Supporting Information

Modular Nanopore Immunotoxins with caged cytotoxic activity tailored by directed evolution

Natalie L. Mutter[1], Misha Soskine[1], Gang Huang[1], Inês S. Albuquerque[2], Gonçalo J. L. Bernardes[2,3] and Giovanni Maglia[1*]

[1]Groningen Biomolecular Science & Biotechnology Institute, University of Groningen, 9747 AG, Groningen, Netherlands

[2]Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Avenida Professor Egas Moniz, 1649-028, Lisboa (Portugal)

[3]Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW (UK)

Materials and Methods

Unless otherwise specified, all chemicals were bought from Sigma-Aldrich. DNA was purchased from Integrated DNA Technologies (IDT). Enzymes were acquired from Fermentas. All errors are given as standard deviations.

Cloning

ClyA-nanobody cloning

A Xho I restriction site (CTCGAG) was introduced at the 3' end of ClyA-AS gene [1] inserted into a T7 expression plasmid (pT7-SC1) [2] by PCR using ClyAXr and ClyAXf primers (Table S4, PCR conditions 1, Table S3). Separately, the synthetic gene encoding for the a EGFR recognizing nanobody 7d12 [3], which included a His_6- at the 3' end and a 16 amino acid glycine, alanine and serine linker (underlined in the sequence) at the 5' end, was amplified by PCR using Nbr and Nbf as primer (Table S4, PCR condition 1, Table S3). Then the newly constructed DNA plasmid containing ClyA-AS and the DNA construct containing the nanobody were purified with QIAquick PCR Purification Kit (Qiagen), digested with Xho I and Hind III, and ligated together using T4 ligase (Fermentas). 0.5 µL of the ligation mixture was transformed into 50 µL of *E. cloni*® 10G cells (Lucigen) by electroporation. The transformed bacteria were grown overnight at 37 °C on ampicillin (100 µg mL^{-1}) LB agar plates. The identity of the clones were confirmed by sequencing.

Construction of saturation mutagenesis library of ClyA-nanobody

The library was constructed by saturation mutagenesis of position 5 and 6 of ClyA-nanobody. For mutagenesis we used the plasmid encoding for ClyA-nanobody as a template. DNA amplification was performed using PCR condition 2 (Table S3), the T7 terminator primer (Table S4), and a primer containing a degenerated codon at position 5 and 6 encoding for the complete set of amino acids (Table S4). The PCR products were pooled together, gel purified (QIAquick Gel Extraction Kit, Qiagen) and cloned into pT7-SC1 by the MEGAWHOP procedure [4]: ~500 ng of the purified PCR product was mixed with ~300 ng of pT7-SC1 plasmid containing ClyA-nanobody gene and the amplification was carried out with Phire Hot Start II DNA polymerase (Finnzymes) using PCR condition 3 (Table S3). The circular template was eliminated by incubation with Dpn I (1 FDU) for 2 hours at 37 °C. 0.6 µL of the resulting mixture was transformed into 50 µL of *E. cloni*® 10G cells (Lucigen) by

electroporation. The transformed bacteria were grown overnight at 37 °C on ampicillin (100 μg mL^{-1}) LB agar plates typically resulting in $>10^5$ colonies which were harvested for plasmid DNA library preparation.

Construction of ClyA S272C

ClyA S272C was obtained from a pT7-SC1 plasmids containing the ClyA-AS gene which served as templates for Quick-Change PCR (PCR condition 1, Table S3) using the primers S272Cf and S272Cr (Table S4). The PCR product was purified using QIAQuick PCR purification kit (Qiagen) and the circular template was eliminated by incubation with DpnI (1FDU) for 1 hour at 37 °C. The mixture (0.5 μL) was transformed into 50 μL of *E. cloni*® 10G cells (Lucigen) by electroporation. The transformed bacteria were grown overnight at 37 °C on ampicillin (100 μg mL^{-1}) LB agar plates. The identity of the clones was confirmed by sequencing.

Construction of S-FraC

S-FraC was prepared by using pT7-SC1 plasmid containing WtFraC gene [5] as template for a PCR reaction using W112SFf and T7 terminator primers (Table S4), and PCR condition 2 (Table S3). The PCR product was purified using QIAQuick PCR purification kit (Qiagen) and cloned into pT7-SC1 by the MEGAWHOP procedure using pT7-SC1 plasmid containing WtFraC gene as template and the purified PCR product as primers.

Construction of S-FraC-nanobody

Part of the 16 amino acid glycine, alanine and serine linker (underlined in the sequence) was introduced at the 3' end of S-FraC by PCR using the T7 promoter and primer FNlinkr (Table S4, PCR condition 2, Table S3). The DNA construct was then gel purified (QIAquick Gel Extraction Kit, Qiagen). ~500 ng then served as primers for a second PCR reaction that used the pT7-SC1 plasmid containing the His_6-tagged ClyA-nanobody gene as template for cloning by the MEGAWHOP procedure.

DHFR-FraC cloning

A synthetic gene encoding for DHFR from *E.coli* (N-terminus end, [6]) and FraC containing the additional mutations D10G and K159E spaced by a 18 amino acid long linker (underlined in the DNA sequence at the end of the method section) and a His_6-tag at the C-terminus was bought from IDT. The synthetic gene was amplified by PCR using primers DFf and DFr

(Table S4) and PCR condition 1 (Table S3). The PCR product was purified by QIAQuick PCR purification kit (Qiagen), digested with Nco I and Hind III (FastDigest, Fermentas), ligated into a pT7-SC1 expression plasmid using T4 ligase (Fermentas) transformed into 50 µL of *E. cloni*® 10G cells (Lucigen) by electroporation.

Construction of DHFR-FraC-nanobody

The gene of MD-DHFR-FraC was amplified using T7 promotor, primer DFnHr (Table S4), and PCR condition 2 (Table S3). In a second PCR reaction FraC-nanobody gene was amplified using T7 terminator, primer DFnHr (Table S4) and PCR condition 2 (Table S3). Both PCR products were purified with QIAQuick PCR purification kit (Qiagen). 50 ng of both purified PCR products, which contain an overlapping sequence, were used as template for a third PCR reaction using T7 promotor, T terminator (Table S4) and PCR condition 2 (Table S3). The resulting PCR product encoding His_6-tagged DHFR-FraC-nanobody gene was digested, ligated and transformed as described in section: "construction of DHFR-FraC"**.**

Construction of DHFR-ClyA

The gene of DHFR-FraC was amplified by PCR using the T7 promoter, primer DHFRlinkr (Table S4), and PCR condition 2 (Table S3). In a second PCR reaction the gene of ClyA was amplified using LinkClyA and ClyAr as primers (Table S4), and PCR condition 2 (Table S3). 50 ng of both constructs, which contain an overlapping sequence, are then the template for a third PCR reaction (PCR condition 2, Table S3) using T7 promotor and ClyAr (Table S4) as primers. The final construct was digested with Nco I and Hind III (FastDigest, Fermentas) and cloned into pT7-SC1 expression plasmid.

Construction of DHFR-FraC library by error-prone PCR

Libraries were constructed by amplifying the DHFR-FraC gene from plasmid DNA using T7 promoter and T7 terminator (Table S4). In the first round of mutagenesis we used a plasmid encoding for DHFR-FraC as template. DNA amplification was performed by error-prone PCR with 0-0.2 mM $MnCl_2$ using T7 promoter and T7 terminator (Table S4) primers and PCR condition 2 (Table S3). The PCR products were pooled together, gel purified (QIAquick Gel Extraction Kit, Qiagen) and cloned into a pT7 expression plasmid (pT7-SC1) by the MEGAWHOP procedure using the purified PCR product and pT7-SC1 plasmid containing DHFR-FraC gene for amplification as described above in section: "construction of ClyA-nanobody library". Typically, $>10^5$ colonies were obtained, which were harvested for plasmid

DNA library preparation. For the second round of mutagenesis we used the DNA plasmids that were derived from the previous round of selection (section: "Hemolytic screening DHFR-FraC libraries"), and used the conditions for DNA amplification by error-prone PCR using template concentrations of 0.1–400 ng [7], and PCR condition 2 (Table S3) using T7 promoter and T7 terminator (Table S4) as primers. These conditions typically yielded in 1 – 4 mutations per gene in the final library. The PCR products were pooled together and handled as described in the previous paragraph. For the third round of mutagenesis we used the DNA plasmids that were derived from the previous round of selection and we changed the protease cleavage site from TEV to furin. In contrast to TEV, furin is cancer associated protease. DNA amplification was performed by PCR condition 2 (Table S3) using primer Furf and T7 terminator (Table S4). These conditions typically yielded in 0 – 2 mutations per gene in the final library. The PCR products were pooled together, gel purified (QIAquick Gel Extraction Kit, Qiagen) and cloned into a pT7 expression plasmid (pT7-SC1) by the MEGAWHOP procedure using the purified PCR product and pT7-SC1 plasmid containing pT7-SC1 plasmid containing DHFR-FraC gene for amplification.

Protein expression

E. cloni® EXPRESS BL21 (DE3) cells were transformed with the pT7-SC1 plasmid containing the appropriate gene. Transformants were selected after overnight growth at 37 °C on LB agar plates supplemented with 100 mg L^{-1} ampicillin. The resulting colonies were inoculated in 10 mL 2xYT medium containing 100 mg L^{-1} of ampicillin. The culture was grown at 37 °C, with shaking at 200 rpm, until it reached an OD_{600} of ~0.6. The expression of protein was induced by the addition of 0.5 mM IPTG and the growth was continued at 21 °C. The next day the bacteria were harvested by centrifugation at 6000*xg* for 30 minutes and pellets were stored at -80 °C.

Protein purification

Periplasmic purification of ClyA and ClyA-nanobody

The pellets containing ClyA-AS or ClyA-nanobody were resuspended in 25 mL of 15 mM Tris.HCl pH 7.5, 1 mM Ethylenediaminetetraacetic acid (EDTA) and 20 % sucrose. Then the bacteria suspension was subjected to vigorous shaking at ambient temperature for 30 minutes. The bacterial cells were harvested by centrifugation at 6000x*g* for 15 minutes. The supernatant was discarded and the pellet resuspended in 10 mL 5 mM $MgCl_2$ supplemented with 0.05 units mL^{-1} of DNase I (Fermentas) and shacked for 1 hour. The cell suspension was

centrifuged at 6000x*g* for 20 minutes. The supernatant was supplemented with 10 mM imidazole and mixed with 200 µL (bead volume) of Ni-NTA resin (Qiagen) that was pre-equilibrated with wash buffer (10 mM imidazole 150 mM NaCl, 15 mM Tris.HCl pH 7.5). After 45 minutes the resin was loaded into a column (Micro Bio Spin, Bio-Rad) and washed with ~10 mL of wash buffer. ClyA- AS or ClyA-nanobody was eluted with approximately 300 µL of wash buffer containing 300 mM imidazole. Protein concentration was determined by Bradford assay and toxin monomers were stored at 4 °C until further use.

Cytosolic purification of proteins (ClyA-S272C, S-FraC, S-FraC-nanobody, DHFR-(TEV)-FraC, MD-DHFR-FraC, DHFR-ClyA and DHFR-FraC-nanobody)

The pellets containing overexpressed proteins were thawed and resuspended in 20 mL of 15 mM Tris.HCl pH 7.5, 150 mM NaCl, 10 mM imidazole, 1 mM $MgCl_2$ and 0.05 units mL^{-1} of DNase I (Fermentas). Then, to initiate cell disruption, the bacteria suspension was supplemented with 0.2 mg mL^{-1} lysozyme and a cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail tablet (Roche) and was subjected to vigorous shaking at ambient temperature for 1 hour. The remaining bacteria were disrupted by probe sonication. The crude lysates were clarified by centrifugation at 6000*xg* for 30 minutes and supernatant mixed with 200 µL (bead volume) of Ni-NTA resin (Qiagen) that was pre-equilibrated with wash buffer (10 mM imidazole 150 mM NaCl, 15 mM Tris.HCl pH 7.5). After 45 minutes of gentle mixing at ambient temperature, the resin was loaded onto a column (Micro Bio Spin, Bio-Rad) and washed with ~10 mL of wash buffer. S-FraC or S-FraC-nanobody was eluted with approximately 300 µl of wash buffer containing 300 mM imidazole. Protein concentration was determined by Bradford assay. All monomers were stored at 4 °C until further use.

Purification of ClyA-AS-S272C was performed as described in the section above, however, to avoid oxidation of the cysteine 5 mM Tris(2-carboxyethyl)phosphine (TCEP) was added until the final step, which included the washing of the Ni-NTA resin with 10 mL wash buffer supplemented with 5 mM TCEP. One additional step was then performed, in which the resin was washed with the same buffer (~10 mL) but without the reducing agent. ClyA-AS-S272C was eluted with approximately 150 µL of wash buffer containing 300 mM imidazole. Immediately after elution from the Ni-NTA resin, the ClyA-AS-S272C monomer was mixed with 20-fold molar excess of Folate-5k PEG-maleimide(Nanocs), dissolved in 15 mM Tris.HCl pH 7.5, 150 mM NaCl and the reaction was allowed to proceed for 2 hours at room

temperature or at 4 °C overnight. According to SDS-PAGE analysis, ~50 % of the ClyA monomers were modified. ClyA-monomers conjugated to folate were separated from unmodified monomers and unreacted Folate-5k PEG-maleimide by gel filtration using 15 mM Tris.HCl pH 8.0, 150 mM NaCl buffer.

Purification of DHFR-ClyA was performed as described for S-FraC above however an additional purification step was necessary, because DHFR-ClyA forms inclusion bodies. Therefore after clarifying the crude lysate by centrifugation, the resulting pellet was resuspended in ~20 mL wash buffer supplemented with 8 M urea. The cell suspension was centrifuged at 6000*xg* for 20 minutes and the supernatant His-Tag purified as described above, while washing the urea concentration was decreased gradually from 8M to 0M.

DHFR-(TEV)-FraC and MD-DHFR-FraC was expressed and lysed as described for S-FraC above. Instead of His-Tag purification using gravity-flow chromatography, the clarified lysate was applied to a HisTrap HP column (GE Healthcare Life Science) connected to Äkta pure chromatography system (GE Healthcare Life Science) which was equilibrated before with wash buffer (30 mM imidazole, 15 mM Tris and 500 mM NaCl). DHFR-(TEV)-FraC and MD-DHFR-FraC was eluted with wash buffer containing 500 mM imidazole. DHFR-(TEV)-FraC and MD-DHFR-FraC was eluted with wash buffer supplemented with 500 mM imidazole. FraC was then obtained from either DHFR-(TEV)-FraC or MD-DHFR-FraC by injecting 0.5 mL trypsin (1 mg mL^{-1}) from bovine pancreas, which cleaves off DHFR and then the column was washed to elute free DHFR and trypsin. Cut FraC was eluted with wash buffer supplemented with 500 mM imidazole.

Purification of DHFR-ClyA and DHFR-FraC-nanobody was performed as described for S-FraC above. FraC-nanobody could also be obtained by proteolysis from DHFR-FraC-nanobody, in which case 1 unit furin was added per 25 µg DHFR-FraC-nanobody and incubated overnight at ambient temperature.

Hemolytic activity assay

Defibrinated sheep blood (ThermoFisher Scientific) was washed with 150 mM NaCl, 15 mM Tris.HCl pH 7.5 until the supernatant was clear. The erythrocytes were then resuspended with the same buffer to ~1 % concentration (OD_{650} 0.6 – 0.8). The suspension (120 µL) was then mixed with the solutions containing toxin. Hemolytic activity was measured by monitoring

the decrease in OD_{650} using the MultiskanTM GO Microplate spectrophotometer (ThermoFisher Scientific). Hemolytic activity of 1 µg ClyA-AS, 1 µg ClyA-nanobody, 1 µg S-FraC and 1 µg S-FraC-nanobody was measured as described above. Hemolysis rate was calculated as inverse of the time elapsed till 50% decrease in turbidity.

To determine hemolytic activity of DHFR-ClyA or DHFR-FraC, 10 µg of toxin was mixed with 0.1 µg trypsin in 100 µL buffer (150 mM NaCl, 15 mM Tris.HCl pH 7.5, 1 mM $CaCl_2$) and incubated 5 minutes before applying to the erythrocyte solutions. As control the same experiment was performed using the same buffer without trypsin. Similarly, solutions containing DHFR-FraC-nanobody were activated using 0.1 µg of furin (PeproTech) instead of trypsin.

The concentration dependency of hemolytic activity, measured for ClyA and ClyA-nanobody, was tested by preparing different toxins concentrations in a final volume of 100 µL buffer (150 mM NaCl, 15 mM Tris.HCl pH 7.5). Then the addition of 120 µL washed 1 % erythrocyte solution was added. After 60 minutes incubation at room temperature hemolysis was recorded by measuring absorbance at 650 nm.

Hemolytic screening of ClyA-nanobody libraries

For screening, plasmid DNA of the library was transformed into *E. cloni*® EXPRESS BL21 (DE3) (Lucigen). 384 clones were randomly picked and individually grown in 96-deep-wells plates overnight by shaking at 37 ºC (0.5 mL 2xYT medium containing 100 µg mL^{-1} ampicillin). The obtained cultures were used as starters for protein overexpression. 50 µL of the overnight starter cultures were inoculated into 500 µL of fresh medium in new 96-deep-well plates and cultures were grown at 37 °C until an optical density of 600 nm (OD_{600}) of about 0.6. Then, Isopropyl-β-D-thiogalactopyranosid (IPTG ,0.5 mM) was added to induce overexpression and the temperature was reduced to 25 °C for an overnight incubation. Bacteria were harvested the following day by centrifugation at 3000*xg* for 15 minutes at 4 °C. The supernatant was discarded and pellets were frozen at -80 °C for two hours to facilitate cell disruption. Cell pellets were then resuspended in 500 µL of lysis buffer (15 mM Tris.HCl pH 7.5, 1 mM $MgCl_2$, 10 µg mL^{-1} lysozyme, 0.2 units mL^{-1} DNAse I) and lysed by shaking at 1300 rpm for 1 hour. The cell suspension was centrifuged at 3000*xg* for 20 minutes. Hemolytic activity using 10 µL of the crude lysate was determined as above described in section: “hemolytic activity assay”. Active variants that showed slower hemolysis rates than ClyA-Nb were selected and the corresponding starter culture was used to obtain the plasmid

DNA. The identity of the clones was confirmed by sequencing. Sequence changes that occurred in the corresponding genes are summarized in Table S1.

Hemolytic screening DHFR-FraC libraries

Libraries of DHFR-FraC were created and transformed into *E. coli* BL21(DE3) and grown in 96 microwell plates as described above in the section: "screening of ClyA-nanobody libraries". In each round of selection, 768 individual clones were tested for hemolytic activity. In the first two selection rounds, two aliquots of 10 µL of crude lysate were tested for hemolytic activity. One aliquot was pre-incubated with trypsin (10 µL, 0.5 mg mL^{-1}, 5 min incubation time), the other was tested directly on red blood cells as described in "screening of ClyA-nanobody libraries". Clones with no or low background activity (t_{50} > than ~60 minutes) and high hemolytic activity after proteolysis (t_{50} < 10 min) were selected and the corresponding starter culture was used to obtain the plasmid DNA. In the third round of screening furin, (3 hours pre-incubation time at 37 °C, 0.01 mg mL^{-1} final furin concentration) was used instead of trypsin. The identities of the clones were confirmed by sequencing. Sequence changes that occurred in the corresponding genes are summarized in Table S2.

Cell viability assay

The cell viability assays were performed with the Human squamous carcinoma cell line A431 (Sigma-Aldrich). Detached cell number was determined by cell counting using an improved Neubauer chamber. The viability of cells was measured using (2-2-methoxy-4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) based cell viability assay included in the Cell Counting Kit-8 (CCK,-8, Sigma-Aldrich) according to the manufactures instructions.

The human squamous carcinoma cell line A431 (Sigma-Aldrich) and the human lung adenocarcinoma cell line Calu-6 (Cell Lines Service) were routinely maintained at 37 °C, in a humidified incubator under 5 % CO_2. A431 cells and Calu-6 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % Fetal Bovine Serum (FBS), 2 mM glutamine, 100 µg mL^{-1} gentamycin.

A431 cells were first detached with 5 mL of 0.25 % Trypsin/EDTA solution, centrifuged at 400*xg* for 5 minutes, and suspended in 10 mL growth medium, counted as described above and seeded 20.000 cells per well in 96-well tissue culture plates in 200 µL growth medium and incubated overnight at 37 °C in 5 % CO_2 atmosphere. Either 2 µL epidermal growth

factor (EGF, final concentration 15 nM) or PBS was added to each well. In various concentrations 10 µL toxin was distributed to the cells and incubated 24 hours at 37 °C in 5 % CO_2 atmosphere. Cells with only medium were included as control. Next, 5 µL CCK-8 solution was added to each well and the plates were incubated 2 hours before measuring absorbance at 450 nm using the MultiskanTM GO Microplate spectrophotometer (ThermoFisher Scientific).

Calu-6 were first detached with 5 mL of 0.25 % Trypsin/EDTA solution, centrifuged at 300*xg* for 5 minutes, and suspended in 10 mL growth medium, counted as described above and seeded 20.000 cells per well in 96-well tissue culture plates in 200 µL growth medium and incubated overnight at 37 °C in 5 % CO_2 atmosphere. In various concentrations 10 µL toxin was distributed to the cells and incubated 48 hours at 37 °C in 5 % CO_2 atmosphere. Cells with only medium were included as control. Next, 5 µL CCK-8 solution was added to each well and the plates were incubated 2 hours before measuring absorbance at 450 nm using the MultiskanTM GO Microplate spectrophotometer (ThermoFisher Scientific).Results are shown as percentage of control cells and represented as average ± standard deviation of 3 independent experiments with triplicates.

For the kinetic experiments A431 cells were seeded in 96-well tissue culture plates as described above. Either 2 µL epidermal growth factor (EGF, final concentration 15 nM) or PBS was added to each well. Each hour one column of the plate was treated with 16 nM SE-ClyA-nanobody toxin. The viability of the cells was measured one hour after the last toxin addition.

Cell viability assay ClyA-folate

KB (folate receptor positive, FR+) cells (kindly supplied by Dr Andreia Gomes, Universidade do Minho, Portugal) and A549 (FR-) cells (kindly supplied by Dr Maria João Amorim, Instituto Gulbenkian de Ciência, Portugal) were routinely maintained at 37 ºC, in a humidified incubator under 5 % CO_2. Both cell lines were grown in RPMI 1640 supplemented with 10 % FBS, 1 % GlutaMAX, 1 % NEAA, 1 % HEPES, 1 % Pen-Strep (all reagents from Gibco, LifeTechnologies, USA), a medium further referred to as “complete RPMI”.

KB cells or A549 cells were seeded in a 96 well plate, at a density of 10 000 cells/well and incubated for 24 hours to allow for cell attachment. Cells were treated with either ClyA-AS (stock solution in 25% glycerol, 7.5 Tris-HCl pH 8.0 75 mM NaCl and 150 mM imidazole) or

ClyA-AS-S272C (stock solution in 25% glycerol, 7.5 Tris-HCl pH 8.0 75 mM NaCl) for 24 hours. Upon end of the incubation period, 50 μg/well of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added to all wells (except for 3 wells, which contained only medium and were used as blank controls), and the cells were returned to the incubator for another 4 hours. After this time, the medium was removed by inverting the plate and 200 μL of dimethyl sulfoxide (DMSO) to solubilize the resulted formazan was added per well. Absorbance at 540 nm was measured (and 690 nm was used as reference wavelength) using a Tecan Infinite M200 plate-reader. Results are shown as percentages of controls (i.e. vehicles) and represent, unless otherwise stated, average ± SEM (of 2 or 3 independent experiments with technical triplicates).

For the Folate Competition Assays, cells were seeded as described above, in "complete RPMI" medium without folate. After 24 hours of incubation, cells were incubated in medium supplemented with 0.002 (i.e. [folate] commonly found in RPMI 1640) or 0.5 mM of folic acid and treated with 11.5 nM ClyA-AS-S272C. After 24 hours of treatment, either a MTT assay or a propidium assay was performed (see below).

Propidium iodide assay

Cells were seeded in 24 well-plates at 30,000 cells/well in RPMI 1640 without folate, supplemented as described above, and incubated for 24 hours to allow for attachment. After this time, the medium was switched according to the above description of the Folate Competition Assays. After 24 hours of treatment, cells were harvested after trypsinization (TrypLE Express, LifeTechnologies, USA) into FACs tubes and washed twice with 1x Phosphate Buffered Saline (PBS). Cells were then resuspended in 5 μg mL^{-1} of propidium iodide solution (in 10 % FBS in 1x PBS) and after 15 minutes fluorescence was measured using a LSR Fortessa cytometer equipped with a 488 nm laser and a 575/26BP + 550LP combination of filters. Results are shown as percentages of controls (i.e. "vehicles") and represent, unless otherwise stated, average ± SEM (of 2 or 3 independent experiments with technical triplicates).

Tables

ClyA-nanobody variant	Sequence changes relative to ClyA-nanobody
1	I5C , F6G
SE-ClyA-nanobody	I5S , F6E, R309Q
3	I5A , F6H, Y264C
4	I5S , F6A
5	I5P , F6H
6	I5F , F6R, I251T
7	I5G , F6P, E19G,Q155R
8	I5Q , F6R
9	I5G , F6G

Table S1. Mutations accumulated during directed evolution of ClyA-nanobody gene.

Round	variant	Sequence changes relative to DHFR-TEV-FraC
2	1	R318C
2	2	S329T, H362L
2	3	S139C, S329T, H362L
2	4	R102H, R318C
2	5	S154N, Y170N
2	6	S154N, Y170N, N325D
3	C-DHFR-FraC	R318C, **L169R, Y170A, F171R, Q172Y, S173K, K174R**
3	HC-DHFR-FraC	R102H, R318C, **L169R, Y170A, F171R, Q172Y, S173K, K174R**
3	MD-DHFR-FraC	T50M, N325D, **L169R, Y170A, F171R, Q172Y, S173K, K174R**

Table S2. Mutations accumulated during directed evolution rounds of the DHFR- FraC gene. In bold sequence changes regarding the conversion from TEV to furin cleavage site.

Condition	PCR composition	Cycling conditions
1	Phire Hot Start II DNA polymerase 25 ng template DNA 2 µM of each primer in 50 µL final volume	pre-incubation 98°C for 30 seconds 30 cycles of: denaturation 98 °C for 5 seconds extension 72 °C for 1 minute
2	REDTaq ReadyMix 100 ng template DNA 2 µM of each primer	pre-incubation at 95 °C for 3 minutes 30 cycles of: denaturation at 95 °C for 15 seconds

	in 200 µL final volume for 4 reactions	annealing at 55 °C for 15 seconds extension at 72°C for 3 minutes
3	Phire Hot Start II DNA polymerase 500 ng template DNA 300 ng primer in 50 µL final volume	pre-incubation 98°C for 30 seconds 30 cycles of: denaturation 98 °C for 5 seconds extension 72 °C for 1.5 minute

Table S3. PCR conditions used for cloning and construction of libraries.

Name of primer	DNA sequence
ClyAXr	TATATATCTCGAGTGCTGCTGCCCACATCCGGGAC
ClyAXf	CACCACTAAAAGCTTGGATCCGGCTGCTAAC
Nbf	ATATATACTCGAGCGGGCTCCTCGGGTTCG
Nbr	TATATATAAGCTTTTATCAGTGGTGATGGTGATGGTGC
5NNN6	GATATAGCCATGGGTACGGGTNNNNNNGCGGAACAGACGGTGGAAG
S272Cf	CTGATGCTGTGCCTGCTGAAAGGTGCCG
S272Cr	CTTTCAGCAGGCACAGCATCAGGTCATCG
DHFRlinkr	TACCTGCACTACTACCGCGTTTATAGCGC
LinkClyA	CCGCGCGCGCTATAAACGCGGTAGTAGTGCAGGTACGGGTATCTTTGCGGAAC
ClyAr	ATATATATAAGCTTACACATCCGGGACTTCAAACAG
W112SFf	ACGATTATAATAGCTATAGCAATTGGTGG
FNlinkr	CAGAGCCAGAGCTGCCCGCCTGAGTTGAGCCAGCCTTAGTGACATGAATTTCTAAG
DFf	ATATCCATGGCTTCAGCTATGATTTCCC
DFr	ATATAAGCTTATTAATGGTGGTGATGATGGC
Furf	GCGTCGCGGCTCCAGTGAGAACCGCGCGCGCTATAAACGCGGTAGTAGTGCAAGTGCTG
DFnHr	AGCCTTAGTGACATGAATTTCTAAG
FraCf	AGTGCTGATGTAGCAGGAGCA
T7-terminator	GCTAGTTATTGCTCAGCGG
T7-promoter	TAATACGACTCACTATAGGG

Table S4. Primers table. N stands for A,G,C or T.

DNA sequences

>ClyA-nanobody (protein sequence)

MGTGIFAEQTVEVVKSAIETADGALDLYNKYLDQVIPWKTFDETIKELSRFKQEYSQE
ASVLVGDIKVLLMDSQDKYFEATQTVYEWAGVVTQLLSAYIQLFDGYNEKKASAQK
DILIRILDDGVKKLNEAQKSLLTSSQSFNNASGKLLALDSQLTNDFSEKSSYYQSQVD
RIRKEAYAGAAAGIVAGPFGLIISYSIAAGVIEGKLIPELNNRLKTVQNFFTSLSATVKQ
ANKDIDAAKLKLATEIAAIGEIKTETETTRFYVDYDDLMLSLLKGAAKKMINTSNEYR
QRHGRKTLFEVPDVGSSTQAGSSGSAGSAGQVKLEESGGGSVQTGGSLRLTCAASGR
TSRSYGMGWFRQAPGKEREFVSGISWRGDSTGYADSVKGRFTISRDNAKNTVDLQM
NSLKPEDTAIYYCAAAAGSAWYGTLYEYDYWGQGTQVTVSSGSAGSSHHHHHH**

>ClyA-nanobody (DNA sequence)

CCATGGGTACGGGTATCTTTGCGGAACAGACGGTGGAAGTTGTGAAAAGTGCGA
TTGAAACGGCTGACGGTGCGCTGGACCTGTATAATAAATATCTGGATCAGGTCAT
CCCGTGGAAAACCTTTGACGAAACGATTAAAGAACTGAGCCGTTTCAAACAGGA
ATACAGTCAAGAAGCGTCCGTCCTAGTGGGCGATATCAAAGTGCTGCTGATGGAT
TCTCAGGACAAATATTTTGAAGCTACCCAAACGGTTTACGAATGGGCGGGTGTGG
TTACCCAGCTGCTGTCCGCATATATTCAGCTGTTCGATGGATACAATGAGAAAAA
AGCGAGCGCGCAGAAAGACATTCTGATCCGCATTCTGGATGACGGCGTGAAAAA
ACTGAATGAAGCCCAGAAATCGCTGCTGACCAGCTCTCAATCATTTAACAATGCC
TCGGGTAAACTGCTGGCACTGGATAGCCAGCTGACGAACGACTTTTCTGAAAAAA
GTTCCTATTACCAGAGCCAAGTCGATCGTATTCGTAAAGAAGCCTACGCAGGTGC
CGCAGCAGGTATTGTGGCCGGTCCGTTCGGTCTGATTATCTCATATTCAATTGCTG
CGGGCGTTATCGAAGGTAAACTGATTCCGGAACTGAACAATCGTCTGAAAACCGT
TCAGAACTTTTTCACCAGTCTGTCTGCTACGGTCAAACAAGCGAATAAAGATATC
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>S-FraC-nanobody (protein sequence)

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>DHFR-(TEV)-FraC (protein sequence)

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>DHFR-(TEV)-FraC (DNA sequence)

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DHFR-FraC-nanobody sequences

>DHFR-(Furin)-FraC-nanobody (protein sequence)

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>DHFR-(Furin)-FraC-nanobody (DNA sequence)

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Figures

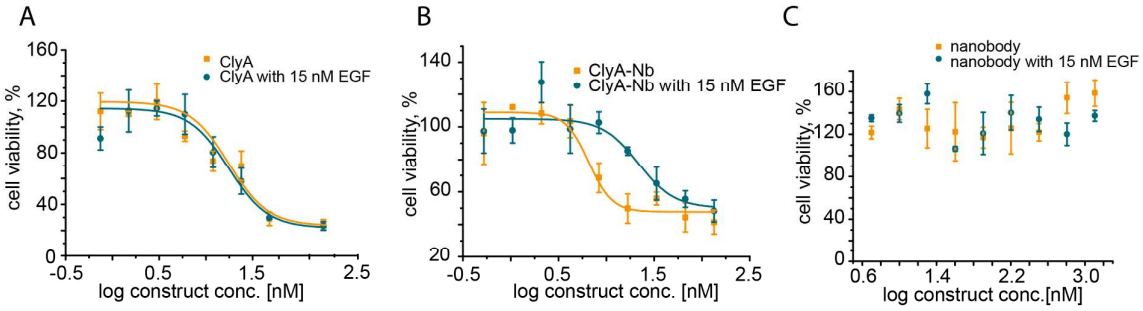

Figure S1. Toxicity of ClyA variants in the presence and absence of 15 nM EGF on A431 epidermoid carcinoma cells. (**A**) ClyA toxicity shows no response on EGF. (**B**) In the presence and absence of 15 nM EGF ClyA-Nb is reduced about two-fold. (**C**) Nanobodies without the toxin have no effect on cell viability.

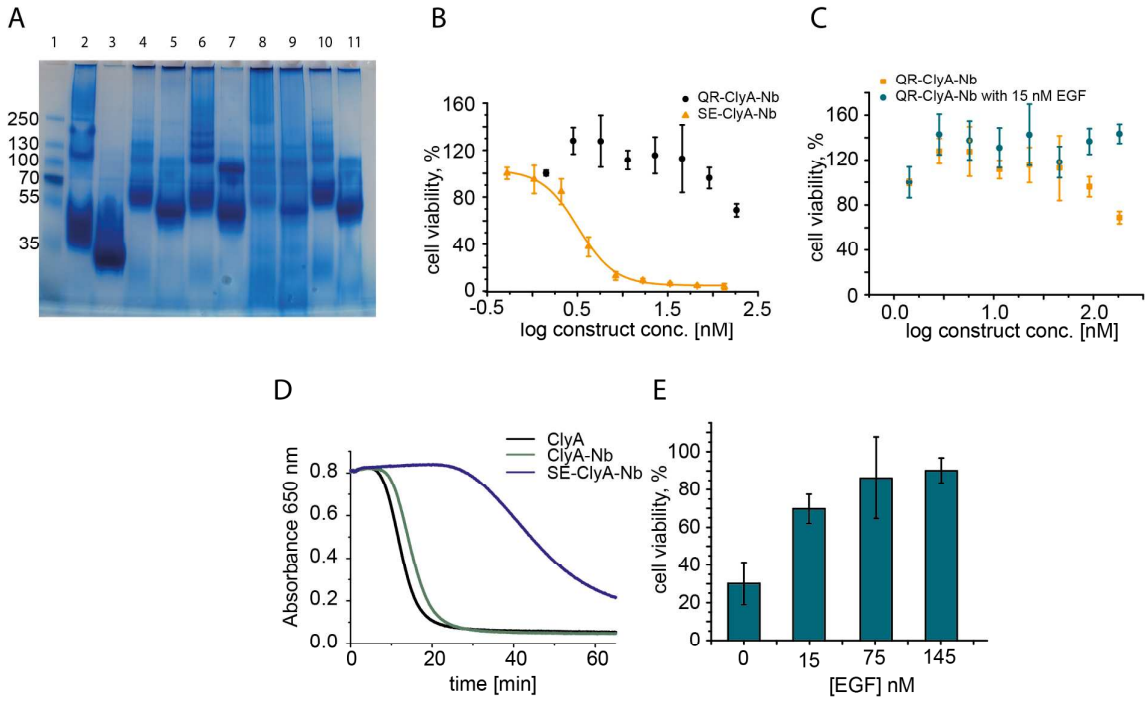

Figure S2. Selection of evolved immunotoxins. (**A**) ClyA-Nb library analysed by a 4-20% BN-PAGE. Lane 1: Protein ladder, lane 2: ClyA-AS, lane 3: ClyA-AS with 0.2% SDS, lane 4: SA-ClyA-Nb, lane 5: SA-ClyA-Nb with 0.2% SDS, lane 6: KW-ClyA-Nb, lane 7: KW-ClyA-Nb with 0.2% SDS, lane 8: GG-ClyA-Nb, lane 9: GG-ClyA-Nb with 0.2% SDS, lane 10: SV-ClyA-Nb, lane 11: SV-ClyA-Nb with 0.2% SDS. (**B**) Representative dose-response curves of SE-ClyA-Nb and QR-ClyA-Nb on A431 cells. SE mutation increases toxicity, while QR mutation decreases toxicity. (**C**) Representative dose-response curves of QR-ClyA-Nb in the presence and absence of 15 nM EGF. (**D**) Comparison of the hemolytic activity of ClyA and ClyA-Nb constructs. Fusion of ClyA to the Nb extends the time necessary for complete hemolysis of red blood cells, the mutation I5S and F6E of ClyA decreases hemolysis speed even further. (**E**) Comparison of cell viability at 16 nM SE-ClyA-Nb concentrations with increasing EGF concentrations. Cell viability increases up to 90% with increasing EGF concentrations.

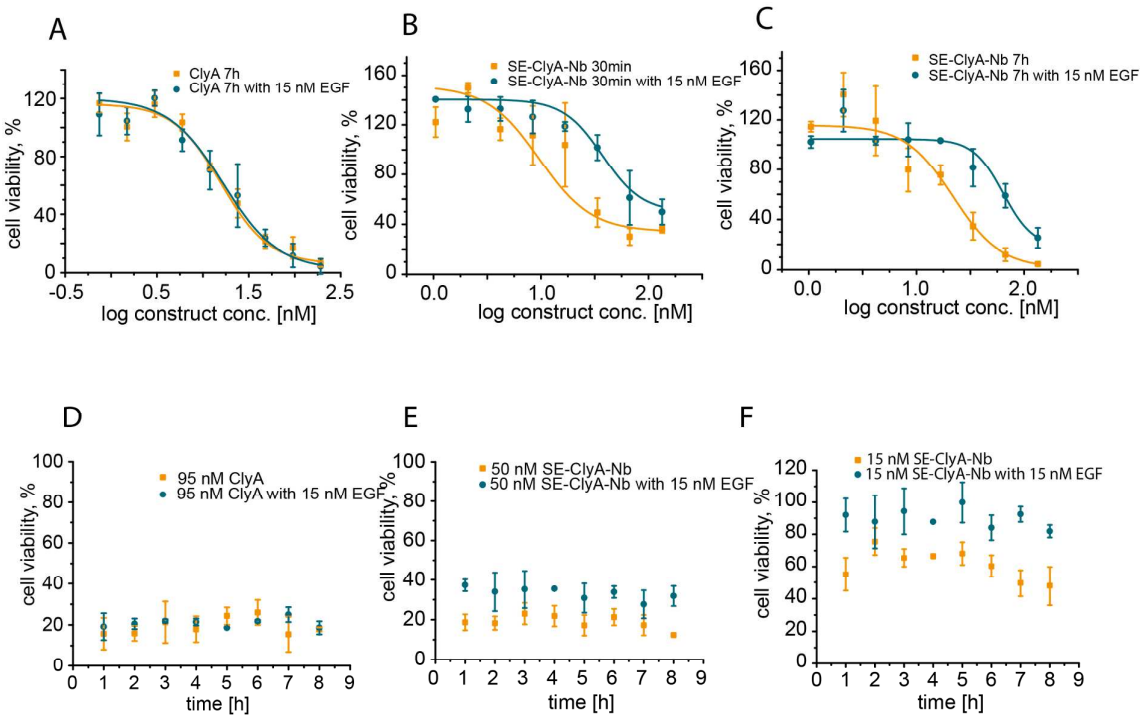

Figure S3. Time and concentration dependence of ClyA cytotoxicity towards A431 cells (**A**) Toxicity of ClyA measured after a 7-hour incubation in the presence and absence of 15 nM EGF. (**B-C**) Representative dose-response curves of SE-ClyA-Nb measured after 30 minutes (**B**) or 7 hours (**C**) incubation, before addition and 2 hour incubation of WST-8, in the presence and absence of 15 nM EGF. The addition of EGF reverses the effect of the nanobody and decreases toxicity for the cells. Representative response curves measured after different incubation times using 95 (**D**), 50 (**E**) and 15 (**F**) nM ClyA in the presence and absence of 15 nM EGF. Different incubation times had no effect on the toxicity towards the cells.

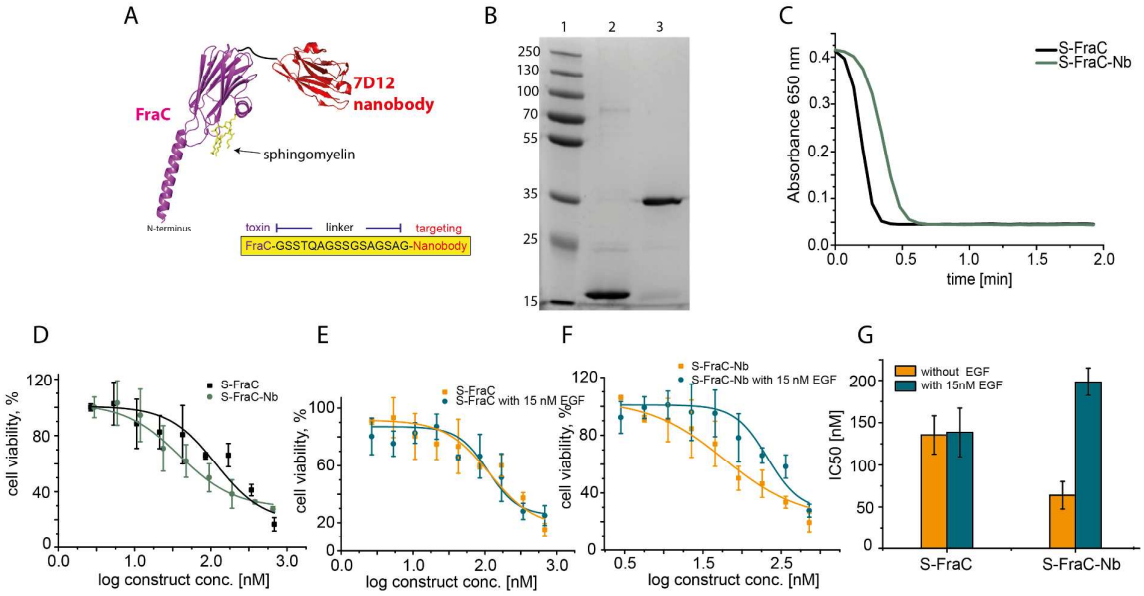

Figure S4. FraC targeting cancer cells. (**A**) Schematic representation of FraC conjugated to nanobody showing FraC (purple, PDB: 4TSY) fused at the C-terminal with the anti-EGFR nanobody 7D12 (red, PDB:4KRL) spaced by a 16 amino acid long linker (sequence indicated in yellow box). (**B**) S-FraC-nanobody examined by 12% SDS-PAGE. Lane 1: Protein ladder, Lane 2: S-FraC, Lane 3: S-FraC-Nb. (**C**) Comparison of the hemolytic activity of S-FraC and S-FraC-Nb. (**D**) Comparison between the toxicity of S-FraC and S-FraC-Nb for EGFR overexpressing A431 cells. (**E-F**) Toxicity of S-FraC and (**E**) S-FraC-Nb (**F**) in the presence and absence of 15 nM EGF. (**G**) Comparison of IC_{50} values of S-FraC and S-FraC-Nb in the presence and absence of EGF.

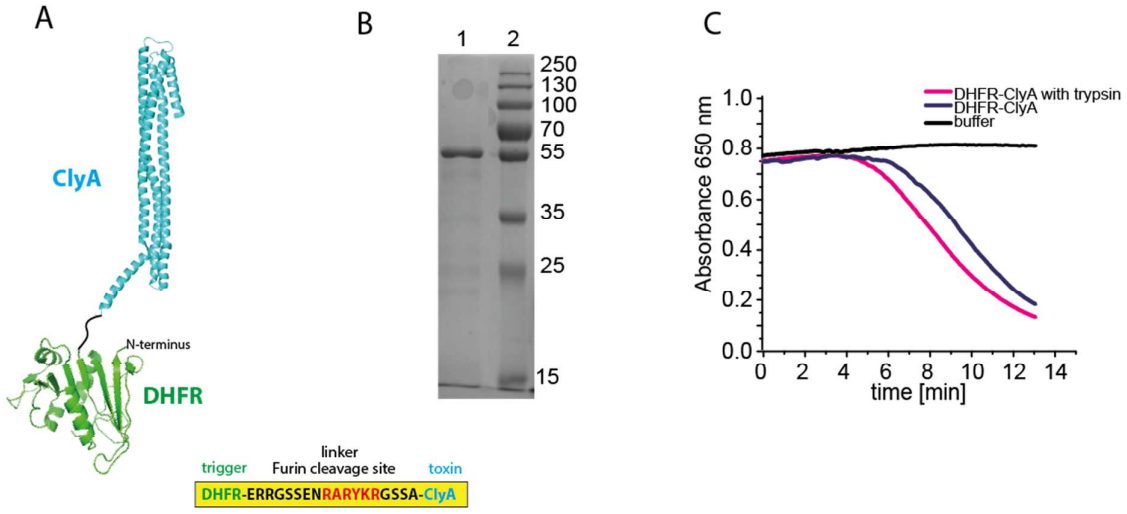

Figure S5. DHFR-ClyA fusion. (**A**) Schematic representation of the conjugate showing ClyA (blue, PDB: 2WCD) fused N-terminal to DHFR (green, PDB: 1RH3) by a 18 amino acid long linker (sequence indicated in yellow box) including a protease cleavage site for furin (indicated in red in yellow box). (**B**) 12% SDS-PAGE gel of the purified DHFR-ClyA chimera examined. Lane 1:DHFR-ClyA, Lane 2: Protein ladder. (**C**) Comparison of the hemolytic activity of DHFR-ClyA and proteolysed DHFR-ClyA. Fusion of ClyA to DHFR did not deactivate the toxin.

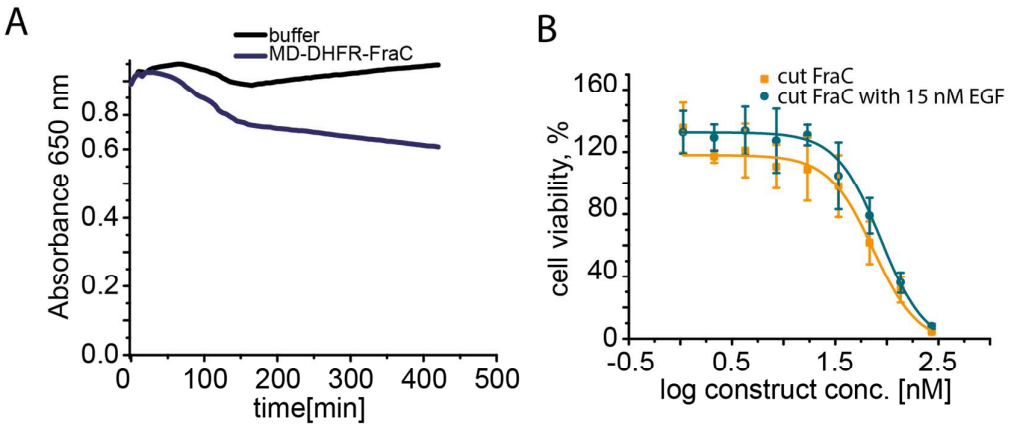

Figure S6. DHFR-FraC fusion. (**A**) Hemolytic activity measurement of MD-DHFR-FraC over 6 hours. No hemolysis of red blood cells could be observed. (**B**) Representative dose-response curves of proteolysed DHFR-FraC in the presence and absence of 15 nM EGF. The addition of EGF had no effect on the toxicity towards the cells.

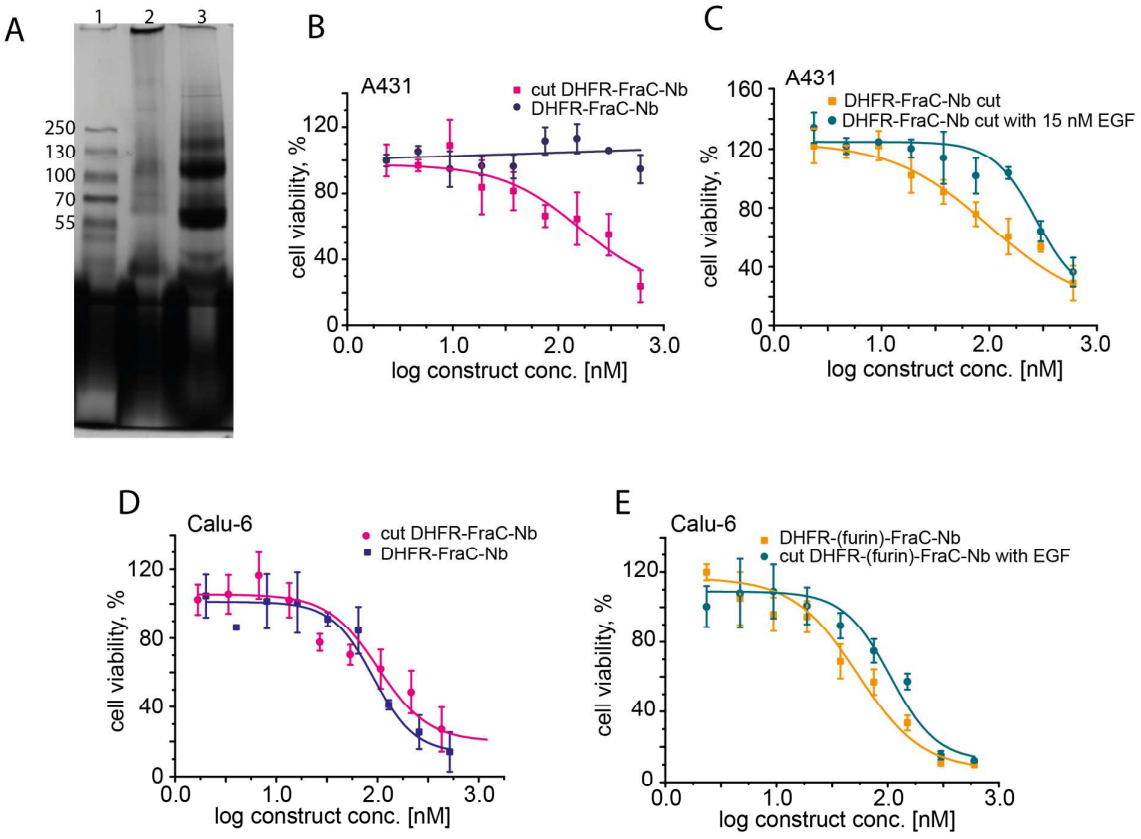

Figure S7. DHFR-FraC fusion. (A) DHFR-FraC-Nb analyzed by a 4-20% blue-native PAGE. [1] Lane 1: Protein ladder, lane 2: DHFR-FraC-Nb, lane 3: DHFR-FraC-Nb with 0.2% SDS. Several bands are observed corresponding to monomeric DHFR-FraC-Nb (56 kDa) and, most likely, to multimeric DHFR-FraC-Nb. Interestingly the oligomeric forms of the toxin appear to be SDS stable. FraC is positively charged, hence the faded bands in line 2 (no SDS) are most likely due to protein construct inability of running in native conditions (**B**) Representative dose-response curves of DHFR-FraC-Nb and proteolysed DHFR-FraC-Nb towards A431 cells, showing that only proteolysed DHFR-FraC-Nb is active. (**C**) Representative dose-response curves of proteolysed DHFR-FraC-Nb in the presence and absence of 15 nM EGF tested on A431 cells, showing that the addition of EGF reverses the effect of the nanobody and decreases toxicity for the cells. (**D**) Representative dose-response curves of DHFR-FraC-Nb and proteolysed DHFR-FraC-Nb towards Calu-6 cells, showing that DHFR-FraC-Nb proteolysed by furin expressed by Calu-6 cells gets activated and cause cell death. (**E**) Representative dose-response curves of proteolysed DHFR-FraC-Nb in the presence and absence of 15 nM EGF tested on Calu-6 cells, showing that the addition of EGF reverses the effect of the nanobody and decreases toxicity for the cells.

References

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