Receptor-directed chimeric toxins created by sortase-mediated protein fusion

Andrew J. McCluskey* and R. John Collier

Department of Microbiology and Immunobiology, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115

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*Address correspondence to: Andrew J. McCluskey, Department of Microbiology and Immunobiology, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, Tel: (617) 432-1931, Fax: (617) 432-0115, E-mail: andrew_mccluskey@hms.harvard.edu

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Abstract

Chimeric protein toxins that act selectively on cells expressing a designated receptor may serve as investigational probes and/or antitumor agents. Here we report use of the enzyme sortase A (SrtA) to create 4 chimeric toxins designed to selectively kill cells bearing the tumor marker, HER2. We first expressed and purified: (i) a receptor recognition-deficient form of diphtheria toxin (DT) that lacks its receptor binding domain and (ii) a mutated, receptor-binding-deficient form of anthrax protective antigen (PA). Both proteins carried at the C terminus the sortase recognition sequence LPETGG and a H₆ affinity tag. Each toxin protein was mixed with SrtA plus either of two HER2-recognition proteins—a single-chain antibody fragment or an Affibody—both carrying an N terminal G₅ tag. With wild-type SrtA the fusion reaction between the toxin and receptor-recognition proteins approached completion only after several hours, whereas with an evolved form of the enzyme, SrtA*, the reaction was virtually complete within 5 minutes. The four fusion toxins were purified and shown to kill HER2-positive cells in culture with high specificity. Sortase-mediated ligation of binary combinations of diverse natively folded proteins offers a facile way to produce large sets of chimeric proteins for research and medicine.
Introduction

Many proteinaceous toxins, such as diphtheria and anthrax toxins, act on mammalian cells through a complex sequence of events that begins with binding to a cell-surface receptor.

For diphtheria toxin (DT) to act on mammalian cells it must be proteolytically cleaved into two, disulfide-linked polypeptide chains, A and B, before or after binding to its receptor HB-EGF (1). The DT:HB-EGF complex is endocytosed and delivered to the endosomal compartment, where the B chain inserts into the membrane under the influence of acidic pH (2). This insertion mediates translocation of the catalytic A chain (DTA) across the membrane to the cytosolic face, where reduction of the disulfide linking it to the B chain allows it to be released into the cytosol (3,4). There, the DTA ADP-ribosylates eukaryotic elongation factor-2, blocking protein synthesis and inducing apoptotic cell death (5-7).

Like DT, anthrax toxin acts by modifying intracellular targets and enters cells via the endosomal compartment. However, anthrax toxin is structurally different from DT and is composed of three large (80-90 kDa) nontoxic, monomeric proteins, which interact noncovalently in binary or ternary combinations to cause toxic responses (8-10). Two of the proteins are enzymatic effectors, Lethal Factor (LF) and Edema Factor (EF), which modify cytosolic targets (11-13). The third protein, Protective Antigen (PA), is responsible for binding multiple receptors (14-16), pore-formation, and transport of LF and EF from the extracellular milieu to the cytosol (17).

In these two toxins and others the receptor, or receptors, are widely distributed among tissues, allowing the toxin to act on a variety of cell types within a mammalian host. If the receptor-binding function can be disrupted by mutation or chemical modification, there is the possibility of changing receptor specificity by linking the toxin to a heterologous receptor-
binding protein (RBP). This fundamental strategy has served as the basis of efforts over many years to develop antineoplastic toxins that selectively kill tumor cells displaying high levels of a specific receptor on their surface (18,19).

While chemical methods have also been used to join polypeptides, recombinant DNA has become the method of choice in recent years to generate homogeneous populations of chimeric toxin fusions (19). For recombinant chimeric proteins to be produced in active form, however, neither of the fused entities must interfere with folding of the other. Also, because the physical chemical properties of no two chimeric proteins are identical, purification of each requires special tailoring. Finally, production of some chimeric toxins may be restricted by biosafety concerns.

The discovery of sortases (20) and ways to adapt them to fuse polypeptides presents an alternative path to create chimeric proteins that avoids these disadvantages. Sortases are sequence-specific transpeptidases that covalently anchor surface proteins to the cell wall of gram-positive bacteria (20,21). Sortase A (SrtA) from *Staphylococcus aureus* catalyzes the cleavage of a short peptide recognition motif (LPXTG) with the concurrent formation of a covalent linkage between the protein carrying this sequence and an oligoglycine-containing substrate (22-24).

Our group and others have demonstrated that targeting the actions of protein toxins to cancer cells, using chemical and recombinant technologies, is an effective approach to treat cancer (18,19,25). In the current study, we have used SrtA-mediated fusion of appropriately tagged, natively folded substrate proteins to create 4 discrete chimeric toxin proteins, all directed to HER2, a cell-surface marker overexpressed on cells in a variety of human cancers (breast, ovarian, gastric). We first synthesized two HER2-specific RBPs in *Escherichia coli* and purified
them. Both RBPs — a small antibody mimic, or Affibody, known as ZHER2 (26), and a single-chain antibody fragment (scFv), termed 4D5 (27) — carried a penta-Gly sequence at the N terminus. We then used SrtA to fuse each RBP to either a receptor recognition-deficient form of DT (mDT) carrying a SrtA-recognition sequence at its C terminus, or alternatively, to mPA, a mutated receptor-binding-deficient form of PA carrying the same C-terminal SrtA-recognition sequence. Our findings demonstrate both the ability of SrtA to fuse structurally diverse pairs of proteins and the value of an evolved form of SrtA, SrtA*, to bring the fusion reaction to completion within a few minutes. The approach described offers the possibility of creating large sets of chimeric toxins or other fusion proteins rapidly from subsets of tagged substrate polypeptides.
Materials and Methods

Materials - Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). A plasmid encoding the gene sequence for anti-HER2 4D5 scFv (4D5) was a generous gift from Gregory Poon (Washington State University, Pullman, WA). The WT SrtA and SrtA* expression plasmids were supplied by Brad Pentelute (MIT, Cambridge, MA). All chemicals were from Sigma Aldrich, unless otherwise stated. The A431 cell line was from ATCC (cat. no. CCL-1555; Manassas, VA) and the JIMT-1 cell line was from AddexBio (cat. no. C0006005; San Diego, CA). BT-474 and MDA-MB-468 cell lines were provided by Jean Zhao (Dana Farber Cancer Institute, Boston, MA) and MDA-MB-231 line by Gregory Poon. The authors did not authenticate the cell lines, but fluorescence-activated cell sorting (FACS) validated HER2 receptor levels. Cells were frozen upon receipt and only low passage number cells were used.

Cell line maintenance - A431 and JIMT-1 cells were maintained in DMEM supplemented with 10% FCS, 500 units/ml penicillin G and streptomycin sulfate (Invitrogen, Carlsbad, CA). All other cell lines were grown in RPMI medium (Invitrogen) supplemented with 10% FCS, 500 units/ml penicillin G and streptomycin sulfate.

Molecular cloning - mDT (residues 1-387 of DT) was cloned into the petSUMO vector (Invitrogen) with a C-terminal glycine-serine repeat ([GS]₃) linker, SrtA recognition motif (LPETGG), and hexa-histidine tag, following standard procedures. mPA, harboring a double mutation (N682A/D683A), was created as described (28,29) and cloned into the pet22b vector (Novagen) with the same C-terminal [GS]₃-linker, SrtA recognition peptide, and His₆-tag.
LPETGG was chosen as the SrtA recognition motif because SrtA more rapidly turns over substrates with a G in the P2’ position (30).

Aminoglycine pentapeptides (G₅) were recombinantly fused to RBPs: a HER2 specific Affibody, ZHER2₃₄₂ (abbreviated ZHER2), and an anti-HER2 scFv (termed 4D5) containing a 24 amino acid peptide linker between the V₅ and V₇ domain (31), by PCR and cloned into the petSUMO vector (Invitrogen).

Protein expression and purification - All proteins were expressed and purified from the BL21(DE3) strain of E. coli (New England Biolabs, Ipswich, MA), under the induction of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), for 2 h at 30 ºC. WT SrtA and SrtA* (harboring mutations P94S/D160N/K196T]) both lacking the membrane-spanning domain (residues 1-58) were expressed and purified as described by Pentelute and co-workers (32). mPA-LPETGG-His₆ was purified from the periplasm as previously described (28,33).

mDT-LPETGG-His₆, G₅-ZHER2, and G₅-4D5 were expressed from the petSUMO vector (Invitrogen) as His₆-SUMO fusions. Cell pellets were lysed by sonication in lysis buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10mM imidazole, 10 mg lysozyme, 2 mg DNAse I, supplemented with a Roche complete protease inhibitor). His-tagged proteins were bound to Ni²⁺-NTA resin, washed with wash buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, and 20 mM imidazole), and eluted with wash buffer supplemented with 250 mM imidazole. The resulting purified proteins were exchanged into imidazole-free buffer (20 mM Tris-HCl, pH 8.0, and 150mM NaCl) and cleaved by SUMO protease for 1 h at room temperature to generate mDT-LPETGG-His₆ and RBPs displaying free N-terminal oligoglycine peptides. G₅-ZHER2 and G₅-4D5 were freed from the His₆-SUMO tag by Ni²⁺ affinity chromatography. mDT-LPETGG-His₆
was separated from the His<sub>6</sub>-SUMO tag by size exclusion chromatography on a HiLoad 16/60 Superdex 75 prep grade column attached to an automated Äkta® purifier (GE Healthcare Biosciences, Pittsburgh, PA).

**SrtA reactions** - mDT-LPETGG-His<sub>6</sub> or mPA-LPETGG-His<sub>6</sub> (50 μM) was incubated with an excess of either G<sub>5</sub>-ZHER2 or G<sub>5</sub>-4D5 (200 μM). Reactions were catalyzed by 5 μM WT SrtA or SrtA* in sortase reaction buffer (50 mM Tris-HCl, 10 mM CaCl<sub>2</sub>, 150 mM NaCl pH 7.5) at room temperature.

**Purification of mTx-RBP conjugates** - mTx-RBP fusions were purified from 0.5 ml reactions by sequential Ni<sup>2+</sup>-NTA and size exclusion chromatography steps (Fig. 1a). Ni<sup>2+</sup>-NTA resin (250 μl) was added to the ligation reactions to bind the His<sub>6</sub>-tagged unreacted mTx substrate and SrtA* enzyme. The flow-through fraction was collected, and the resin was washed with an additional 1 ml of wash buffer. The flow-through and wash fractions were pooled and mTx-RBP fusions were separated from unreacted RBP using a HiLoad 16/60 Superdex 200 prep grade size exclusion chromatography column.

**Cytotoxicity and competition assays** - Cells were plated in appropriate medium at densities of 3-3.5x10<sup>4</sup> cells/well in 96 well plates and incubated overnight at 37 °C. The following day, cells were exposed to medium supplemented with the toxin conjugate or toxin mixture. For mDT-variants, cells were exposed to eight 10-fold serial dilutions (starting with a final concentration of 100 nM) for 24 h. For mPA-variants, cells were exposed to 20 nM mPA-ZHER2 or mPA-4D5 plus a 10-fold serial dilution of LF<sub>N</sub>-DTA (starting with a final concentration of 100 nM) for 4
hr. After the incubation period, toxin-containing medium was removed and replaced with leucine-deficient medium supplemented with 1 µCi of [3H]-leucine/ml (Perkin Elmer, Billerica, Massachusetts) and incubated for an additional hour. Plates were washed twice with cold PBS (200 µl) prior to the addition of 200 µl of scintillation fluid. The amount of [3H]-leucine incorporated was determined by scintillation counting using a Wallac MicroBeta TriLux 1450 LSC (PerkinElmer, Waltham, MA). Percent protein synthesis was normalized against untreated cells and plotted versus the concentration of LFN-DTA or DT-variant in GraphPad Prism.

Competition assays were performed as described above where increasing concentrations of free G5-ZHER2 or G5-4D5 were added to medium containing 20 nM mPA-ZHER2/mPA-4D5 plus LFN-DTA (1 nM) and exposed to BT-474 cells for 4 h. Percent protein synthesis was normalized against untreated cells and plotted using GraphPad Prism.

**Co-culture cytotoxicity assay** - Cancer cell lines were seeded (3.5x10⁴ cells/well) in partitioned sections of a chambered tissue culture slide (Thermo Scientific, Rockford, IL). After an overnight incubation, the medium was removed, and the partitioning element was discarded. The slides were washed with PBS and incubated for 24 h with RPMI medium containing (i) 20 nM of mPA-ZHER2 with 10 nM LFN-DTA, (ii) 20 nM mPA-4D5 plus 10 nM LFN-DTA, (iii) 100 nM mDT-ZHER2, or (iv) 100 nM mDT-4D5. Following toxin exposure, cells were processed as previously described (25).
Results and Discussion

HER2 is overexpressed in several cancers (34-37) and is the target of FDA-approved protein therapeutics (e.g., trastuzumab and T-DM1), as well as receptor-rediredcted protein toxins in pre-clinical stages (25,38-40). Some classes of toxins, such as diphtheria toxin (DT) and anthrax toxin, have evolved an active mechanism of crossing the endosomal membrane and delivering bioactive proteins to the cytosol (17). This endosomal escape mechanism can be exploited to deliver a cytocidal enzymatic “payload”, such as the catalytic domain of DT (DTA) (5,6) used in the current work, or other bioactive polypeptides that modulate intracellular processes.

Ploegh and others have demonstrated the use of SrtA in vitro to incorporate polypeptides (41-43), biochemical handles (e.g., biotin) (44), fluorescent probes (44,45), peptide nucleic acids (30), sugars (46), lipids (47), unnatural amino acids (43), and chemical groups (32) into a number of structurally distinct proteins. Although SrtA can be expressed in E. coli and purified as a soluble enzyme (23,48), its use for in vitro protein engineering has been limited by long reaction times (typically 16 - 24 h) and the need for large quantities of enzyme (> 30 μM) to circumvent suboptimal kinetics ($k_{cat}/K_m$ LPETG = 100-200 M$^{-1}$ s$^{-1}$) (49).

Recently, Liu and coworkers evolved SrtA by yeast display to generate mutants with improved kinetics (49). Here we describe the use of wild-type SrtA and an evolved SrtA variant (SrtA*) to assemble receptor-directed chimeric protein toxins in vitro (Fig. 1). The approach requires two building blocks: (i) a mutated, receptor recognition-deficient toxin protein (mTx) containing a canonical C-terminal SrtA recognition motif (here, LPETGG), and (ii) a heterologous receptor-binding protein (RBP) carrying an N-terminal oligoglycine peptide (Fig. 1). SrtA catalyzes cleavage of the toxin moiety between Thr and Gly of the recognition peptide
and formation of a covalent bond between the carboxyl group of Thr and the amino group of the oligoglycine peptide of the RBP (Fig. 1) (23,24).

Two HER2-directed single-chain toxins were created by fusing mDT with a HER2 specific Affibody (ZHER2) (26) or humanized a single-chain antibody fragment (4D5) (27); the products were designated mDT-ZHER2 and mDT-4D5, respectively (Fig. 1). The catalytic DTA chain contained within these single-chain toxins served as a cytocidal payload that causes inhibition of protein synthesis and apoptotic cell death upon its delivery to the cytosolic compartment of sensitive cells (5,6).

We also created two HER2-directed binary toxins. First we fused ZHER2 or 4D5 to the C-terminus of mPA(28,29), yielding mPA-ZHER2 and mPA-4D5 (Fig. 1). PA, the receptor-binding pore-forming component of anthrax toxin, noncovalently binds the enzymatic components of the toxin and delivers them to the cytosol (9,10). However, the effector moieties of anthrax toxin are not cytocidal towards most cell types, therefore we combined mPA-ZHER2 or mPA-4D5 with LF_{N}-DTA, an effector protein containing the high affinity N terminal PA-binding domain of the anthrax lethal factor (LF_{N}) with DTA. LF_{N}-DTA binds to mPA-ZHER2 and mPA-4D5 and upon its delivery to the cytosol, the DTA moiety blocks protein synthesis, as with the single-chain toxins. Our previous study (25), using the same panel of cell lines, demonstrated that DTA delivery by recombinantly fused mPA-ZHER2 resulted in rapid protein synthesis inhibition and subsequent cell death via apoptosis. In the current study we used protein synthesis inhibition as a sensitive read-out for delivery of DTA to the cytosol to monitor the functions of the SrtA fusions. DTA thus served as the enzymatic effector moiety of all of the targeted toxins in our study.
The fusion reaction creating mDT-ZHER2, mDT-4D5, mPA-ZHER2 or mPA-4D5 was virtually complete within 5 min when 5 μM SrtA* was used as catalyst, whereas the same concentration of wild-type SrtA required more than 4 h to achieve the same level of fusion (compared Fig. 2A and 2B). Reaction rates showed no significant dependence on specific substrate proteins, indicating that the nature of the folded polypeptide entities to which the LPETGG and G5 tags were attached mattered little in the Srt-catalyzed reactions. SrtA* reaction rates were consistent with results reported by Ling et al. to ligate peptides with chemical groups to proteins for use in semi-synthesis strategies (32).

SrtA*-ligated fusions were purified by sequential Ni²⁺-NTA and size exclusion chromatography steps to give of virtually pure products (yield 20-65 %) (Fig. 2C; Table 1). Cancer cell lines expressing various levels of HER2, including a trastuzumab-resistant line isolated from a HER2-positive patient clinically resistant to trastuzumab (50), were incubated with either mPA-ZHER2/mPA-4D5 plus LF₅-NTA for 4 h or mDT-ZHER2/mDT-4D5 for 24 h. Following toxin exposure, protein synthesis was measured over a 1 h period. All four fusions were able to direct toxin action to HER2-positive cells, and the degree of cell killing was dependent on the level of cell surface HER2 (Fig. 3) (25,51). Cells expressing the highest levels were the most sensitive (BT-474; Fig. 3, Table 1), and HER2-negative cells, MDA-MB-468, were unaffected (Fig. 3). The specificity of SrtA*-generated toxin fusions for cells bearing the cognate receptor was confirmed, and the absence of bystander effects on cells lacking HER2 demonstrated, in experiments conducted with mixed cell populations of HER2-positive and -negative cells (Fig. 4). Some non-specific toxicity was observed for mDT-4D5 towards HER2-negative MDA-MB-468 cells in mixed cell populations. Unlike mPA and LF₅-NTA, mDT retains some residual non-specific activity towards even toxin resistant cells (52), an effect that is
reduced when the protein is attached to an RBP. The decrease in protein synthesis may indicate that 4D5, when fused to mDT, does not have the same steric effects as ZHER2 in shielding off-target toxicity of mDT in mixed populations.

Free ZHER2 Affibody competitively inhibited mPA-ZHER2-dependent cell killing, but not mPA-4D5-dependent killing (Fig. 5); and free 4D5 protected cells against mPA-4D5 plus LF_N-DTA, but not against mPA-ZHER2-dependent killing under the same conditions (Fig. 4). These findings are consistent with structural data demonstrating ZHER2 and a Fab fragment of trastuzumab (from which 4D5 is derived) recognize nonoverlapping HER2 epitopes (53).

The combinations of mPA-ZHER2 or mPA-4D5 with LF_N-DTA were 10-100 fold more potent than the corresponding DT fusions (Table 1), as the mPA chimeric toxins acted in a shorter incubation period (4 h versus 24 h) and were able to kill MDA-MB-231, a cell line expressing low levels of HER2, (Fig. 3). The difference in EC_{50} for the toxin conjugates could be a result of more efficient effector delivery by mPA, combined with its ability to deliver multiple enzymatic effectors (54).

The results we report here have demonstrated the use of Srt-based fusion to modify the receptor specificity of both a single-chain and a binary toxin. Each of 2 structurally diverse toxins was fused to two equally diverse HER2-binding proteins, underlining the versatility of SrtA-based protein fusion and its potential use for fusing a broad array of appropriately tagged proteins. Consistent with this versatility, Ploegh and coworkers were able to link DTA to cholera holotoxin to identify host factors required for intoxication (55). The fact that fusion reactions in our study reached completion within a few minutes when SrtA* was used makes this evolved form of SrtA particularly attractive.
SrtA-based protein fusion is appealing from many perspectives. i) It can circumvent potential problems in expression and/or folding of recombinantly fused polypeptides into their respective active configurations. Thus, whereas we were unable to express and purify mPA-4D5 as a single polypeptide fusion, the individual mPA and 4D5 proteins expressed, folded, and underwent rapid Srt*-mediated fusion to yield a biologically active product. ii) Srt-based fusion avoids the need to tailor a purification protocol for each individual chimeric protein, as is required when such proteins are produced recombinantly. iii) Preparation of subsets of pure, appropriately tagged fusion partners opens the possibility of easily preparing large numbers of fusions (the algebraic product of the numbers of entities in the subsets) for testing. With the protocol described here, in which both the sortase enzyme and the toxin protein substrate carried a His₆ tag, we were able to remove the enzyme, unreacted toxin protein, and the GGH₆ peptide product with a Ni²⁺-NTA column. Subsequent removal of unreacted receptor binding protein on a size exclusion column yielded the desired chimeric proteins in substantially pure form. iv) With chimeric toxins in which the individual fusion moieties are nontoxic and only the fusion product displays toxic properties, sortase-based fusion of the purified tagged substrates avoids biosafety issues that may arise in expressing the fused polypeptide *in vivo*. 
Acknowledgements

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References


Therapeutics Targeting Her2/neu. Mol Cancer Ther 2013. doi: 10.1158/1535-7163.MCT-13-0002


Table 1. SrtA* ligation reaction yields and *in vitro* activities of HER2-targeted toxin fusions on various cell lines

<table>
<thead>
<tr>
<th>Toxin</th>
<th>% Yield</th>
<th>BT-474</th>
<th>JIMT-1</th>
<th>A431</th>
<th>MDA-MB-231</th>
<th>MDA-MB-468</th>
</tr>
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<tbody>
<tr>
<td>mPA-ZHER2 + LFN-DTA c</td>
<td>35 +/- 6</td>
<td>$9.1 \times 10^{-13}$</td>
<td>$2.3 \times 10^{-12}$</td>
<td>$2.3 \times 10^{-11}$</td>
<td>$6 \times 10^{-10}$</td>
<td>$&gt;1 \times 10^{-7}$</td>
</tr>
<tr>
<td>mPA-4D5 + LFN-DTA c</td>
<td>20 +/- 3</td>
<td>$1.5 \times 10^{-12}$</td>
<td>$1.6 \times 10^{-11}$</td>
<td>$8.6 \times 10^{-11}$</td>
<td>$2.2 \times 10^{-9}$</td>
<td>$&gt;1 \times 10^{-7}$</td>
</tr>
<tr>
<td>mDT-ZHER2 d</td>
<td>67 +/- 4</td>
<td>$1.7 \times 10^{-11}$</td>
<td>$1.2 \times 10^{-9}$</td>
<td>$5.4 \times 10^{-9}$</td>
<td>$&gt;1 \times 10^{-7}$</td>
<td>$&gt;1 \times 10^{-7}$</td>
</tr>
<tr>
<td>mDT-4D5 d</td>
<td>38 +/- 9</td>
<td>$1.3 \times 10^{-11}$</td>
<td>$5.9 \times 10^{-10}$</td>
<td>$3.0 \times 10^{-9}$</td>
<td>$8.3 \times 10^{-8}$</td>
<td>$&gt;1 \times 10^{-7}$</td>
</tr>
</tbody>
</table>

a. EC$_{50}$ values were calculated in GraphPad Prism from dose-response curves (presented in Fig.3).
b. Molar ratio of the mTx-RBP product versus mTx after purification. Average of 3 independent reactions.
d. Measured by $[^3]$H-leucine incorporation after 24 h toxin exposure
Figure Legends

Fig. 1 Sortase A-mediated protein-protein ligation. A) The modified toxin (mTx) had an optimized C-terminal SrtA recognition motif (LPETGG) and a His$_6$ affinity tag connected to the protein with a $[\text{GS}]_3$-linker. The heterologous receptor-binding protein (RBP) was engineered to contain an N-terminal pentaglycine peptide. SrtA cleaves between the threonine and glycine residues in the recognition sequence of the mTx and the N-terminal pentaglycine on RBP reacts with the newly created mTx C-terminus to yield a transpeptidation product. The fusions were purified by sequential Ni$^{2+}$-NTA and size exclusion chromatography steps. B) By this approach four HER2 receptor-targeted protein toxin fusions were created. Single-chain toxins were assembled by ligating mDT to either a ZHER2 Affibody or 4D5 scFv RBP. Binary toxins were created by ligating the same RBPs to mPA and combining the resulting fusion with a cytocidal effector, LF$_N$-DTA.

Fig. 2. Protein-protein ligation by sortase A. a and b) SrtA* or WT SrtA reactions were stopped at the indicated times, and the reaction mixtures were analyzed by SDS-PAGE (images were cropped from SFig. 1 and SFig. 2). Grey arrows indicate the shift in SDS-PAGE mobility for the ligated mTx-RBP fusions, compared to unligated forms (black arrow). c) Fusions were purified, as described in Fig. 1, and visualized by coomassie blue staining.

Fig. 3. HER2-targeted toxin fusions mediate specific killing of HER2-positive cells. Cells were incubated with mPA-ZHER2 or mPA-4D5 and increasing concentrations of LF$_N$-DTA for 4 h (panels a and b) or the indicated concentrations of mDT-ZHER2 or mDT-4D5 (panels c and d) for 24 h. Cells were washed and exposed to medium containing $[^3]$H-leucine for 1 h. Protein
synthesis was measured by scintillation counting and normalized against cells treated with mPA-ZHER2 or mDT. Results with cells expressing high, medium, low, or no HER2 receptor are colored red, blue, purple, and black respectively. Each point on the curves represents the average of 4 experiments.

Fig. 4. **Receptor Redirected protein toxins specifically kill HER2-positive tumor cells in a heterogeneous population.** Cells were plated in separate compartments of a chambered slide and incubated at 37 °C. The next day, the partition was removed, and the slide was incubated with a) mPA-ZHER2 plus LF_N-DTA, b) mPA-4D5 and LF_N-DTA, c) mDT-ZHER2, or d) mDT-4D5. After 24 h, cells were incubated for 1 h with medium supplemented with [3H]-leucine and dissolved in 6 M guanidine-HCl. The incorporated radiolabel was quantified by scintillation counting and percent protein synthesis was normalized against untreated cells.

Fig. 5. **Competition by ZHER2 and 4D5 for mPA-ZHER2- and mPA-4D5 -dependent killing.** Cells overexpressing HER2 (BT-474) were exposed to mPA-ZHER2 (solid lines) or mPA-4D5 (broken lines) and LF_N-DTA, plus free G5-ZHER2 (solid symbols) or G5-4D5 (open symbols). After 4 h, medium was replaced with medium supplemented with [3H]-leucine for 1 h. Protein synthesis was measured by scintillation counting and normalized against untreated cells. Each point on the curves represents the average of 4 experiments.
Figure 1

(a) Schematic representation of the conjugation process involving mTx, mPA, and mDT.

(b) Two pathways for the conjugation of mPA and mDT with ZHER2 and 4D5 antibodies.

Pathway I: mPA-[GS]3-LPETGG-His6-COOH + N2H-G5- → RBP

Pathway II: mDT-[GS]3-LPETGG-His6-COOH + RBP

Sub-pathways:

Ia) mPA-[GS]3-LPETGG-His6-COOH + ZHER2 + LFN-DTA

Ib) mPA-[GS]3-LPETGG-His6-COOH + 4D5

Ila) mDT-[GS]3-LPETGG-His6-COOH + ZHER2

IIb) mDT-[GS]3-LPETGG-His6-COOH + 4D5
Figure 2
Figure 3
Figure 4
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