°C, the fusion protein was internalized into cytoplasmic vesicle structures (Fig. 2D), which were identified as early endosomes by their pattern of distribution (26). These observations indicated that the fusion proteins bound the cell surface and were internalized.

**Purified e23sFv-TD-tBID protein recognized breast cancer cells from patients and preserved the ability to kill HER2-positive tumor cells after incubation in human serum.** Binding of e23sFv-TD-tBID to freshly isolated breast cancer cells was investigated by immunofluorescence microscopic analysis of paraformaldehyde-fixed primary cells after confirming the expression level of HER2 (Supplementary Fig. S1; Fig. 3A). With his-NusA protein serving as a negative control, specific staining with e23sFv-TD-tBID protein was observed.
was detected on primary breast cancer cells (Fig. 3B), suggesting that the hexahistidine tag at the NH$_2$ terminus of e23sFv-TD-tBID did not affect its binding properties.

The stability of immunoproapoptotic protein under physiologic conditions is required for their potential as therapeutic agents. To estimate the stability of e23sFv-TD-tBID, we incubated purified e23sFv-TD-tBID proteins in human serum at 37°C for varying time periods before testing their integrity as a functional protein determined by cytotoxicity assays on SK-BR-3 cells. The e23sFv-TD-tBID proteins retained nearly full cytotoxic activity after incubation in human serum for up to 48 hours (Supplementary Fig. S2), suggesting that they were folded properly and very stable under those conditions.

Purified e23sFv-TD-tBID protein could induce HER2-positive tumor cell death, which was dependent on furin activity and could be blocked by endosome/lysosome inhibitor bafilomycin A1. To characterize the cytotoxicity of the recombinant e23sFv-TD-tBID in vitro, we evaluated the proliferation of several HER2-positive cells after incubation with escalating doses of e23sFv-TD-tBID. Growth inhibition of HER2-positive cells was documented by MTT assays. Cell-killing effects were observed against a variety of HER2-positive cell lines, with a calculated IC$_{50}$ of ~25 ng/mL on SK-BR-3 cells, 48 ng/mL on MDA-MB-453 cells, 35 ng/mL on SKOV-3 cells, and 40 ng/mL on SGC7901 cells (Fig. 4A). Furthermore, e23sFv-TD-tBID did not affect the growth of HER2-negative HUVECs at concentration up to 1 µg/mL (Fig. 4A) or even up to 10 µg/mL (data not shown). These results suggest a correlation between surface expression levels of HER2 and sensitivity of cells to e23sFv-TD-tBID.

The effects of e23sFv-TD-tBID on the induction of apoptosis of both HER2-positive breast cancer cell lines and primary breast cancer cells were examined by flow cytometry. Treatment of HER2-positive and HER2-negative cells with 1 µg/mL e23sFv-TD-tBID for 12 hours resulted in pronounced induction of apoptosis as measured by Annexin V-FITC and PI staining (Fig. 4B). e23sFv-TD-tBID-mediated apoptosis was characterized by the reduction of mitochondrial transmembrane potential (Fig. 4C). This...
suggests that the observed cytotoxic effect of this fusion protein is mediated through an apoptotic mechanism. Immunotoxins bind specifically to the cell surface target, and the pathway of internal translocation to an intracellular compartment for cytotoxic activity is different for different toxins (30–32). Here, we investigated the pathway of e23sFv-TD-tBID from the extracellular space to the cytosol. SK-BR-3 cells were treated with e23sFv-TD-tBID (100 ng/mL) with or without the specific endosome/lysosome inhibitor bafilomycin A1 (baf; 10 nmol/L), the furin inhibitor dec-RVKR-cmk (25 μmol/L), or the protein transport inhibitor brefeldin A (BFA; 10 nmol/L). After 48 hours, baf and dec-RVKR-cmk inhibited the cell-killing activity of e23sFv-TD-tBID, but BFA did not influence its activity (Fig. 4D). Because inactivation of endosome/lysosome or furin blocks its entry to the cytosol and its cytotoxic activity, we conclude that internalized e23sFv-TD-tBID, after cleavage by furin in the endosome, was directly translocated to the cytosol.

**Purified e23sFv-TD-tBID proteins inhibited HER2-positive tumor growth without toxic side effects.** The in vivo therapeutic effects of e23sFv-TD-tBID were tested in nude mice bearing human breast cancer xenografts. SK-BR-3 cells (5 × 10^6) were implanted into the fat pad of nude mice. Treatment was started on day 8 when the tumors reached ∼50 mm^3. Animals were given three single i.p. injections with 1, 5, or 10 mg/kg every other day for a total of five doses. The control groups received PBS. Data shown in Fig. 5A showed that the tumor growth was obviously inhibited in all the three groups after e23sFv-TD-tBID treatment in a significant dose-dependent way as the tumor growth in 5 or 10 mg/kg almost stopped (Fig. 5A).

![Figure 5](cancercancerres.aacrjournals.org) Antitumor activity of e23sFv-TD-tBID in vivo. A, antitumor effect of i.p. injections of e23sFv-TD-tBID doses of 1, 5, or 10 mg/kg on SK-BR-3 tumors growing in nude mice. Mice were s.c. injected with 5 × 10^6 SK-BR-3 cells, and treatment was initiated when tumor volume reached ∼50 mm^3. Points, mean tumor volume versus time; bars, SD. Arrows showed the day intervals of e23sFv-TD-tBID injection. B, effects of combined treatment with e23sFv-TD-tBID and chemotherapeutic drugs on HER2-overexpressing breast carcinoma xenografts. Mice (n = 6/group) were s.c. injected with 5 × 10^6 SK-BR-3 cells, and treatment was initiated when tumor volume reached ∼50 mm^3. C, Kaplan-Meier survival curve. D, TUNEL assay of apoptotic cells in tumor tissues from e23sFv-TD-tBID-treated mice. PBS served as negative control.
Fig. 6. Toxicity and specificity of e23sFv-TD-tBID on mice. A, toxicity of e23sFv-TD-tBID on mice monitored by animal weight loss. B, H&E staining of liver, kidney, spleen, lung, and heart tissues from xenograft mice treated with e23sFv-TD-tBID. Magnification, ×200. C, immunohistochemical staining was done on cancer, liver, kidney, spleen, lung, and heart tissues, and there is no e23sFv-TD-tBID detected besides cancer tissue from e23sFv-TD-tBID–treated group. Magnification, ×200. D, contrast-enhanced three-dimensional MRI of mice bearing human breast tumors treated with 200 μL of MR contrast agent Gold Magnetic nanoparticle coupled with (b) or without (a) e23sFv-TD-tBID (protein to chelator ratio of 1:2) at preinjection and 6, 12, and 24 h after i.v. administration. Note the extensive uptake in the tumors.
The taxane drug paclitaxel and the anthracycline antibiotic doxorubicin are two of the most active chemotherapeutic agents for breast cancer treatment. Treatment with e23sFv-TD-tBID, Herceptin, paclitaxel, or doxorubicin significantly slowed the tumor growth compared with the control group (P < 0.01). Doxorubicin or paclitaxel combined with e23sFv-TD-tBID significantly reduced tumor volumes compared with either agent alone (P < 0.05), indicating that combination therapy with e23sFv-TD-tBID enhanced the antitumor activity of chemotherapy (Fig. 5B). Kaplan-Meier survival analysis showed that mice treated with e23sFv-TD-tBID, Herceptin, or chemotherapeutics lived significantly longer than untreated mice (Fig. 5C). The average survival time of mice treated with chemotherapeutic agents was shorter than e23sFv-TD-tBID–treated or Herceptin-treated groups, likely due to the higher systemic toxicity of chemotherapeutic agents. Apoptotic cells in tumor tissues were identified by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay (Fig. 5D). The data in Fig. 5B and C also showed that treatment with 5 mg/kg of e23sFv-TD-tBID could get the same effect as Herceptin in 10 mg/kg.

The animal toxicity at each of these dose levels was evaluated by body weight of the mice, and no significant differences were seen (Fig. 6A). Compared with untreated controls, the level of alanine aminotransferase/aspartate aminotransferase activity in the plasma of treated mice did not change significantly (data not shown). Consistent with these findings, pathologic changes neither in liver nor in any other tissue were detected with histologic analysis after e23sFv-TD-tBID treatment (Fig. 6B). By immunohistochemical staining, no cross-reactivity was seen in liver, kidney, spleen, lung, and heart tissues (Fig. 6C). However, as shown in Supplementary Fig. S3, e23sFv-TD-tBID could still recognize mouse HER2 on mouse mammary tumor EMT6 cells by immunofluorescence staining.

To measure tumor deposition, mice bearing human breast tumors were injected i.v. with e23sFv-TD-tBID conjugated to Gold Magnetic nanoparticles. Their localization at different time points is shown in Fig. 6D. The low signal intensity in the tumor tissue was shown by three-dimensional MRI within 6 hours after injection. The signal intensity gradually increased 24 hours later. Accumulation of e23sFv-TD-tBID was greatest in tumors with relatively little normal tissue distribution (background levels). These results showed that e23sFv-TD-tBID can quickly accumulate and efficiently penetrate into tumor masses. We conclude that e23sFv-TD-tBID can specifically target the HER2-overexpressing tumors with potent antitumor activity but without damaging normal tissues.

**Discussion**

In this study, human immunopropaoptotic molecules were designed to circumvent some of the problems encountered with earlier immunotoxins, such as dose-limiting toxicities presenting as vascular leak syndrome, thrombocytopenia, hepatic damage, and immunogenicity of powerful bacterial or plant toxins. HER2 has long been recognized and considered as a promising target for immunotherapeutic applications, including mAbs (33), vaccines (34), and immunotoxins (35, 36).

Bid is the molecular linker bridging various peripheral death pathways to the central mitochondria pathway. Bid cleavage and activation can be exerted by different pro tease, such as caspase-8, granzyme B, calpain, or cathepsin B, suggesting Bid as a key factor in many paradigms of cell death (37). Truncated Bid is much more potent than full-length Bid in activating Bax or Bak, leading to mitochondrial function loss and the release of multiple proapoptotic factors. The 15-kDa tBid is smaller than other proapoptotic molecules (e.g., caspase-3, caspase-6, granzyme B, and AIF) and may facilitate better tumor penetration, improve pharmacokinetics, and reduce immunogenicity. Such a protein would be preferred in the construction of an immunopopaoptotic agent.

Our long-term goal is to develop humanized immunotoxins for cancer therapy. The fusion protein we describe here is an improved form of larger immunopropaoptotic molecules previously constructed with exogenous translocation domains. e23sFv-TD-tBID consists of an anti-HER2 scFv and the truncated form of Bid. The 10–amino acid DT translocation domain was chosen to link e23sFv and tBID because the proteolytically cleavable spacer was shown to enhance cell-killing activity (38, 39). We used a prokaryotic expression system to produce biologically active e23sFv-TD-tBID, which retained the binding specificity for HER2 of the parental scFv and was rapidly internalized into HER2-positive target cells.

The intracellular mechanism of e23sFv-TD-tBID was further conducted. The data show that cytotoxicity of e23sFv-TD-tBID was significantly diminished by the endosome/lysosome inhibitor and furin inhibitor but not by the trans-Golgi network inhibitor, indicating that e23sFv-TD-tBID accessed the cytosol depending on endosome/lysosome processing and furin activity and not through the trans-Golgi network (40). Based on the functional studies, we conclude that e23sFv-TD-tBID kills target cells by a complex series of steps that are initiated by binding to cell surface, followed by endocytosis, proteolytic processing, and translocaton into the cytosol. In cytosol, active tBID anchors the mitochondrial membrane, leading to irreversible mitochondrial damage and the release of cytochrome c to induce apoptotic cell death. Here, we report for the first time the mechanism of the antitumor action of this human immunopropaoptotic protein and contribute to the rational design of immunoconjugates with improved properties.

Of therapeutic interest, the immunopropaoptotic proteins we prepared effectively killed HER2-positive cell lines and primary patient-derived breast cancer cells without affecting the endothelial cell growth. e23sFv-TD-tBID inhibited proliferation of HER2-overexpressing SK-BR-3 cells at an IC50 of 25 ng/mL. The antitumor activity of e23sFv-TD-tBID was also shown in a dose-dependent manner in vivo without any obvious drug-related toxic reactions at doses
up to 10 mg/kg. Furthermore, in combination with chemotherapy, e23sFv-TD-tBID therapy of solid tumors may achieve better response, potentially reducing the required drug dosage and lowering systemic toxicity. Of critical importance to the eventual clinical development of e23sFv-TD-tBID will be examination of the maximum tolerated dose toxicity profile and efficacy studies of this fusion protein in other well-characterized human HER2-positive xenograft models.

Based on previous findings (41, 42) that suggested a correlation between MRI signal changes and drug distribution, we initiated the development of tumor-specific contrast agents to evaluate the distribution of the therapeutic agents. MRI confirmed good tumor-targeting properties and penetration of e23sFv-TD-tBID. Because of its small molecular weight, e23sFv-TD-tBID targeted tumors rapidly, and quickly cleared from the blood, leading to a lower exposure overall to normal tissues.

In conclusion, we have produced and characterized a novel immunoproapoptotic protein, e23sFv-TD-tBID. The potent and selective cytotoxicity in vitro, favorable tumor localization properties, as well as the powerful antitumor effect of e23sFv-TD-tBID in vivo suggest its potential in clinical application in the treatment of HER2-positive carcinomas. Because e23sFv-TD-tBID is fully humanized, stable, and selectively cytotoxic to target cells and can be produced in large amounts, it deserves further development for cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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