Clickable Shiga Toxin B Subunit for Drug Delivery in Cancer Therapy

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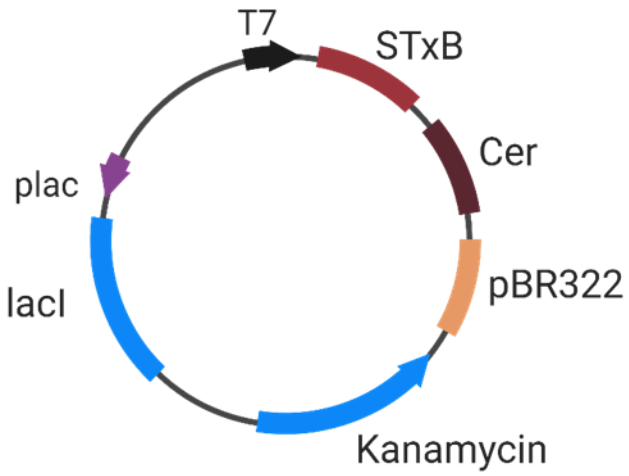
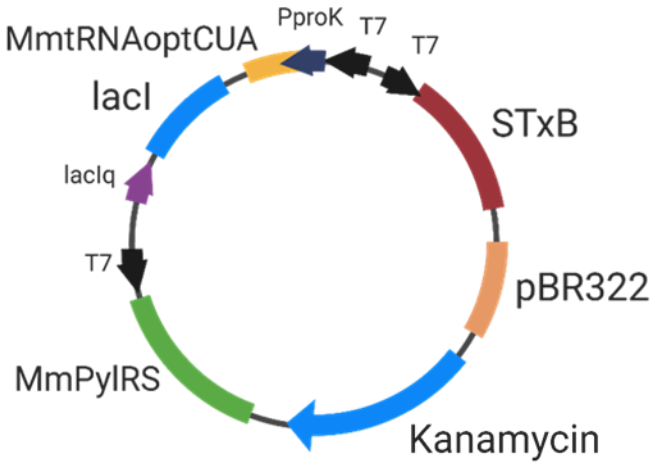
Supplementary

(a)



(b)

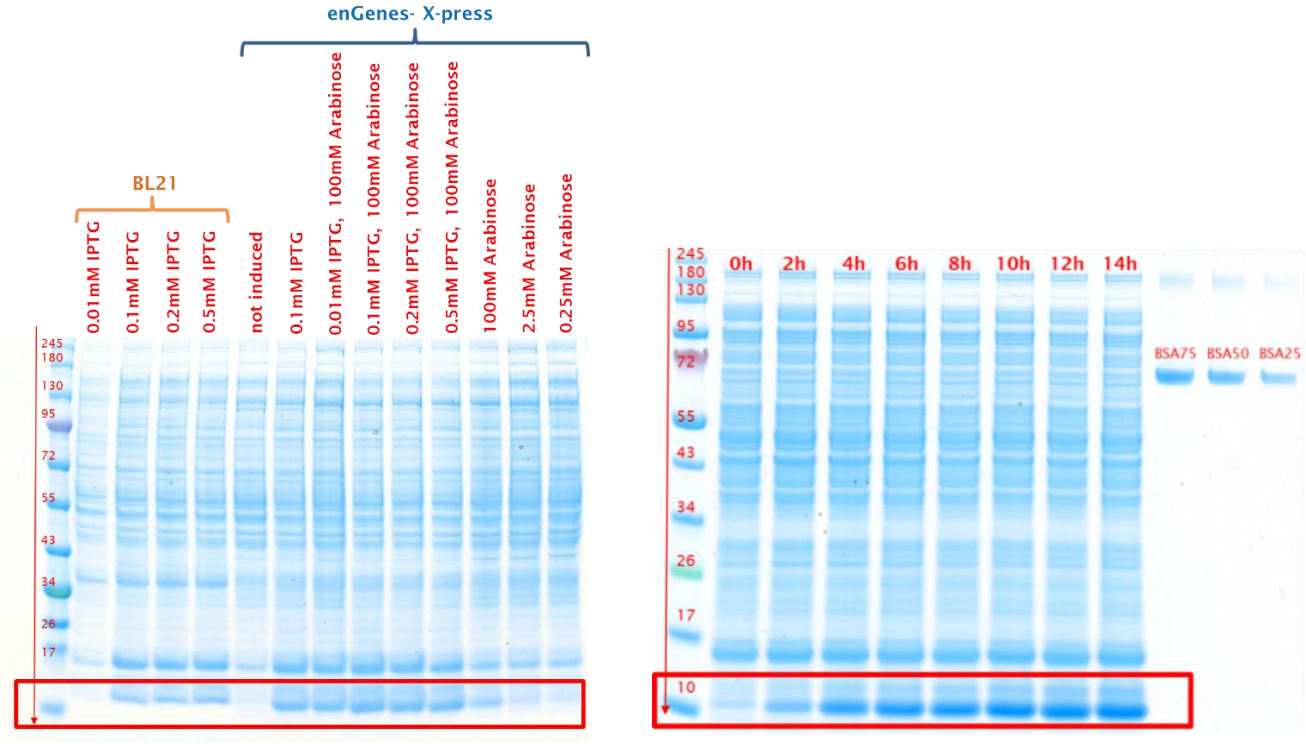


(c)

MTPDCVTGKVEYTKYNDDDTFTVKVGDKELFTNRWNLQSLLLSAQITGMTVTIKTNACHNGG
GFSEVIFRGSGHHHHHH

Supplementary 1. Design of (a) pET30a<STxB$_{wt}$>Cer, (b) pSCS-T7x3<STxB$_{wt}$> or pSCS-T7x3<STxB$_{AzK}$>. Created with BioRender.com. (c) STxB$_{AzK}$ sequence. The incorporated non canonical amino acid, azido lysine (AzK) at position K9, is highlighted.

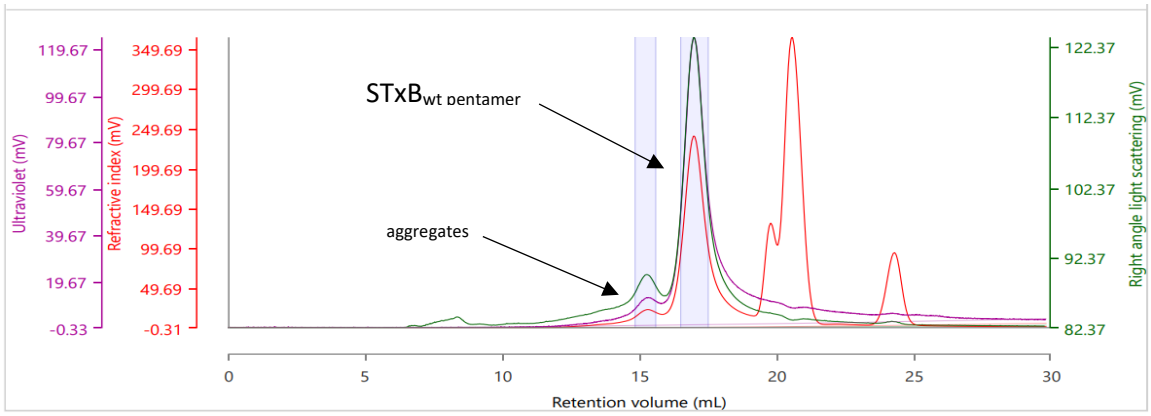
(a) (b)



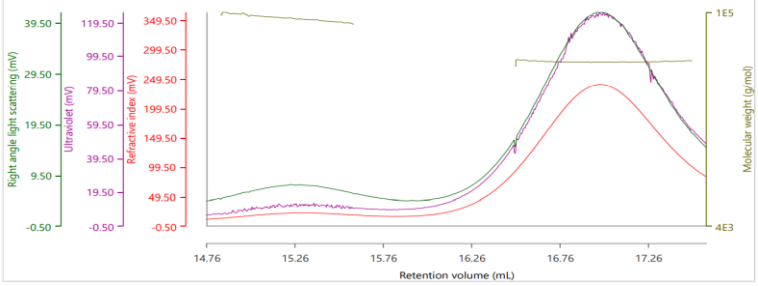
18 **Supplementary 2.** SDS-PAGE gel of $STxB_{wt}$ expression optimization. (a). Expression of BL21 (DE3) versus enGenes-X-press (V1) strain
19 in µ-bioreactor cultivations (800 µL scale). (b). Expression of enGenes-X-press (V1) strain in benchtop bioreactor (1L) fed-batch process.

20

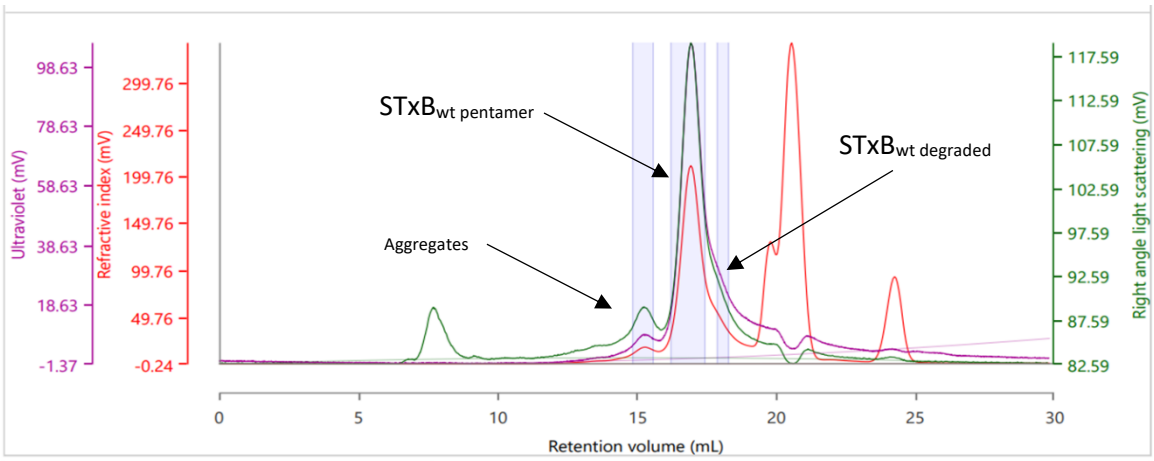
(a)



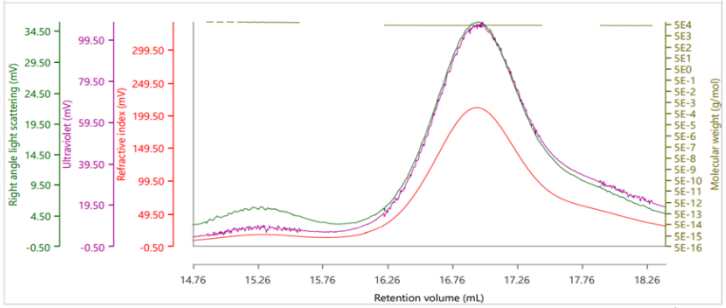
	sampleday0 Injection 1 Peak 1	Peak 2
Mw (g/mol)	89 634	45 776
Mw/Mn	1.002	1
Frac. of sample (%)	7.67	92.33
Recovery (%)	53.32	53.32
Measured conc. (mg/mL)	1.0665	1.0665
Calc. dn/dc (mL/g)	N/C	N/C
Calc. dA/dc (mL/(mg*cm))	N/C	N/C
RV (mL)	15.31	16.99
RI peak (mV·mL)	14.86	178.96
UV peak (mV·mL)	7.96	93.6
RALS peak (mV·mL)	5.07	31.2
LALS peak (mV·mL)	4.58	28.45
Data Processed	Yes	Yes
Peak Measured conc. (mg/mL)	0.0817	0.9847



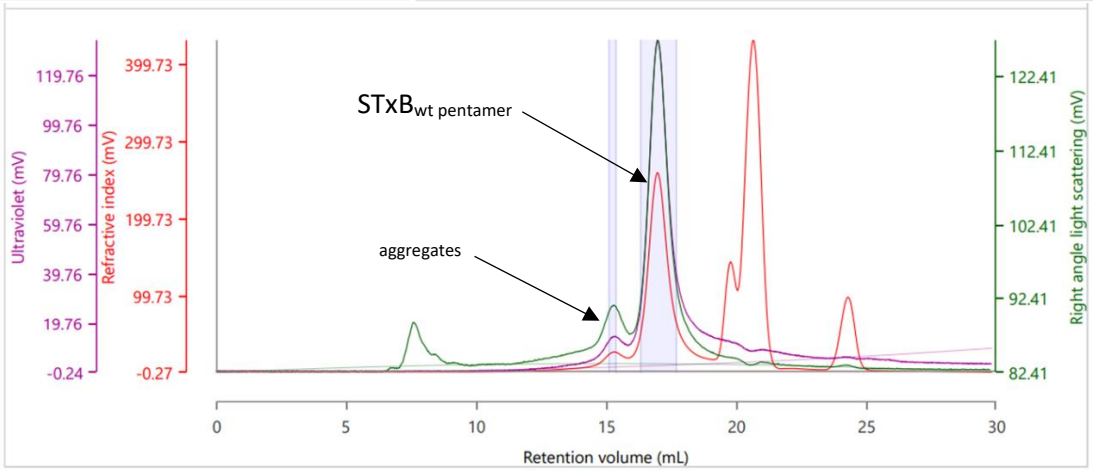
(b)



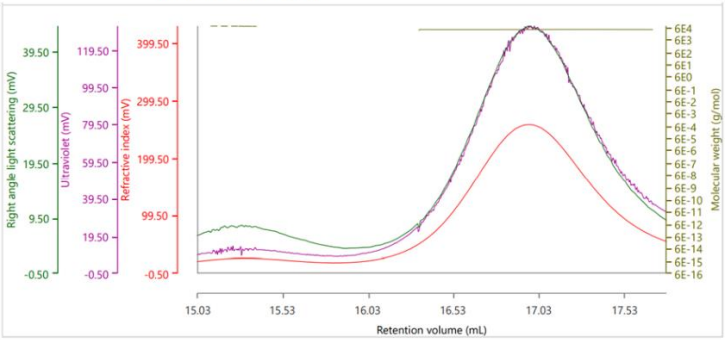
	sampletube Injection 1 Peak 1	Peak 2	Peak 3
Mw (g/mol)	85 176	44 870	42 846
Mw/Mn	1.001	1	1
Frac. of sample (%)	5.87	84.94	9.19
Recovery (%)	61.73	61.73	61.73
Measured conc. (mg/mL)	1.0803	1.0803	1.0803
Calc. dn/dc (mL/g)	N/C	N/C	N/C
Calc. dA/dc (mL/(mg*cm))	N/C	N/C	N/C
RV (mL)	15.3	16.95	17.9
RI peak (mV·mL)	11.53	166.81	17.94
UV peak (mV·mL)	5.48	84.52	10.32
RALS peak (mV·mL)	3.71	28.5	2.93
LALS peak (mV·mL)	6.08	27.07	3.02
Data Processed	Yes	Yes	Yes
Peak Measured conc. (mg/mL)	0.0634	0.9176	0.0992



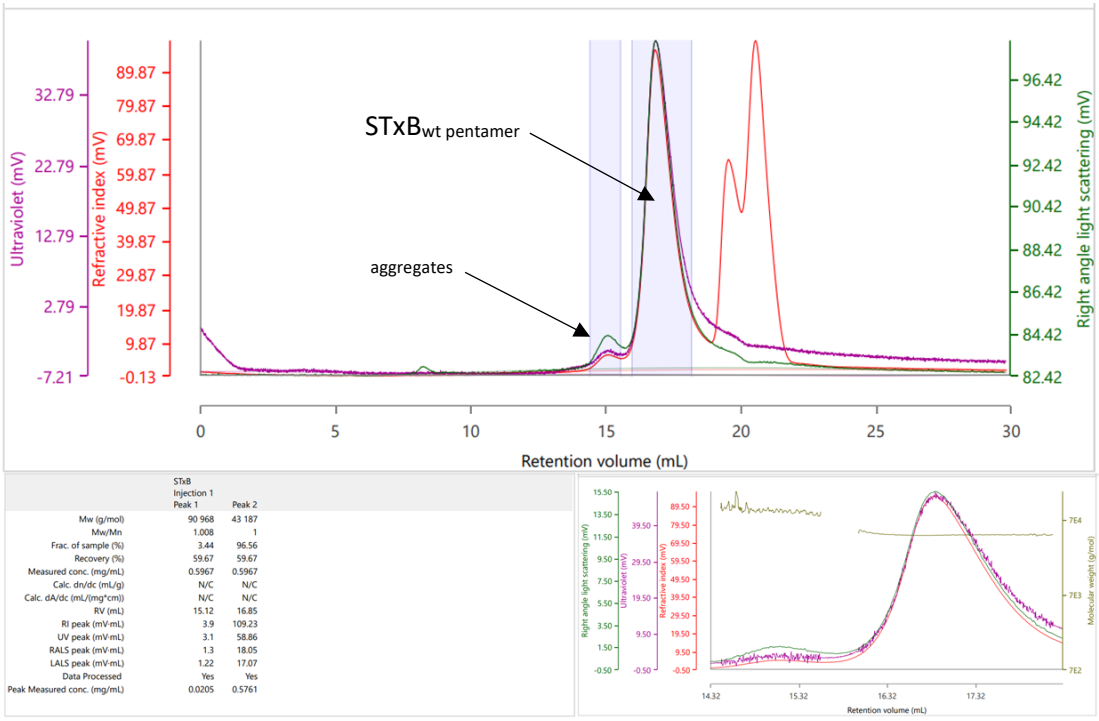
(c)



	alreadfilt Injection 1 Peak 1	Peak 2
Mw (g/mol)	83 455	44 641
Mw/Mn	1	1
Frac. of sample (%)	2.98	97.02
Recovery (%)	58.83	58.83
Measured conc. (mg/mL)	1.2355	1.2355
Calc. dn/dc (mL/g)	N/C	N/C
Calc. dA/dc (mL/(mg*cm))	N/C	N/C
RV (mL)	15.31	16.98
RI peak (mV·mL)	6.67	217.91
UV peak (mV·mL)	3.22	112.26
RALS peak (mV·mL)	2.09	37.05
LALS peak (mV·mL)	4.38	35.32
Data Processed	Yes	Yes
Peak Measured conc. (mg/mL)	0.0369	1.1986



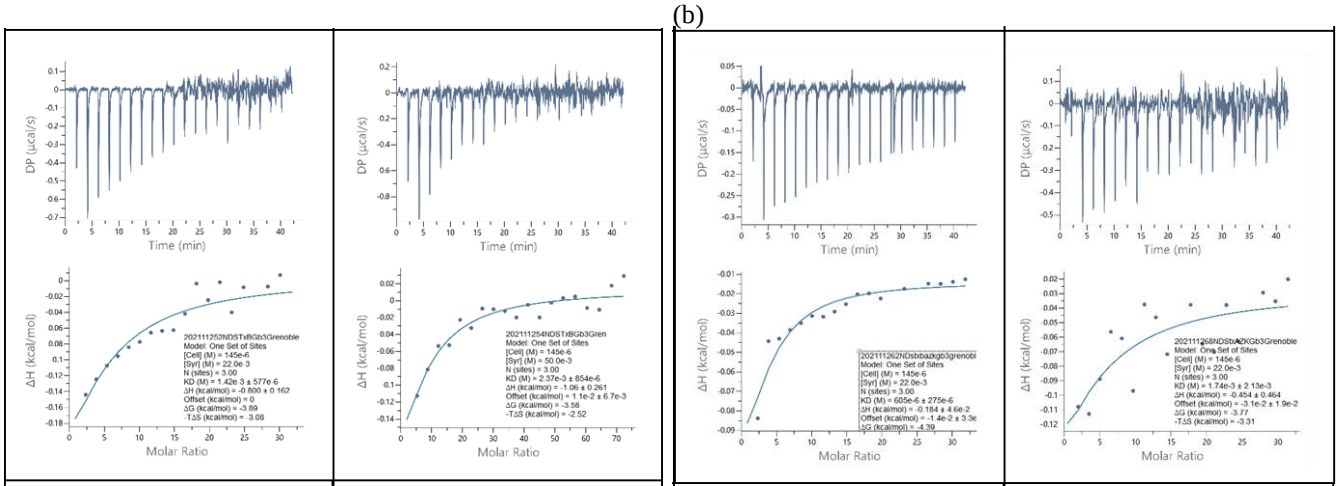
(d)



	STxB Injection 1 Peak 1	Peak 2
Mw (g/mol)	90 968	43 187
Mw/Mn	1.008	1
Frac. of sample (%)	3.44	96.56
Recovery (%)	59.67	59.67
Measured conc. (mg/mL)	0.5967	0.5967
Calc. dn/dc (mL/g)	N/C	N/C
Calc. dA/dc (mL/(mg*cm))	N/C	N/C
RV (mL)	15.12	16.85
RI peak (mV·mL)	3.9	109.23
UV peak (mV·mL)	3.1	58.86
RALS peak (mV·mL)	1.3	18.05
LALS peak (mV·mL)	1.22	17.07
Data Processed	Yes	Yes
Peak Measured conc. (mg/mL)	0.0205	0.5761

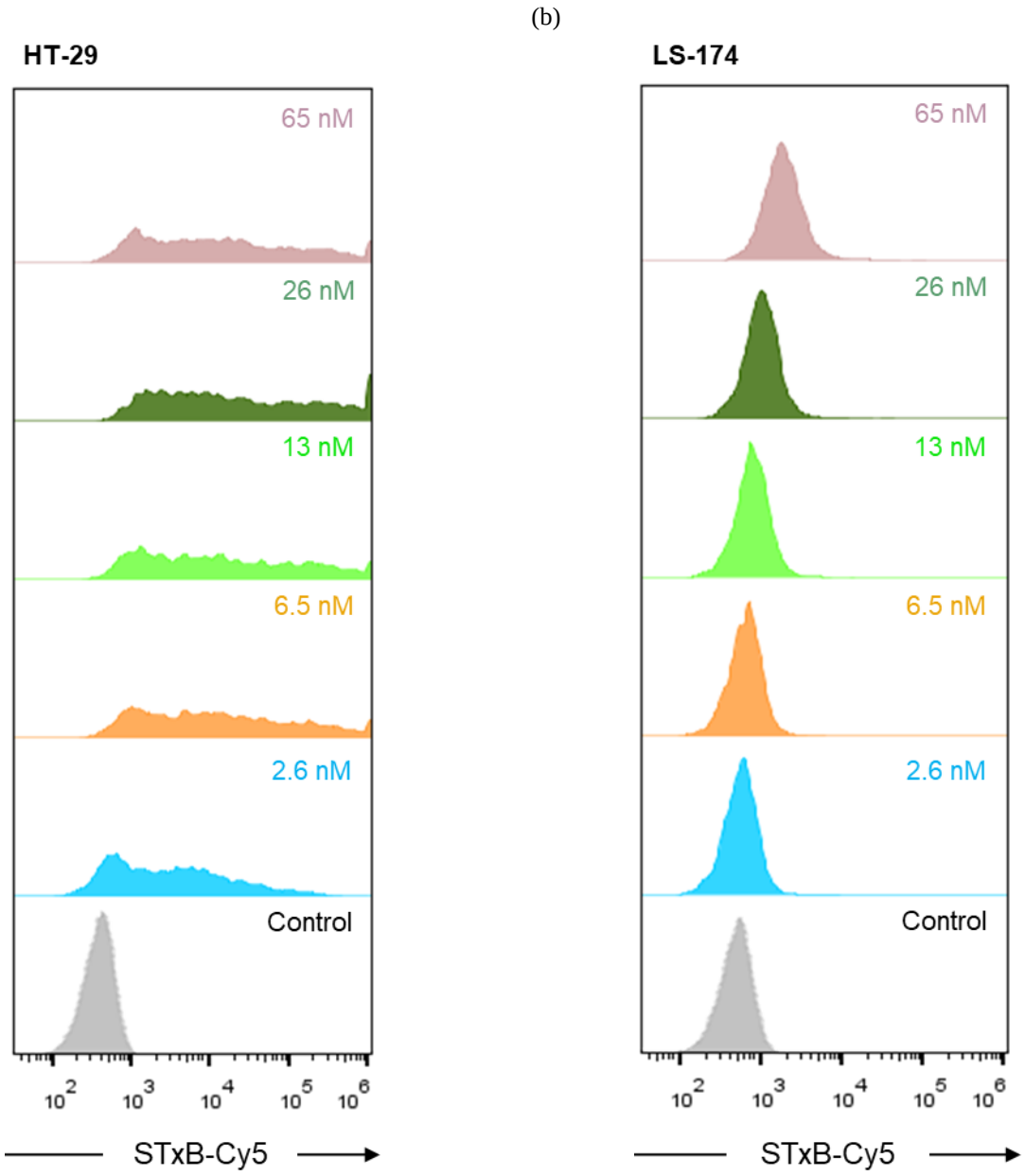
Supplementary 3. SEC-MALS stability measurement of $STxB_{wt}$. (a) $STxB_{wt}$ measured upon defrosting (-80°C). (b) $STxB_{wt}$ measured upon 2 weeks storage (4°C). Degraded $STxB_{wt}$ removed upon filtration and analysis shown in (c), where $STxB_{wt}$ was measured upon 2 weeks storage (4°C). (d) $STxB_{wt}$ defrosted 5 times (-20°C).

(b)



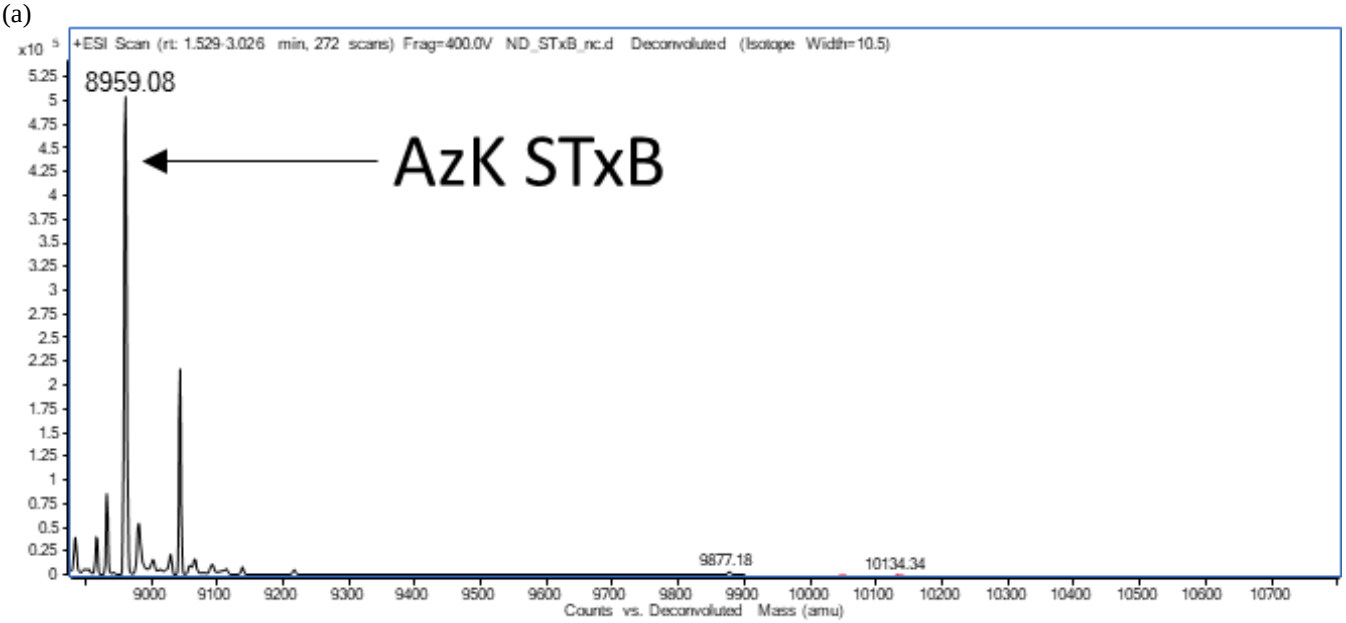
(a)

Supplementary 4. ITC measurement of $STxB_{wt/AzK}$ binding to Gb3 receptor. (a) Duplicate measurement of $STxB_{wt}$ binding to Gb3. (b) Duplicate measurement of $STxB_{AzK}$ binding.

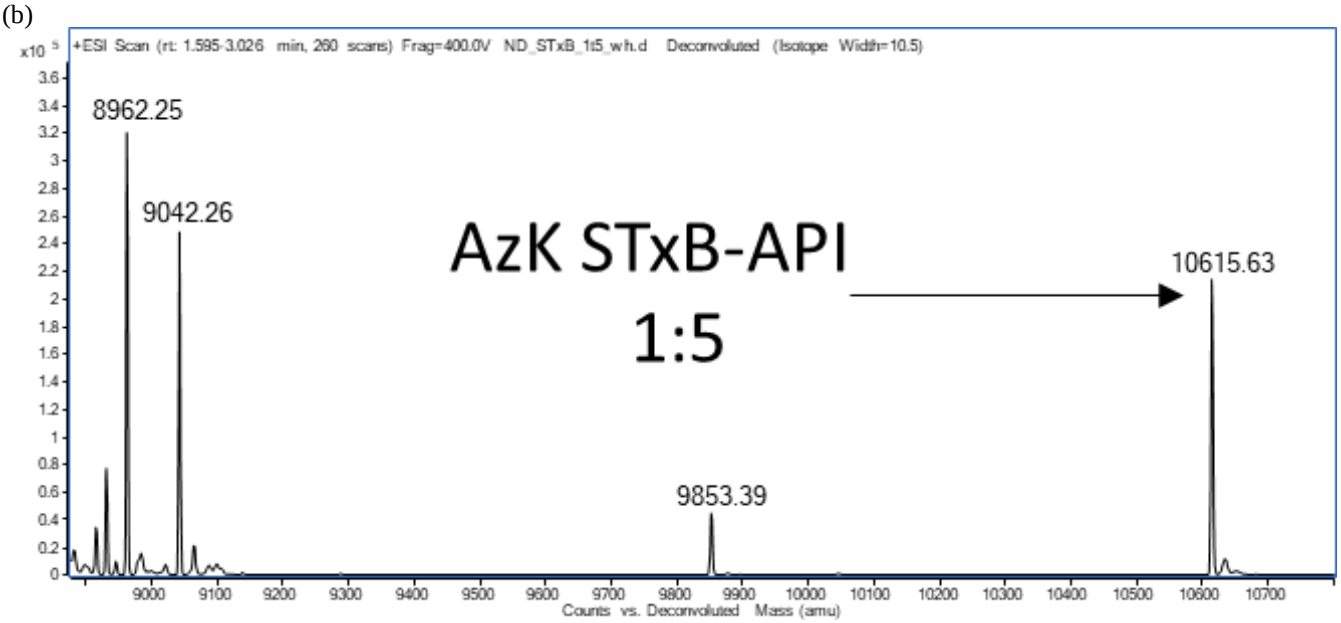


Supplementary 5. Expression of Gb3 antigens at the surface of HT-29 and LS-174 tumour cells. (a) Representative histograms of flow cytometry analysis of gated living HT-29 cells stained with increasing concentrations of STxB obtained from commercial sources. Cells were treated with fluorescently labelled STxB-Cy5 for 30 min on ice. (b) Histograms of fluorescence intensity of gated living LS-174 cells incubated with increasing concentrations of STxB-Cy5 available for commercial use, for 30 min on ice.

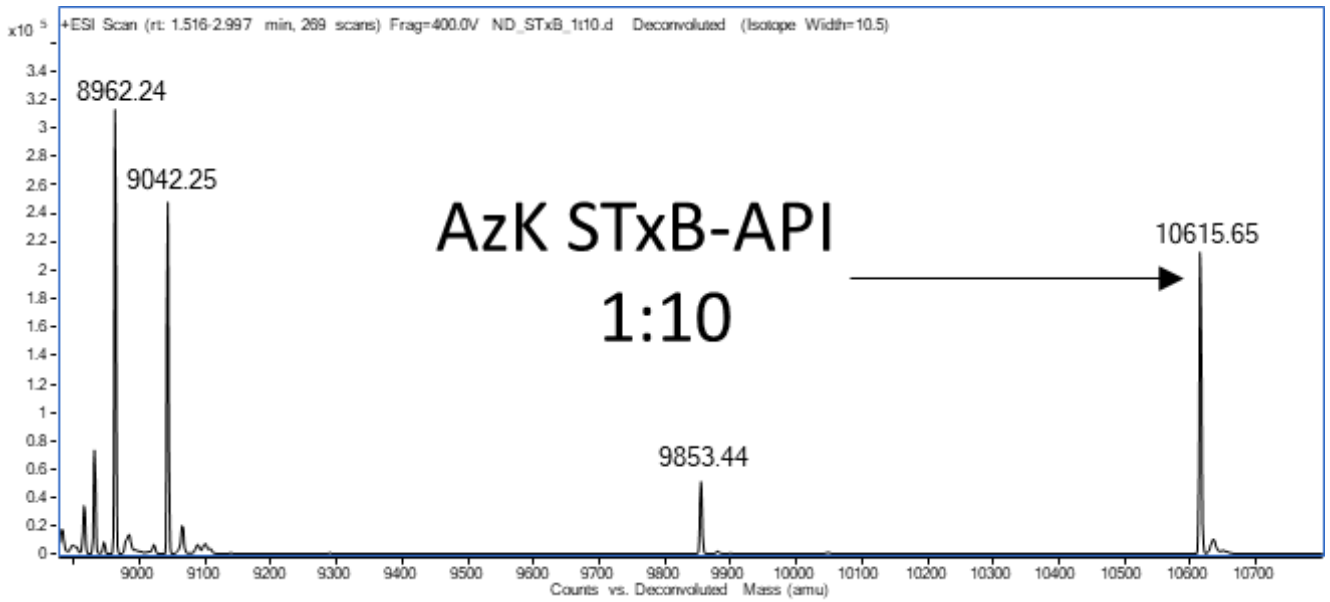
(a)


+ESI Scan (rt: 1.529-3.026 min, 272 scans) Frag=400.0V ND_STxB_nc.d Deconvoluted (Isotope Width=10.5)
x10 5
8959.08
AzK STxB
9877.18
10134.34
Counts vs. Deconvoluted Mass (amu)

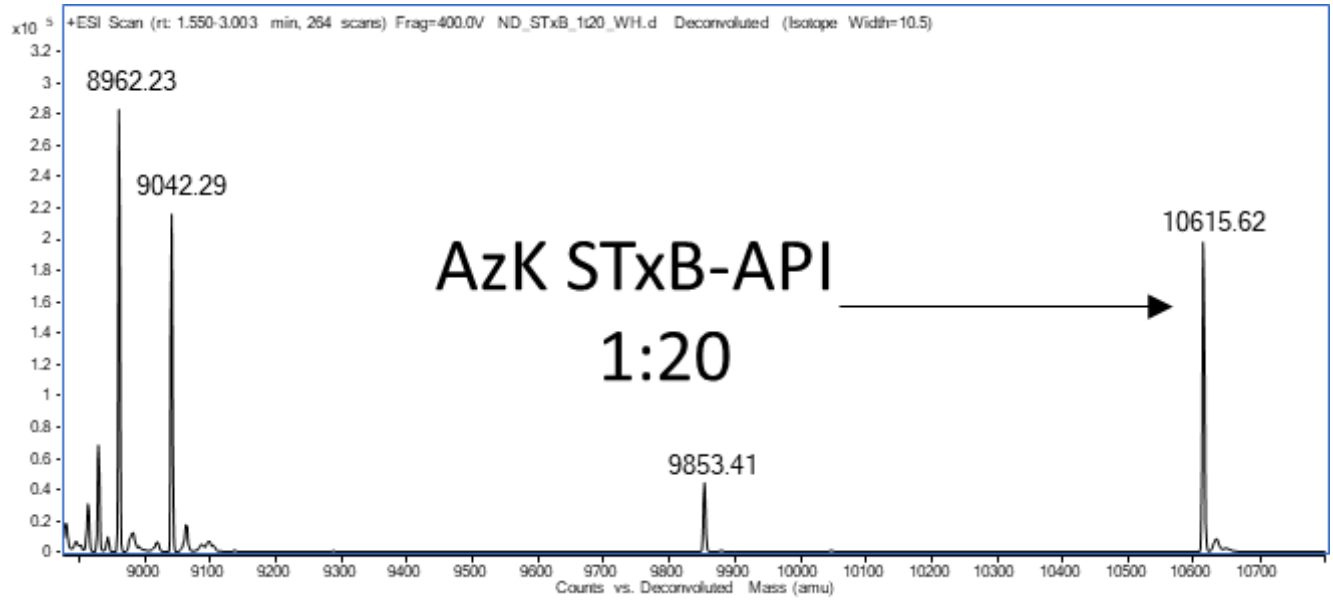
(b)


+ESI Scan (rt: 1.595-3.026 min, 260 scans) Frag=400.0V ND_STxB_1t5_wh.d Deconvoluted (Isotope Width=10.5)
x10 5
8962.25
9042.26
AzK STxB-API
1:5
10615.63
9853.39
Counts vs. Deconvoluted Mass (amu)

(c)

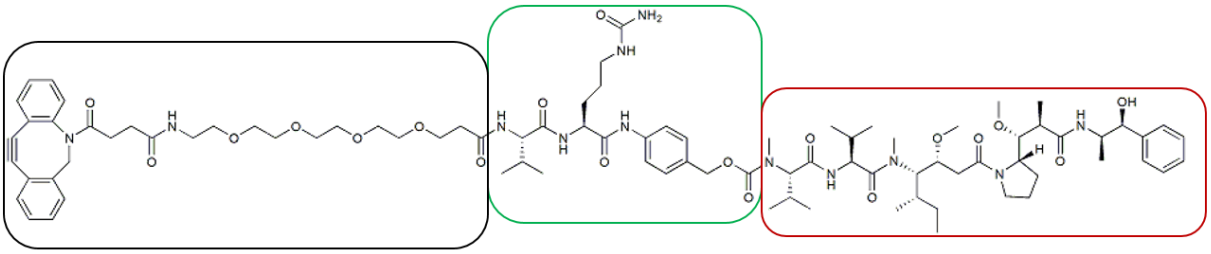


(d)



Supplementary 6. Intact protein MS analysis of $STxB_{AzK}$ (8959 Da) and DBCO-PEG4-Cit-PAB-MMAE drug attachment (10615 Da). The sample of (a) not coupled $STxB_{AzK}$, (b) coupled with the drug to ratio 1:5, (c) coupled with the drug to ratio 1:10 and (d) coupled with drug to ratio 1:20. 8959 MW is measured $STxB_{AzK}$, and 10615.6 MW is mesured $STxB_{AzK}$ attached to DBCO-PEG4-Cit-PAB-MMAE. The remaining peaks are not identified and will require further research.

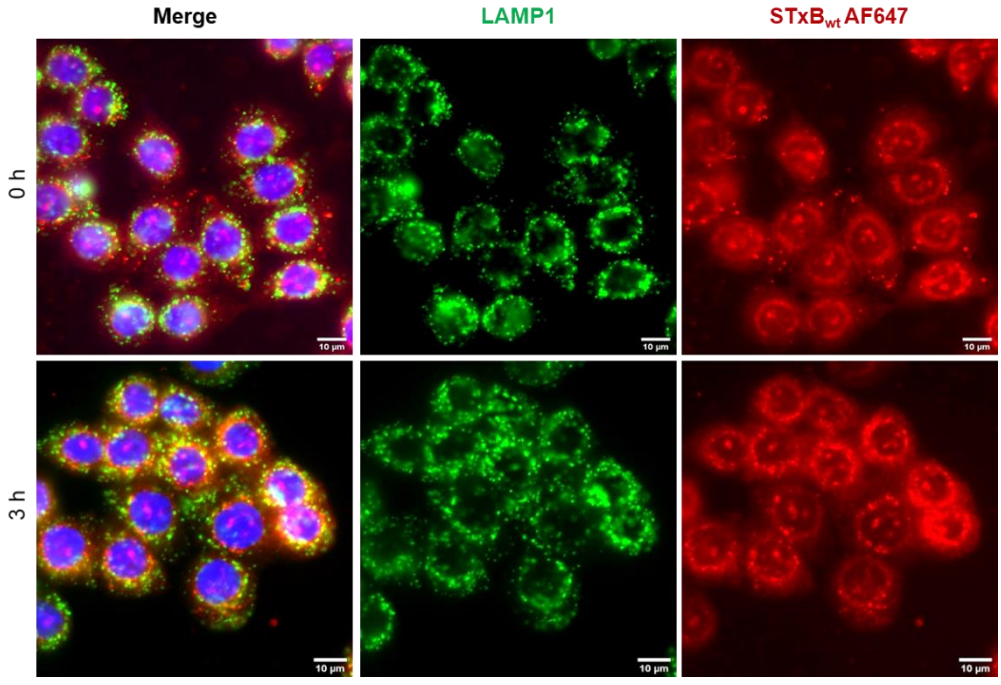
DBCO-PEG4-Val-Cit-PAB-MMAE



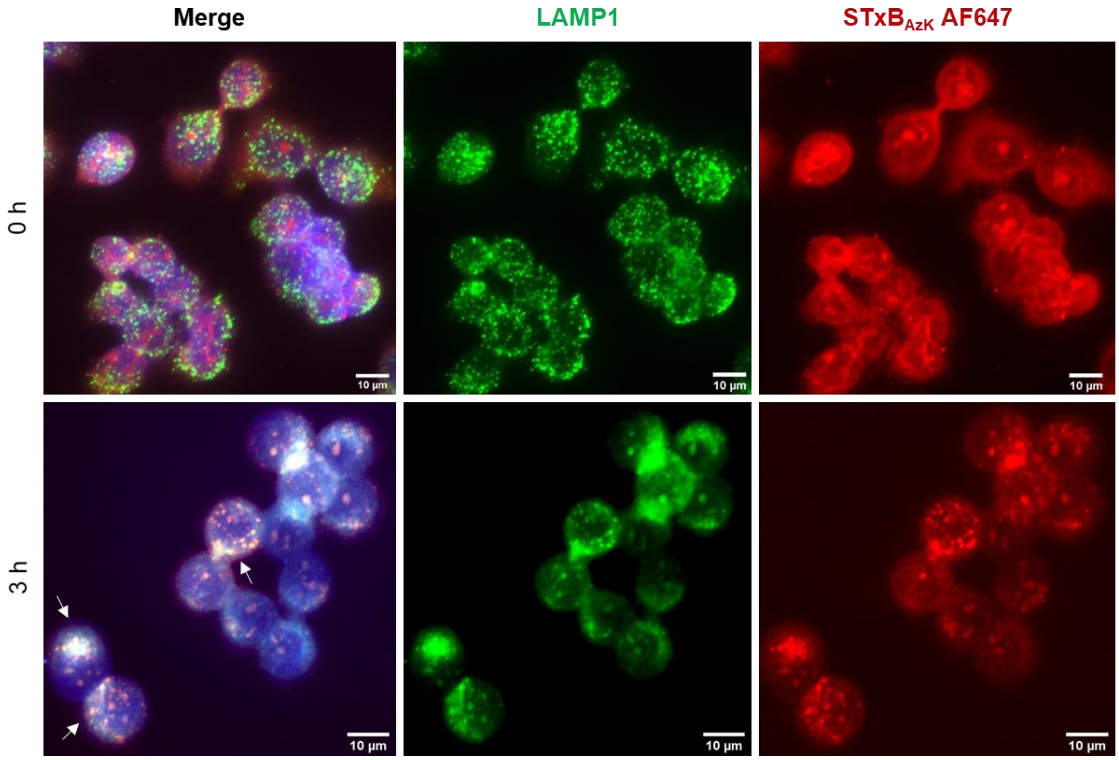
- DBCO-PEG4 enables click-chemistry to $STxB_{AzK}$
- Valine-citrulline-PAB-linker is enzymatically cleaved by intracellular cathepsin B
- Monomethyl Auristatin E (MMAE) is the toxic payload released into the cytosol after cleveage

Supplementary 7. Composition of DBCO-PEG4-Val-Cit-PAB-MMAE toxic payload delivered to Gb3-expressing cancer cells by $STxB_{AzK}$.

(a)

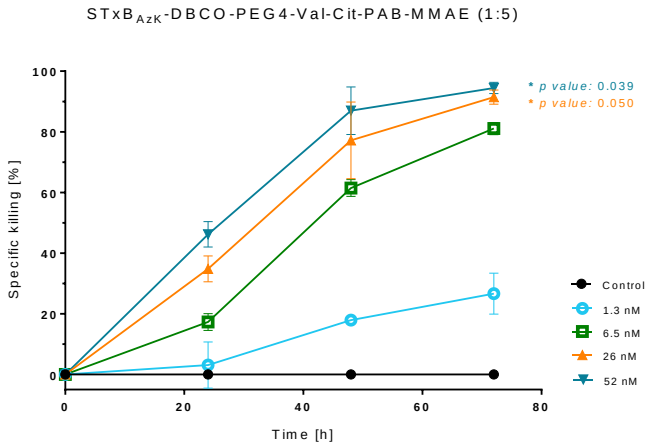


(b)

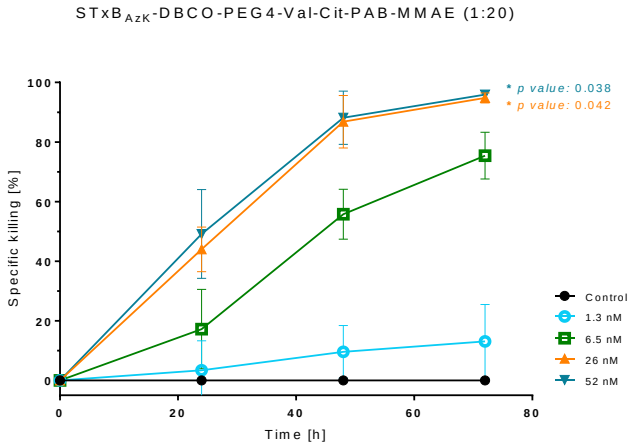


Supplementary 8. Fluorescence imaging of HT-29 cells incubated with STxB$_{wt/AzK}$. (a) Immunofluorescence studies of HT-29 cells treated with STxB$_{wt}$ AF647 (red) for 30 min on ice (0 h) or 3 h at 37°C. Lysosomes were stained with antibodies directed against LAMP1 (green). Nuclei were counterstained by DAPI (blue). Scale bars: 10 µM. (b) Immunofluorescence studies of HT-29 cells incubated with STxB$_{AzK}$ AF647 (red) for 30 min on ice (0 h) or 3 h at 37°C. Lysosomes were stained with antibodies directed against LAMP1 (green). Nuclei were counterstained by DAPI (blue). Scale bars: 10 µM. Images reveal intracellular uptake and accumulation of STxB$_{wt/AzK}$ in HT-29 cells. White arrows in the lower panel (3 h) highlight a potential accumulation of STxB$_{AzK}$ in the lysosomes of target cells, visible as overlap of red (STxB$_{AzK}$ AF647) and green (LAMP1) fluorescent signals.

(a)

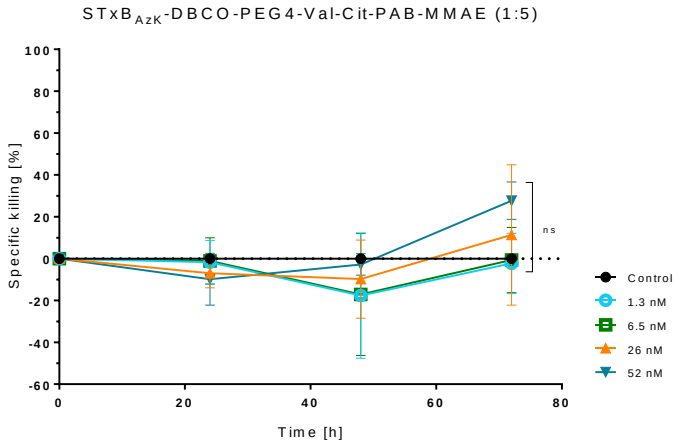


(b)

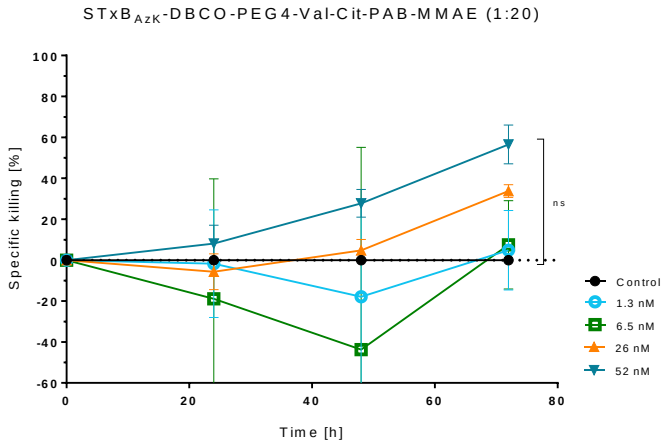


Supplementary 9. $STxB_{AzK}$-MMAE conjugates (1:5) and (1:20) mediate cytotoxic drug delivery to HT-29 tumour cells and the *in vitro*-specific killing. Quantification of specific killing activity upon incubation of $Gb3^+$ HT-29 target cells with $STxB_{AzK}$ after conjugation to MMAE in two different reactions with 1:5 or 1:20 molar ratios. (a) Cytotoxicity assay of HT-29 cells incubated with 1.3, 6.5, 26 or 52 nM $STxB_{AzK}$-DBCO-PEG4-Val-Cit-PAB-MMAE (1:5) for 72 h. (b) Cytotoxicity assay of HT-29 cells incubated with 1.3, 6.5, 26 or 52 nM $STxB_{AzK}$-DBCO-PEG4-Val-Cit-PAB-MMAE (1:20) for 72 h. Percent viability was calculated relative to the luminescence from an equal number of input control cells and used to calculate percent specific killing. Results are expressed as a mean ± SD (n = 3) from 3 separate experiments. Statistical differences in independent, identical samples were determined with a two-tailed, unpaired t-test for control and treatment groups, at each time point. Tests with a p-value ≤ 0.05 are considered statistically significant and marked with an asterisk (*). Non-significant results are not highlighted.

(a)



(b)



Supplementary 10. $STxB_{AzK}$-MMAE conjugates (1:5) and (1:20) mediate cytotoxic drug delivery to LS-174 tumour cells and *in vitro* killing. Quantification of specific killing activity upon incubation of low Gb3-expressing LS-174 target cells with $STxB_{AzK}$ after conjugation to MMAE in two different reactions with 1:5 or 1:20 molar ratios. (a) Cytotoxicity assay of LS-174 cells incubated with 1.3, 6.5, 26 or 52 nM $STxB_{AzK}$-DBCO-PEG4-Val-Cit-PAB-MMAE (1:5) for 72 h. (b) Cytotoxicity assay of LS-174 cells incubated with 1.3, 6.5, 26 or 52 nM $STxB_{AzK}$-DBCO-PEG4-Val-Cit-PAB-MMAE (1:20) for 72 h. Percent viability was calculated relative to the luminescence from an equal number of input control cells and used to calculate percent specific killing. Results are expressed as a mean ± SD (n = 3) from 3 separate experiments. Statistical differences in independent, identical samples were determined with a two-tailed, unpaired t-test for control and treatment groups, at each time point. Tests with a p-value ≤ 0.05 are considered statistically significant and marked with an asterisk (*). Non-significant results are indicated as ns.