Supporting Information
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Delivery of Antibody Mimics into Mammalian Cells via Anthrax Toxin Protective Antigen
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ADDITIONAL METHODS:

Construction of plasmids for recombinant proteins and transfection. The genes for Abl-SH2 was purchased from Addgene (pDONR223-ABL1, 23939). The gene for affibody, ABRaf, DARPin, and 7c12 was purchased from DNA2.0 (Menlo Park, CA). The gene for 10FN3 was kindly provided by K. Dane Wittrup (MIT, Department of Biological Engineering). The 10FN3 mutant, HA4 was generated by mutagenesis. The amino acid sequences are shown in Figure S12. The constructs, pET SUMO-G5-affibody, pET SUMO-G5-10FN3, and pET SUMO-SH2, were prepared using the Champion pET SUMO protein expression system (Life Technologies). AccuPrime Taq DNA polymerase (Life Technologies) was used to PCR amplify the DNA using the primers listed in Table S1. pET SUMO-G5-HA4 with two restriction sites after the stop codon (BamHI and XhoI) was generated by mutagenesis based on pET SUMO-G5-10FN3. 7c12 was PCR amplified, digested with BamHI and XhoI, and ligated in pET SUMO-G5-HA4 vector with a 20-amino acid linker GGSG(GGSGG)3G between HA4 and 7c12. For pET SUMO-LF-DTA (C186S)-LPSTGG-His5 and pET SUMO-LF-LPSTGG-His6, a G2S mutation was introduced to minimize undesired loss of Methionine. The plasmid for transfection of eGFP-ABRaf was generated by a two-step ligation. eGFP was inserted into pcDNA3 using NotI and BamHI restriction sites and ABRaf was then inserted using BamHI and XhoI restriction sites, with a GSGGGGSGGGGG between eGFP and ABRaf.

Protein expression and purification. His6-SUMO-G5-affibody, His6-SUMO-G5-GB1, His6-SUMO-G5-DARPin, His6-SUMO-G5-HA4, His6-SUMO-G5-HA4-7c12, His6-SUMO-G5- HA4-7c12mut and His6-SUMO-SH2 were expressed in E. coli BL21 (DE3)
cells in 1L LB culture and approximately 5 g of cell pellet was obtained for each. His$_6$-SUMO-LF$_N$-DTA (C186S)-LPSTGG-His$_5$, His$_6$-SUMO-LF$_N$-LPSTGG-His$_6$, His$_6$-SUMO-LF$_N$-DTA (C186S), SrtA*-His$_6$, WT anthrax protective antigen (PA), and PA[F427H] were expressed in *E. coli* BL21 (DE3) cells at New England Regional Center of Excellence/Biodefense and Emerging Infectious Diseases (NERCE). For each purification, approximately 40 g of cell pellet was lysed by sonication in 100 ml of 50 mM Tris-HCl, 150 mM NaCl, pH 7.5 buffer containing 200 mg lysozyme, 4 mg Roche DNAase I, and 2 tablet of Roche protease inhibitor cocktail. The suspension was centrifuged at 17,000 rpm for 40 minutes. The supernatant was loaded onto three 5 ml HisTrap FF crude Ni-NTA column (GE Healthcare, UK) and washed with 100 mL of 20 mM Tris-HCl, 150 mM NaCl, at pH 8.5 and 100 mL of 40 mM imidazole in 20 mM Tris-HCl, 500 mM NaCl, at pH 8.5. The protein was eluted from the column with buffer containing 500 mM imidazole in 20 mM Tris-HCl, 500 mM NaCl, pH 8.5, and buffer exchanged into 20 mM Tris-HCl, 150 mM NaCl, pH 8.5 using a HiPrep 26/10 Desalting column (GE Healthcare). WT PA and PA[F427H] was overexpressed in the periplasm of *E. coli* BL21 (DE3) cells and purified by anion exchange chromatography.

**Synthesis of TAT peptide and native chemical ligation (NCL) of TAT-DTA.** The TAT peptide (YGRKKRRQRRRLLG)\textsuperscript{11} was synthesized by Fmoc solid phase peptide synthesis on hydrazide resin (calculated mass: 1856.1 Da; observed monoisotopic mass: 1856.1 ± 0.1 Da). The crude peptides were purified by preparative RP-HPLC on Agilent Zorbax 300SB C$_{18}$ column (9.4 x 250 mm, 5 µm). The hydrazide C-terminus was converted to an MPAA thioester according to the previously published method by Fang,
TAT-MPAA was ligated to $^1$C-DTA by NCL using 1.4 mM TAT-MPAA and 270 µM 1C-DTA in 100 mM phosphate buffer pH 7.0, 150 mM NaCl, 20 mM TCEP in 4 h. The reaction was quenched with 200 mM sodium 2-mercaptoethane sulfonate and desalted to 20 mM Tris buffer pH 7.5, 150 mM NaCl. To alkylate the cysteine, 50 mM bromoacetamide was added to react for 10 minutes. The reaction was again quenched with 200 mM sodium 2-mercaptoethane sulfonate and buffer exchanged into 20 mM Tris buffer pH 7.5, 150 mM NaCl to obtain TAT-DTA protein.

**Synthesis of TAT-HA-LPSTGG peptide and sortagging to G$_5$-proteins.** The TAT-HA-LPSTGG peptide (AGYGRKKRRQRRRGYPYDVPDYAGLPSTGG) was synthesized by Fmoc solid phase peptide synthesis on aminomethyl resin (calculated mass: 3397.8 Da; observed average mass: 3397.8 ± 0.1). The crude peptides were purified by preparative RP-HPLC on Agilent Zorbax 300SB C$_{18}$ column (9.4 x 250 mm, 5 µm). The G$_5$-proteins (1 – 4) were conjugated to TAT-HA-LPSTGG using 10 µM SrtA* in sortase buffer (10 mM CaCl$_2$, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 1 h at RT. For each reaction, the TAT-HA-LPSTGG peptide (230 – 600 µM) was used in two-fold excess compared to the G$_5$-protein (115 – 300 µM). After 1 h, all of ligation reactions were diluted approximately 20-fold with 20 mM Tris-HCl pH 8.5. The samples were purified by anion exchange chromatography (GE HiTrap Q HP) at 0-30% 1 M NaCl in 20 mM Tris-HCl pH 8.5 over 100 mL. The fractions containing the ligated product were concentrated using a 3 kDa or 10 kDa concentrator. For the TAT-HA-1 (affibody) ligation, the product eluted with TAT-HA-LPSTGG peptide in the flow through, which
was subsequently concentrated and buffer exchanged three times to remove excess peptide. Purity of proteins was analyzed by LCMS and SDS-PAGE gel (Figure S4).
SCHEMES AND FIGURES

**Figure S1** One-pot sortagging reaction. N-terminal small ubiquitin-like modifier (SUMO) was first cleaved from the protein substrate, His$_6$-SUMO-LF$_N$-DTA-LPSTGG-His$_5$, using 1 µg SUMO protease per mg of protein substrate at RT for 30 minutes to expose the native N-terminus of LF$_N$-DTA-LPSTGG-His$_5$, then the sortagging reaction was carried out as described.
Figure S2 Coomassie stained SDS-page gel of LDv’s and Lv’s obtained after sortagging and purification.
Figure S3 Western blot analysis of delivered Lv’s. CHO-K1 cells were treated with 250 nM Lv2-5 in the presence of 40 nM PA for 12 hours. After treatment, cells were detached with trypsin and the cytosolic proteins were extracted with digitonin buffer and analyzed by immunoblotting. Lane 1 contains 4 ng of Lv2.
**Figure S4** Coomassie stained SDS-PAGE gel of TAT-HA-1 to -4 (1 µg).
Figure S5 Cellular uptake of TAT-HA-1 to -4. CHO-K1 cells were treated with 2.5 μM TAT-HA-1 to -4 in serum-free medium for 4 hours (a) or 16 hours (b) then lifted with trypsin and washed with PBS. The total lysates were analyzed by immunoblotting. Inset showed even with increased anti-HA intensity there was little amount of TAT-HA-2 and -3 detectable (a).
Figure S6 The level of protein synthesis inhibition in K562 cells treated with varying concentrations of LF$_N$-DTA or LDv5 in the presence of 20 nM PA for 4 hours.
Figure S7 SPR curves for SUMO-SH2 and varying concentrations of Lv5 (a) or HA4-7c12 (b). SPR measurements were taken in 10 mM HEPES, pH 7.4, 150 mM NaCl, 0.005% P20, and 3 mM EDTA with a BIAcore3000 instrument. The SUMO fusion of Abl SH2 domain was immobilized to an ethyl(dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide activated CM5 sensor chip. Purified HA4-7c12 or Lv5 was then flowed over the sensor chip at a rate of 20 µl min⁻¹ and the association and dissociation kinetics were monitored. Surface was regenerated by 10 mM glycine pH 2.0 between different runs. Data was fit to a 1:1 Langmuir binding model using multiple kinetic traces with the BIAevaluation software. The K_d was calculated as 12 nM for Lv5 (a) and 6 nM for HA4-7c12 (b).
Figure S8 Linear relationship between signal intensity of each band and the amount of protein loaded (Lv5: $y = 378000x - 137000$ $R^2 = 0.989$). The western blot images were analyzed in Image Studio Lite program (LI-COR Biosciences). The signal intensity of each band was compared to that of 0.1-7.5 ng of pure protein loaded on the same blot. The amount of protein in each lane was calculated based on the linear relationship between the signal intensity and the amount of protein.
Figure S9 Phosphorylation analysis of Bcr-Abl. a) Western blot analysis of Lv5 and Bcr-Abl pY421. b) The Bcr-Abl pY412 intensity was normalized to GAPDH and was set to 1 in the non-treated condition. K562 cells were treated with 500 nM Lv5 and 60 nM PA or PA[F427H] in serum-free medium for 1 day and FBS (10% v/v) was added to treat the cells for an additional day. Cells were lysed and immunoblotted with antibodies recognizing Abl phosphorylated on Tyr412 or LF. The amount of Lv5 on the blot was quantified as 1 ng (a). The estimation of the molecules per cell and concentration of Lv5 in the cell was based on cell number of ~100,000 cells and cell volume of ~2 pL.
Figure S10 Apoptosis measurement of K562 cells that were not treated or treated with 1 μM imatinib for 3 days. PerCP indicates the DNA fraction and FITC reports the TUNEL positive fraction by detecting the AlexaFluor 488 dye-labeled anti-BrdU antibody.
Figure S11 MTS cell viability assay. CHO-K1 cells were plated in a 96-well plate at 2,500 cells per well and treated with LFN-DTA or LV1 with or without PA, or with PA at the indicated concentration for 48 hours. CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, 10 µL) was added to each well and incubated for 1 hour according the manufacturer’s instructions. The absorbance at 490 nm was normalized to 1 nM LFN-DTA + PA (set as 0) and untreated cells (set as 100).
Affibody

Val^{1}\text{AspAsnLysPheAsnLysGluGlnGln}^{10}\text{AsnAlaPheTyrGluIleLeuHisLeuPro}^{20}

Asn^{21}\text{LeuAsnGluGlnArgAsnAlaPhe}^{30}\text{IleGlnSerLeuLysAspAspProSerGln}^{40}

Ser^{41}\text{AlaAsnLeuLeuAlaGluAlaLysLys}^{50}\text{LeuAsnAspAlaGlnAlaProLys}

GB1

Met^{1}\text{ThrTyrLysLeuIleLeuAsnGlyLys}^{10}\text{ThrLeuLysGlyGluThrThrGluAla}^{20}

Val^{21}\text{AspAlaAlaThrAlaGluLysValPhe}^{30}\text{IleGlnSerLeuLysAspAsnAspValAsp}^{40}

Gly^{41}\text{GluTrpThrTyrAspAspAlaThrLys}^{50}\text{ThrPheThrValThrGlu}

DAPRin

Ser^{1}\text{AspLeuGlyLysLeuLeuGluAla}^{10}\text{AlaArgAlaGlyGlnAspAspGluValArg}^{20}

Ile^{21}\text{LeuMetAlaAsnGlyAlaAspValAsn}^{30}\text{AlaTyrAspAspAsnGlyValThrProLeu}^{40}

His^{41}\text{LeuAlaAlaPheLeuGlyHisLeuGlu}^{50}\text{IleValGluValLeuLeuLysTyrGlyAla}^{60}

Asp^{61}\text{ValAsnAlaAlaAspSerTrpGlyThr}^{70}\text{ThrProLeuHisLeuAlaAlaThrTrpGly}^{80}

His^{81}\text{LeuGluIleValGluValLeuLeuLys}^{90}\text{HisGlyAlaAspValAsnAlaGlnAspLys}^{100}

Phe^{101}\text{GlyLysThrAlaPheAspIleSerIle}^{110}\text{AspAsnGlyAsnGluAspLeuAlaGluIle}^{120}

Leu^{121}\text{GlnLysLeuAsn}

10FN3

Val^{1}\text{SerAspValProArgAspLeuGluVal}^{10}\text{ValAlaAlaThrProThrSerLeuLeuIle}^{20}

Ser^{21}\text{TrpAspAlaProAlaValThrValArg}^{30}\text{TyrTyrArgIleThrTyrGlyGluThrGly}^{40}

Gly^{41}\text{AsnSerProValGlnGluPheThrVal}^{50}\text{ProGlySerLysSerThrAlaThrIleSer}^{60}

Gly^{61}\text{LeuLysProGlyValAspTyrThrIle}^{70}\text{ThrValTyrAlaValThrGlyArgGlyAsp}^{80}

Ser^{81}\text{ProAlaSerSerLysProIleSerIle}^{90}\text{AsnTyrArgThrGluIleAspLysProSer}^{100}

Gln^{101}
HA4
Val¹ SerSerValProThrLysLeuGluVal¹⁰ValAlaAlaThrProThrSerLeuLeuIle²⁰
Ser²¹ TrpAspAlaProMetSerSerSerSer³⁰ ValTyrTyrTyrArgIleThrTyrGlyGlu⁴⁰
Thr⁴¹ GlyGlyAsnSerProValGlnGluPhe⁵⁰ ThrValProTyrSerSerThrAlaThr⁶⁰
Ile⁶¹ SerGlyLeuSerProGlyValAspTyr⁷⁰ ThrIleThrValTyrAlaTrpGlyGluAsp⁸⁰
Ser⁸¹ AlaGlyTyrMetPheMetTyrSerPro⁹⁰ IleSerIleAsnTyrArgThr

7c12
Gly¹ GlySerGlySerSerSerValSerSerVal¹⁰ ProThrAsnLeuGluValValAspAlaThr²⁰
Pro²¹ ThrSerLeuLysIleSerTrpAspAla³⁰ TyrTyrSerSerTrpGlnAsnValLysTyr⁴⁰
Tyr⁴¹ ArgIleThrTyrGlyGluThrGlyGly⁵⁰ AspSerProValGlnGluPheThrValPro⁶⁰
Gly⁶¹ TyrTyrSerThrAlaThrIleSerGly⁷⁰ LeuSerProGlyValAspTyrThrIleThr⁸⁰
Val⁸¹ TyrAlaTyrAspThrPhePheProGly⁹⁰ TyrGluProAsnSerProIleSerIleAsn¹⁰⁰
Tyr¹⁰¹ ArgThr

ABRaf
Val¹ AspAsnLysPheAsnLysGluValAsn¹⁰ LeuValAlaAspGluIleTrpLeuLeuPro²⁰
Asn²¹ LeuAsnAsnGlnGlnValTrpAlaPhe³⁰ IleThrSerLeuLysAspAspProSerGln⁴⁰
Ser⁴¹ AlaAsnLeuLeuAlaGluAlaLysLys⁵⁰ LeuAsnAspAlaGlnGluProLys

Figure S12 Protein sequences
### Table S1 PCR primers

| Primer   | Forward Sequence                                      | Reverse Sequence                                      |
|----------|-------------------------------------------------------|-------------------------------------------------------|
| 10FN3 fwd | 5’-GGCGGTGGCGGTGCGGTTCTGTGCTTCCGAGGGAC-3’            |                                                       |
| 10FN3 rev | 5’-TAACTGGATGGTTGTCAATTTCTGTTC-3’                    |                                                       |
| Affibody fwd | 5’-GGCGGTGGCGGTGCGGTTCTGTGCTTCCGAGGGAC-3’             |                                                       |
| Affibody rev | 5’-TTACTTTGGCGGCTTGTGCG-3’                           |                                                       |
| GB1 fwd  | 5’-GGCGGTGGCGGTGCGGTTCTGTGCTTCCGAGGGAC-3’             | GAACGG-3’                                             |
| GB1 rev  | 5’-TTACTTTGGCGGCTTGTGCG-3’                           |                                                       |
| DARPin fwd | 5’-GGCGGTGGCGGTGCGGTTCTGTGCTTCCGAGGGAC-3’             |                                                       |
| DARPin rev | 5’-TTACTTTGGCGGCTTGTGCG-3’                           |                                                       |
| SH2 fwd  | 5’-TCCCTGGAGAACACTCCTGGTACC-3’                        |                                                       |
| SH2 rev  | 5’-TTACTTTGGCGGCTTGTGCG-3’                           |                                                       |
| 7c12 fwd | 5’-TTTTTTGGGATCCCGGGCGGCGTCTGTGCGGCGGGTGGTAGCG-3’     |                                                       |
| 7c12 Rv  | 5’-TTTTTTGGGATCCCGGGCGGCGTCTGTGCGGCGGGTGGTAGCG-3’     |                                                       |
| Protein                                      | Observed MW (Da) | Calculated MW (Da; average) |
|----------------------------------------------|------------------|----------------------------|
| SrtA*-His<sub>6</sub>                       | 19214.6 ± 0.4    | 19214.5                    |
| LF<sub>N</sub>-DTA                           | 52047.0 ± 0.4    | 52046.2                    |
| His<sub>6</sub>-SUMO-LF<sub>N</sub>-DTA-LPSTGG-His<sub>5</sub> | 66700.0 ± 0.4    | 66700.1                    |
| His<sub>6</sub>-SUMO-LF<sub>N</sub>-LPSTGG-His<sub>6</sub> | 45289.8 ± 0.4    | 45289.8                    |
| WT PA                                        | 83754.1 ± 0.4    | 83751.6                    |
| PA[F427H]                                    | 83742.4 ± 0.4    | 83738.3                    |
| Affibody                                     | 6925.8 ± 0.2     | 6925.6                     |
| GB1                                          | 6481.4 ± 0.2     | 6481.0                     |
| DARPin                                       | 13701.5 ± 0.2    | 13701.3                    |
| 10FN3                                        | 11022.4 ± 0.2    | 11022.2                    |
| HA4                                          | 10977.1 ± 0.2    | 10977.1                    |
| HA4-7c12                                     | 23244.1 ± 0.4    | 23244.2                    |
| HA4-7c12mut                                   | 23099.1 ± 0.4    | 23099.1                    |
| SUMO-SH2                                      | 24626.6 ± 0.4    | 24626.4                    |
| ABRaf                                         | 6864.9 ± 0.2     | 6864.6                     |
| ¹C-DTA                                        | 20840.60 ± 0.4   | 20840.1                    |
| DTA                                           | 20897.5 ± 0.4    | 20898.2                    |
| TAT-DTA                                       | 22722.84 ± 0.4   | 22723.4                    |
Table S3 Isolated yields of sortagging ligations from SrtA* reaction.

| Protein | Isolated Yield (%) |
|---------|--------------------|
| LDv1    | 21                 |
| LDv2    | 33                 |
| LDv3    | 34                 |
| LDv4    | 28                 |
| LDv5    | 22                 |
| Lv1     | 42                 |
| Lv2     | 47                 |
| Lv3     | 38                 |
| Lv4     | 35                 |
| Lv5     | 37                 |
| Lv5mut  | 38                 |
| Lv6     | 42                 |
**Table S4** EC$_{50}$ values of 30-minute protein synthesis inhibition assay (Fig. 1d). The errors represent fitting errors from Sigmoidal Boltzmann Fit.

| Protein    | EC$_{50}$ (pM) |
|------------|----------------|
| LF$_N$-DTA | 41 ± 8         |
| LDv1       | 33 ± 11        |
| LDv2       | 56 ± 29        |
| LDv3       | 62 ± 9         |
| LDv4       | 38 ± 4         |
### Table S5 List of variants

| Abbreviation | Variant |
|--------------|---------|
| LDv1         | ![LDv1 Diagram](image1) (affibody) |
| LDv2         | ![LDv2 Diagram](image2) (GB1) |
| LDv3         | ![LDv3 Diagram](image3) (DARPin) |
| LDv4         | ![LDv4 Diagram](image4) (HA4) |
| LDv5         | ![LDv5 Diagram](image5) (HA4-7c12) |
| Lv1          | ![Lv1 Diagram](image6) (affibody) |
| Lv2          | ![Lv2 Diagram](image7) (GB1) |
| Lv3          | ![Lv3 Diagram](image8) (DARPin) |
| Lv4          | ![Lv4 Diagram](image9) (HA4) |
| Lv5          | ![Lv5 Diagram](image10) (HA4-7c12) |
| Lv5mut       | ![Lv5mut Diagram](image11) (HA4(Y87A)-7c12(Y62E/F87K)) |
| Lv6          | ![Lv6 Diagram](image12) (ABRaf) |
LCMS Appendix

LDv1

LDv2

LDv3

LDv4

LDv5
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