**Reductive Methylation and Mutation of an Anthrax Toxin Fusion Protein Modulates its Stability and Cytotoxicity**

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## Supplemental Material and Methods

*Cell culture*

RAW264.7 (murine leukemic monocyte / macrophage cell line), CHO CL6 (Chinese hamster ovary cells), HN6 (human head and neck squamous carcinoma cell line), HeLa (human cervical carcinoma cell line), and PC-3 (human prostate adenocarcinoma cell line) cells were used for cytotoxicity studies. CHO CL6cells were maintained in modified Eagle's medium alpha with Glutamax™-1 (Gibco, Life Technologies, Grand Island, NY) and all other cells in Dulbecco's modified Eagle's medium with Glutamax™-1 (Gibco, Life Technologies, Grand Island, NY), respectively. All media were supplemented with 10 % fetal bovine serum (Gibco, Life Technologies, Grand Island, NY) and 50 µg/mL gentamicin (Quality Biological, Gaithersburg, MD). All cells were grown at 37 °C and 5 % $CO_2$.

*Cytotoxicity assays*

Cells were sub-cultured in 96-well plates (10,000 cells/well) over night. Cells were treated with various concentrations of FP59 variants in combination with 250 ng/mL PA for 48 h. For toxin neutralization assay, toxin solutions (25 ng/mL PA and 10 ng/mL of the FP59 variants) were pre-incubated with varying dilutions of polyclonal mouse anti-PA or mouse anti-LF antibodies for 60 min at 37°C prior to addition on CHO CL6 cells. Cells were incubated with toxin-antibody

solution for 48 h. Cell viability was assessed by the addition of 3-(4,5-dimethylthiazo-2-yl-02,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO) at a final concentration of 0.5 mg/mL, incubation for another 60 min at 37°C, and release of the blue pigment produced by viable cells using 50 µL 0.5% (w/v) sodium dodecyl sulfate, 25 mM HCl in 90 % (v/v) isopropanol. A plate reader was used to read the $A_{570}$ values for each well, and percent viability was calculated relative to wells which were not treated with toxin.

For toxin neutralization assay, toxin solutions (0.25 µg/mL PA and 10 ng/mL of the FP59 variants) were pre-incubated with varying dilutions of polyclonal mouse anti-PA or mouse anti-LF antibodies for 60 min at 37°C prior to adding the toxins on CHO CL6 cells. Cells were incubated with toxin-antibody solution for 48 h and relative cell survival was determined as described above.

*β-lactamase activity assay*

LFn-β-lactamase (LFnBla [1]) fusion protein (100 µL of 0.1 µg/mL LFnBla or LFnBla rm) was tested for β-lactamase enzymatic activity by incubation with the substrate Nitrocefin (100 µg/mL Nitrocefin in 100 µL PBS) in a 96-well plate. The absorbance was measured at 486 nm for 1 h every 5 min.

Cellular activity of LFnBla and LFnBla rm was tested on CHO CL6 cells. 40,000 CHO CL6 cells/well were seeded overnight and subsequently treated for 1 h at 37 °C with 250 ng/mL PA and 0.05-5 nM LFnBla or LFnBla rm. Cells were washed twice with PBS and incubated with 100 µL cell medium and 20 µl CCF2-AM loading solution (Life Technologies, Grand Island, NY) for 3 h at room temperature. β-lactamase activity in cells was determined by fluorescence measurement at 447 and 520 nm on a Safire spectral fluorescence photometer using the Magellan 5 software (Tecan, Maennedorf, Switzerland).

*Cytotoxicity assay for LFnCdtB and SE*

LFn-cytolethal distending toxin B (LFnCdtB) and saporin-epidermal growth factor (SE [2]) where reductively methylated as described for FP59 and FP59AGG. Human cervical carcinoma HeLa cells were seeded overnight in 96-well plates (10,000 cells/well) were exposed to different concentrations of LFnCdtB and LFnCdtB rm (both fusion proteins in the presence of 250 ng/mL of PA ) or SE and SE rm for 48 h and viable cells were quantitated in an MTT assay. Relative survival was calculated as the percentage of living cells after treatment in relation to untreated cells.

*cAMP release assay for EF*

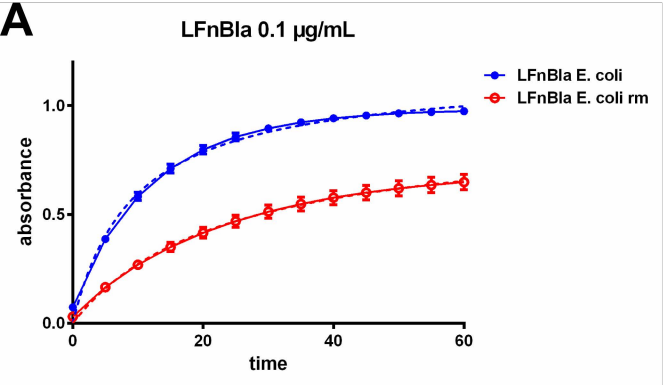
Anthrax edema factor (EF) was reductively methylated as described for FP59 and FP59AGG. Two different EF variants (1 ng/mL) have been used for the reductive methylation, EF with an N-terminal alanine (EF-A) and with an N-terminal arginine (EF-R). EF-A was reported as one of the most stable EF-variants, while EF-R is one of the least stable EF variant in cells [3]. Furthermore, both variants have been reductively methylated in the presence of 2 mM rat calmodulin (CaM) and 2 mM $CaCl_2$ to protect lysines at CaM-binding sites from dimethylation. cAMP generation by the EF variants was determined in the presence of 1 mM ATP, 80 µg/mL bovine serum albumin, and 2 mM $MnCl_2$ for 30 min at room temperature. cAMP detection was performed with the HTRF cAMP HiRange Kit (Cisbio US, Bedford, MA) according to the manufacturer's protocol.

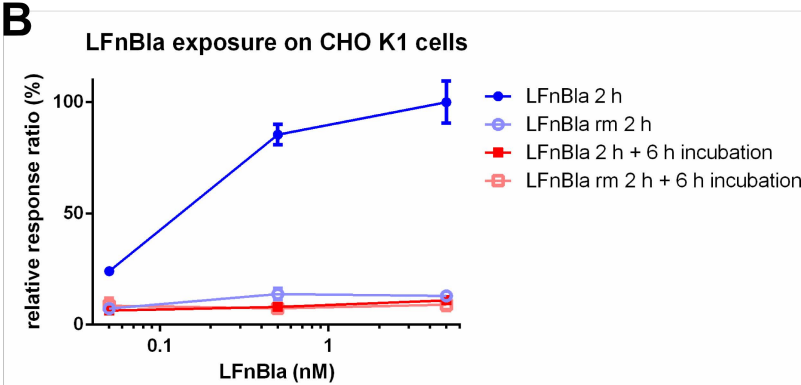
Reference List

1 Hobson, J. P., Liu, S., Rono, B., Leppla, S. H. & Bugge, T. H. Imaging specific cell-surface proteolytic activity in single living cells. *Nature methods* **3**, 259-261, doi:10.1038/nmeth862 (2006).

2 Bachran, C. *et al.* A lysine-free mutant of epidermal growth factor as targeting moiety of a targeted toxin. *Life sciences* **88**, 226-232, doi:10.1016/j.lfs.2010.11.012 (2011).
3 Leysath, C. E. *et al.* Anthrax edema factor toxicity is strongly mediated by the N-end rule. *PloS one* **8**, e74474, doi:10.1371/journal.pone.0074474 (2013).

# Figure S1


A
LFnBla 0.1 µg/mL
LFnBla E. coli
LFnBla E. coli rm
absorbance
1.0
0.5
0.0
0
20
40
60
time


B
LFnBla exposure on CHO K1 cells
relative response ratio (%)
100
50
0
0.1
1
LFnBla (nM)
LFnBla 2 h
LFnBla rm 2 h
LFnBla 2 h + 6 h incubation
LFnBla rm 2 h + 6 h incubation

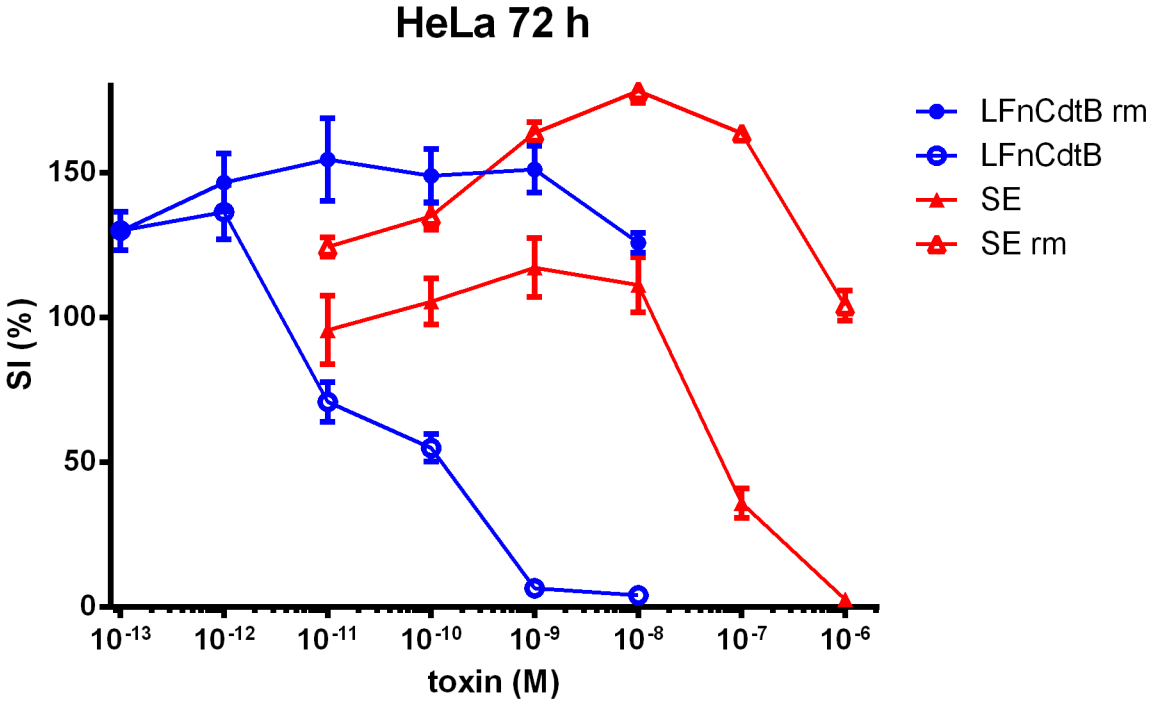

HeLa 72 h
SI (%)
150
100
50
0
$10^{-13}$
$10^{-12}$
$10^{-11}$
$10^{-10}$
$10^{-9}$
$10^{-8}$
$10^{-7}$
$10^{-6}$
toxin (M)
LFnCdtB rm
LFnCdtB
SE
SE rm

Table S1: cAMP amounts released by EF variants and EF variants after reductive methylation.

| EF variant | cAMP (nM) |
|---|---|
| EF-R | 2800 |
| EF-R rm | 8 |
| EF-R rm + CaM | 7 |
| EF-A | 1201 |
| EF-A rm | 8 |
| EF-A rm + CaM | 1313 |