Supplementary Materials for

Molecular dissection of cobra venom highlights heparinoids as an antidote for spitting cobra envenoming

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References (*66* - *70*)

Other Supplementary Materials for this manuscript include the following:

Data files S1 to S12

Supplementary Materials and Methods

Chemicals, drugs and biological materials

Sydney: Resazurin Sodium Salt (R7017) and Iscove's modified Dulbecco's medium (IMDM; I3390) were purchased from Sigma-Aldrich (Merck). Dulbecco's modified Eagle's medium (DMEM; 11960-044), Puromycin (A11138-03), TrypLE Express (12605-028), and penicillin-streptomycin (15140-122) were purchased from Gibco (Thermo Fisher Scientific). Fetal bovine serum (FBS; SFBS-F) was purchased from Bovogen Biologicals. Heparin sodium (H3393), tinzaparin sodium (T1490000), dalteparin sodium (D0070000) and N-acetyl-heparin sodium (A8036) were purchased from Sigma-Aldrich (Merck). Working stocks were 3mM in PBS, heparin (monomer ~1135 g/mol, 3.4 mg/mL), tinzaparin (~6500 g/mol, 19.5 mg/mL), dalteparin (~6000 g/mol, 18 mg/mL), and N-acetyl-heparin (~1500 g/mol, 4.6 mg/mL).

Liverpool: Thiazolyl blue methyltetrazolium bromide (MTT; M5655), dimethyl sulfoxide (DMSO; 276855), and propidium iodide (PI; P4170) were purchased from Sigma-Aldrich (Merck). Dulbecco's modified Eagle's medium (DMEM; 11574516), fetal bovine serum (FBS; 11573397), FluoroBrite DMEM (A1896701), glutaMAX supplement (35050038), penicillin-streptomycin (11528876), phosphate buffered saline (11503387), and TrypLE Express were purchased from Gibco (Thermo Fisher Scientific). Heparin sodium (H0200000), tinzaparin sodium (T1490000), and dalteparin sodium (D0070000) were purchased from Sigma-Aldrich (Merck). Working stocks were 50 mg/mL in PBS, stored at -20°C and thawed prior to use for experiments.

Cell culture

HAP1 cells were generously provided by Thijn R. Brummelkamp. HAP1 and HEK293T cells were cultured in Iscove's Modified Dulbecco Media (Gibco) and Dulbecco's Modified Eagle Medium (Sigma-Aldrich) respectively, both containing 10% FBS and 1X Penicillin-Streptomycin at 37°C, 5% CO_2.

Immortalized human epidermal keratinocyte cells, HaCaT cells (*66*, *67*), were purchased from Caltag Medsystems. Cells were cultured in phenol red-containing DMEM with GlutaMAX supplemented with 10% FBS, 100 IU/mL penicillin, 250 µg/mL streptomycin, and 2 mM sodium pyruvate (Standard HaCaT medium). For the MTT and PI multiplexed assays, a minimally fluorescent medium was used instead: FluoroBrite DMEM supplemented with 1% GlutaMAX 100X supplement, 1% FBS, 100 IU/mL penicillin, 250 µg/mL streptomycin, and 2 mM sodium pyruvate (Low background fluorescence HaCaT medium). The cells were split and medium changed twice per week up to a maximum of 30 passages. Cells were maintained in a humidified, 95% air/5% CO_2 atmosphere at 37°C (standard conditions).

Cell viability and death assays

HAP1 cells were trypsinized and seeded in 96 well plates at a density of 3.5 x10^4 cells/well. After 24 h, serial dilutions of spitting cobra venom (spanning 0.02-50 mg/mL) were added for a further 24 h. After incubation resazurin solution was added to a final concentration of 30 µg/ml and incubated for 2.5-3 h at 37 °C. The fluorescence was measured at 544 nm excitation and 590 nm emission using a microplate spectrophotometer (FLUOstar Omega, BMG Labtech).

HaCaT cell viability and death were quantified with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) and PI (propidium iodide) multiplexed assays, respectively, as previously described (*37*) with the following alterations: On day 1, cells were seeded (10,000 cells/well in half-volume, black-sided, & clear-bottomed 96-well plates [Greiner; 675090]); On day 2, cells were treated (50 µL/well) with: (a) serial dilutions of Nigerian *N. nigricollis*, Tanzanian *N. nigricollis*, or *N. pallida* (4.74-47.4 µg/mL), or (b) these same three venoms (15 µg/mL) combined with Heparin, dalteparin, or tinzaparin (1,000 µg/mL) in PI-containing medium for 24 hours. On day 3, PI fluorescence (Ex_{544}/Em_{612}) and MTT absorbance (A_{550}), after 2 h of MTT exposure, were measured on a CLARIOstar Plus Microplate Reader (BMG Labtech).

Lentivirus production

Toronto KnockOut Library v3 (TKOv3) library plasmid production. HEK293T cells were seeded at 25 x10^6 per T-175 flask. Once cells reached 70-90% confluency after 24 h they were transfected using Lipofectamine 3000 (Thermo) with pCAG-VSVG (Addgene plasmid 35616), psPAX2 (Addgene plasmid 12260) and the TKOv3 plasmid library (Addgene plasmid 90294) at a 1:3:3 ratio. 16 h after transfection the medium was replenished with fresh medium. At 48 h post transfection the lentivirus-containing supernatant was collected and filtered through a 0.45 µm ultra-low protein binding filter (Merck Millipore) and concentrated with 10 000 MW Pierce protein concentrators (ThermoFisher Scientific). The concentrated lentivirus media was aliquoted and stored at -80°C.

The concentrated lentiviral library was titrated by transducing HAP1 cells with a dilution series of virus supplemented with polybrene (8 µg/ml). After 24 h, the viral medium was replaced with normal medium to allow cell recovery. Medium containing puromycin (1 µg/ml) was added to the transduced cells and selection was conducted for 72 h with puromycin-supplemented medium being refreshed daily. The multiplicity of infection (MOI) of the virus was determined through comparison of percentage of surviving cells to non-infected control cells by resazurin viability assay.

Cell transduction using TKO v3 library

Using an MOI of 0.3 as previously titrated, 70 x10^6 HAP1 cells were transduced with the TKOv3 lentivirus library with 8 µg/ml of polybrene for ~300-fold library coverage after selection. After 24 h, viral media was refreshed with normal media for cell recovery. At 48 h post transduction,

cells were split into two replicates and selected for seven days with medium containing puromycin (1 µg/ml).

In vitro CRISPR screen for spitting cobra venom modifiers

Pooled library transduced HAP1 cells were split across four T-175 flasks with 19 x10^6 cells per flask. After 24 h, medium was replaced with spitting cobra venom containing medium (5 µg/ml) with one flask refreshed with normal medium as a control. Cells were treated for 3 days then allowed to recover in normal media for 1-3 days. Cells were then replated and treated again with venom containing medium and this process was repeated three times with cells collected at 9 days of selection for genomic DNA extraction.

Genomic DNA sequencing

Genomic DNA was extracted from cell pellets with the ISOLATE II Genomic DNA Kit (Bioline). Samples were then prepared for Next Generation Sequencing (NGS) via PCR as previously described (*68*). Samples of gDNA (25 µg for controls and 5 µg for selected samples) were added to NEBNext High-Fidelity 2X PCR Master Mix (New England Biolabs) along with the following primers to bind the sgRNA region: NGSCRISPRv2F1 5'-GGACAGCACAGATCCAGTTTGGT-3' and NGSCRISPRv2R1 5'-GAGCCAATTCCCACTCCTTTCAA-3'. A second PCR reaction with a staggered primer mix of P5 and P7 indexing primers unique to each sample was prepared as outlined in (*69*). Reactions were isolated by gel electrophoresis and resultant products sent to NovogeneAIT Genomics Singapore for NGS. Data was analyzed using the MAGeCK (v05.9.2) pipeline (*17*) to identify genes that sensitize or protect when compared to diversity controls.

Ingenuity pathway analysis (IPA)

Ingenuity Pathway Analysis (Qiagen v01-21-03) was utilized to identify the significant biological pathway(s) enriched by the knockout screens. Canonical pathway analysis was conducted with IPA using a restricted set of significant genes (absolute $\log_2$FC > 1 and FDR of < 0.1).

Generation of single KO cells and gene validation

To validate candidate genes from the screen, top sgRNAs from the parent library were selected and cloned by traditional restriction digestion cloning into pLentiCRISPRv2 (Addgene plasmid 52961) in parallel to a non-targeting control sgRNA. Lentiviruses carrying these sgRNAs were produced as above, and transduced HAP1 cells were selected with 2 µg/ml puromycin for three days. Cells were allowed to recover for a week before analysis. Successful knockout was confirmed by PCR amplification and Sanger sequencing of each sgRNA target site and comparison to wild type through Synthego ICE (*70*). sgRNA and PCR/sequencing primer sequences are in **data file S3**.

Isolation of heparin-binding venom components

Heparin Affinity Chromatography. To isolate heparin binding components of the venoms, crude *N. pallida*, TZN *N. nigricollis*, and NGA *N. nigricollis* venoms were fractionated using heparin affinity chromatography. Initially, crude venoms were dissolved in 0.1X PBS at 1 mg/mL and loaded onto a 5 mL heparin column (HiTrap Heparin HP, Cytiva). Toxins were eluted with a 0-1.2 M NaCl gradient in PBS. Fractionation was assessed by SDS-PAGE and concentration was estimated from absorbance at 280 nm on a NanoDrop (ThermoFisher Scientific). Protein aliquots were snap frozen in liquid nitrogen and stored at -80 °C until required.

Cation Exchange. The major venom constituents of *N. pallida* and TZN *N. nigricollis* were further isolated using cation exchange chromatography. P3 of each venom was dialysed against 50 mM sodium phosphate, pH 6 and loaded onto a 1 mL cation exchange column (HiTrap SP HP, Cytiva). The toxins were then separated with a 0-0.6 M gradient of NaCl in 50 mM sodium phosphate, pH 6. 3FTx and $bPLA_2$ eluted in that order. Venom components were dialysed in PBS and used for further assays and taken for mass spectrometry, or in 20 mM HEPES pH 7.5, 150 mM NaCl, 0.05% Tween 20 for surface plasmon resonance. Protein purification was analyzed by SDS-PAGE and measured via NanoDrop (ThermoFisher Scientific) and Qubit (ThermoFisher Scientific). Protein aliquots were snap frozen in liquid nitrogen and stored at -80 °C until required.

Additional isolation of 3FTx cytotoxins 3 and 4, 20 mg of venom was dissolved in 2 mL 50 mM sodium phosphate, pH 6.0 and then applied to a 4.7 mL HPSP cation exchange chromatography column (Cytiva) equilibrated in the same buffer. Elution was carried out using a 15-column volume (CV) gradient of 0 - 0.7 M NaCl in 50 mM sodium phosphate, pH 6.0. The flow rate was 0.5 mL/min and 1 mL fractions were collected from the start of the NaCl gradient. The peak containing cytotoxins 3 and 4 was made up to 1.2 M in NaCl and loaded onto a 1 mL Phenyl Superose hydrophobic interaction chromatography column. Proteins were then eluted in a 5 CV gradient of 1.2 M NaCl in 25 mM sodium phosphate pH 7.2 to 30% (v/v) ethylene glycol in 25 mM sodium phosphate pH 7.2. The flow rate was 0.5 mL/min and elution was monitored at 214 nm. RP-HPLC and SDS-PAGE analysis showed that the eluted cytotoxins 3 and 4 were pure and were used in the present study after dialysis into PBS.

Mass spectrometry

1D liquid chromatography-mass spectrometry (LC-MS) was carried out on an Oritrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Scientific). Raw data were processed and protein sequences identified using Mascot and database derived from (*32*). Tables of identified sequences are in **data files S4-12**.

Surface plasmon resonance (SPR)

Heparin, tinzaparin and dalteparin were biotinylated by reaction of EZ-link NHS-LC-LC-Biotin with the free amino groups of unsubstituted heparin glucosamine residues. 100 μM of heparin and heparinoids were prepared in 0.1 M sodium bicarbonate pH 8.5. A five-fold excess of NHS-LC-LC-Biotin (570 g/mol in 100% DMSO) was added and the reaction was incubated on ice for 3 h. To remove unreacted biotin, the mixtures was dialysed in 20 mM HEPES pH 7.5 and 150 mM NaCl, with 3.5 k MWCO SnakeSkin Dialysis Tube (Thermo Fisher Scientific). Biotinylated heparins were snap-frozen and stored at -80 °C until required.

SPR measurements were taken using a BIAcore T200 instrument (Cytiva) and analyzed using the BIAcore Evaluation software (v3.2). Experiments were performed at 25 °C using the multi cycle kinetics mode. Biotinylated heparins were immobilized onto a CM5 chip (Cytiva) amine-coupled with streptavidin at around 100 response units (RU). 20 mM HEPES pH 7.5, 150 mM NaCl and 0.05% Tween-20 was used as the running buffer. Regeneration of the chip with 2M NaCl PBS followed each venom/fraction injection. Non-specific binding of toxins to heparins was determined by injecting the toxins over a control flow cell with streptavidin prepared without bound heparins. Binding to this surface was subtracted in all binding curves. Affinities measured for protein mixtures are concentration weighted means.

Conjugation of venom with fluorophores

Whole venoms were conjugated to Alexa Fluor™ 488 as per the protein labeling kit manufacturer's instructions (Invitrogen). Briefly, 50 µl of 1 M sodium bicarbonate was added to 500 µl of 2 mg/ml crude venom. The solution was then added to Alexa Fluor™ 488 reactive dye and stirred for 1 h at room temperature. Conjugated venom was then loaded into Zeba™ Dye and Biotin Removal Spin Columns and eluted via centrifugation. Tagging efficiency (~4-5 moles dye per mole protein) and protein concentrations were determined via NanoDrop (ThermoFisher Scientific).

Flow cytometry

Cell surface attachment of tagged venoms was detected by flow cytometry. Briefly, HAP1 cells were incubated with TrypLE at 37 °C and neutralized with IMDM once dissociated. 5 $x10^5$ cells were collected and incubated with Alexa Fluor™ 488-conjugated venom (100 µM/mL) with and without heparin (20 µM) or tinzaparin (20 µM) for 30 min on ice. The cells were washed twice with FACS buffer consisting of PBS, 2% BSA and 0.5mM EDTA before resuspending in the same buffer with DAPI (0.1 µg/mL) and analyzed on the Cytek Aurora (Cytek Biosciences).

A

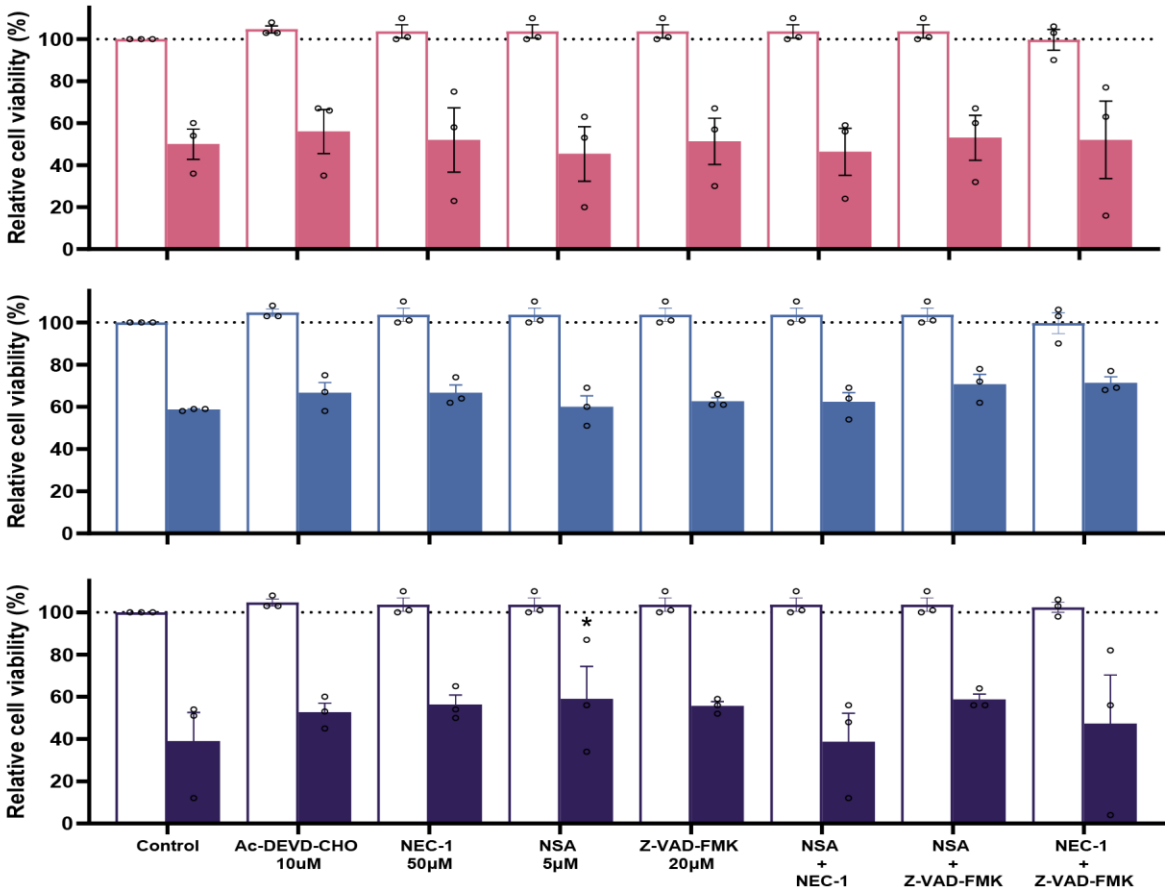

Fig. S1. Apoptosis and necroptosis inhibition

(**A**) Inhibition of apoptosis (Ac-DEVD-CHO; Z-VAD-FMK), necroptosis (NSA, Nec-1), or a combination on HAP1 cells treated with spitting cobra venom (10 µg/ml). Significance was determined by ordinary one-way ANOVA and Dunnett test, *P<0.05.

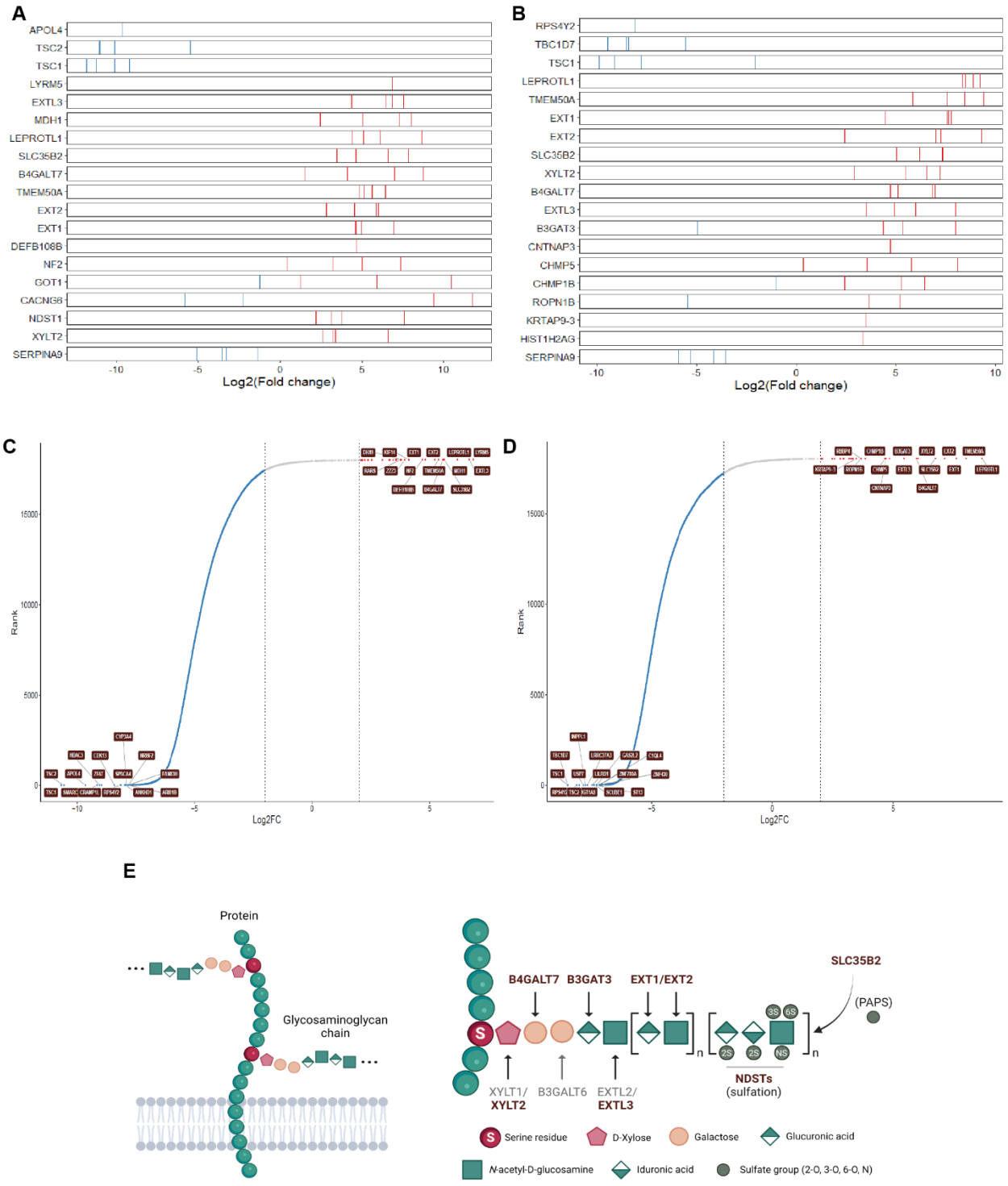

Fig. S2. CRISPR KO screen additional information.

(**A**) Performance in *N. pallida* screen of example sgRNAs. Top 3 sgRNAs targeting sensitizing genes, Top 15 sgRNAs targeting resistance genes and control. (**B**) Performance in *N. nigricollis* screen of example sgRNAs. Top 3 sgRNAs targeting sensitizing genes, Top 15 sgRNAs targeting resistance genes and control. (**C**) Rank plot showing $\log_2$(fold changes) (LFCs) in the

N. pallida screen. (**D**) Rank plot showing $\log_2$(fold changes) (LFCs) in the *N. nigricollis* screen. (**E**) Schematic representation of the heparan/heparin sulfate biosynthesis pathway, with screen hits shown in bold. Created with BioRender.com.

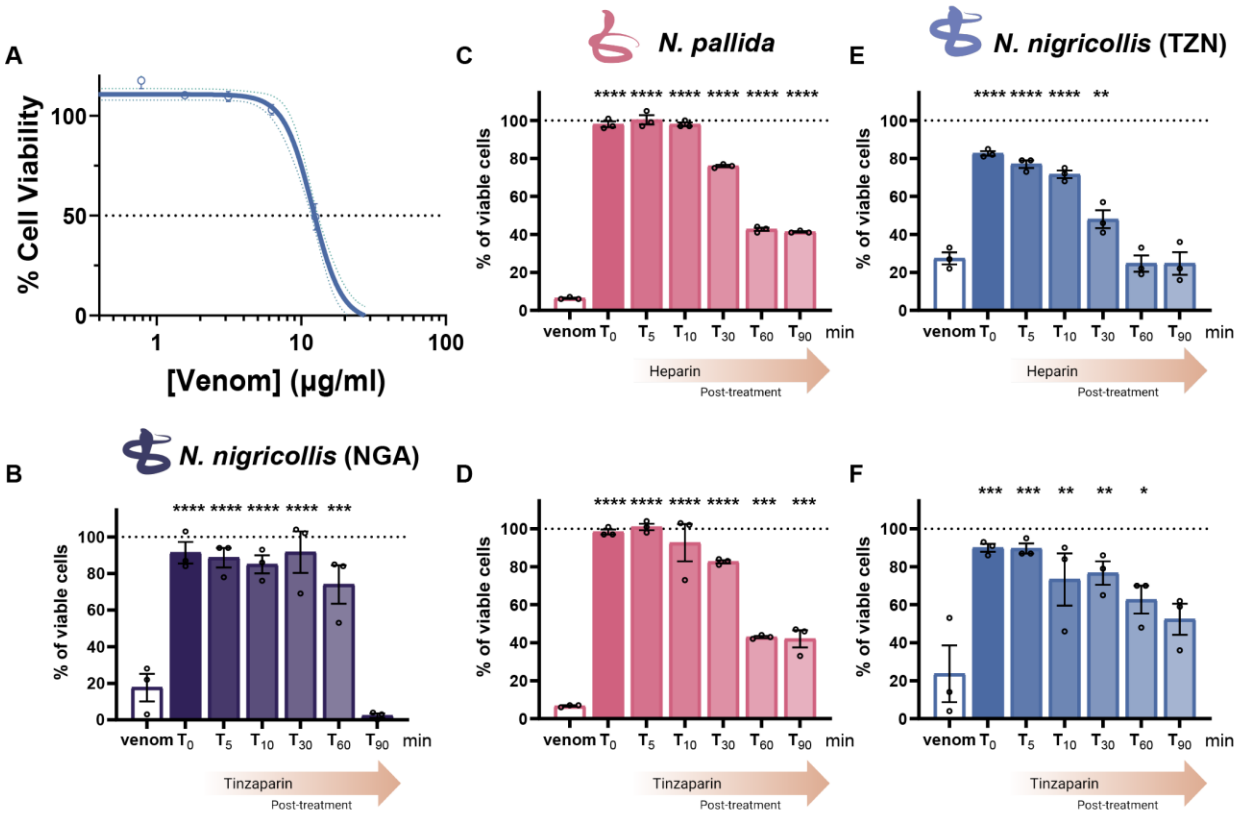

Fig. S3. Additional heparin and tinzaparin time course information.

(**A**) HAP1 cell viability as determined by resazurin assays after 24 h treatment with serial dilutions of Tanzanian *N. nigricollis* venom (*n* = 3). (**B**) HAP1 cells were treated with 10 µg/mL *N. nigricollis* (TZN) venom before addition of 20 µM tinzaparin immediately after, or 5-, 10-, 30-, 60- or 90-min post venom application. (**C**) HAP1 cells were treated with 10 µg/mL *N. pallida* venom before addition of 20 µM heparin or (**D**) 20 µM tinzaparin immediately after, or 5-, 10-, 30-, 60- or 90-min post venom application. (**E**) HAP1 cells were treated with 10 µg/mL *N. nigricollis* (TZN) venom before addition of 20 µM heparin or (**F**) 20 µM tinzaparin immediately after, or 5-, 10-, 30-, 60- or 90-min post venom application. Significance determined by Ordinary one-way ANOVA and Dunnett test, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (*n* = 3)

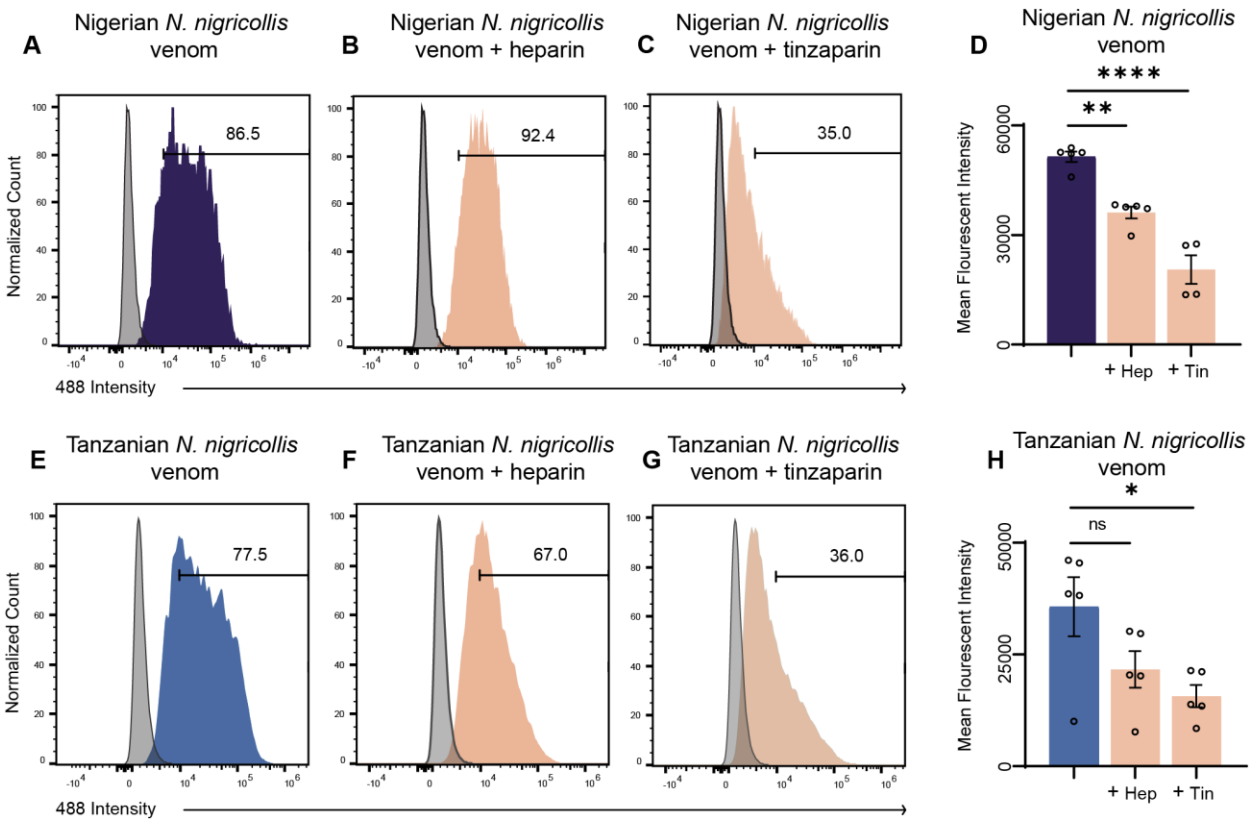

Fig. S4. Additional flow cytometry information.

(**A**) Representative flow cytometry histograms of WT HAP1 in gray and cells exposed to Alexa488-tagged Nigerian *N. nigricollis* venom (**B**) with heparin (**C**) or with tinzaparin. (**D**) Quantification of binding intensity (n = 5). Significance was determined by one-way ANOVA and Dunnett test, **P<0.01, ****P<0.0001. (**E**) Tanzanian *N. nigricollis* venom (**F**) with heparin (**G**) or with tinzaparin. (**H**) Quantification of binding intensity (n = 5). Significance was determined by One-way ANOVA and Dunnett test, *P<0.05.

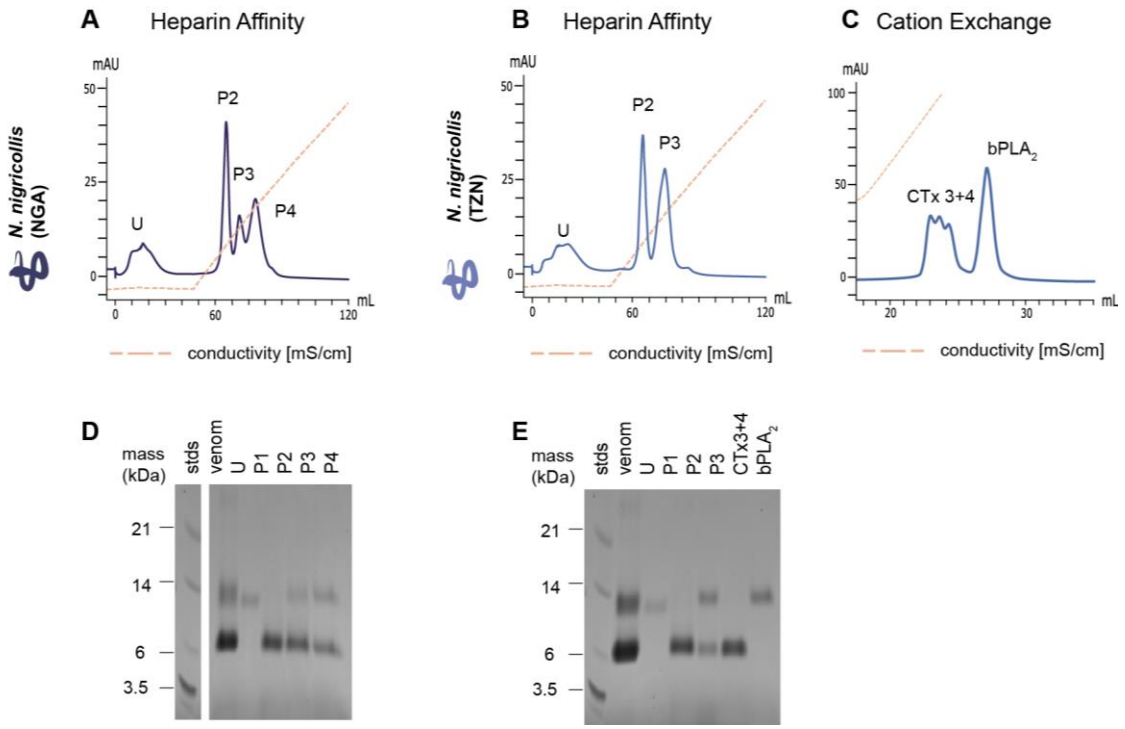

Fig. S5. *N. nigricollis* venom chromatography.

(**A**) Heparin affinity chromatography of Nigerian *N. nigricollis* venom. Unbound (U), Peak 2 (P2) and Peak 3 (P3). (**B**) Heparin affinity chromatography of Tanzanian *N. nigricollis* venom. Unbound (U), Peak 2 (P2) and Peak 3 (P3). (**C**) Cation exchange chromatography of Tanzanian *N. nigricollis* Peak 3. (**D**) SDS-PAGE gel of whole venom and resulting toxin fractions of Nigerian *N. nigricollis* venom. (**E**) SDS-PAGE gel of whole venom and resulting toxin fractions of Tanzanian *N. nigricollis* venom.

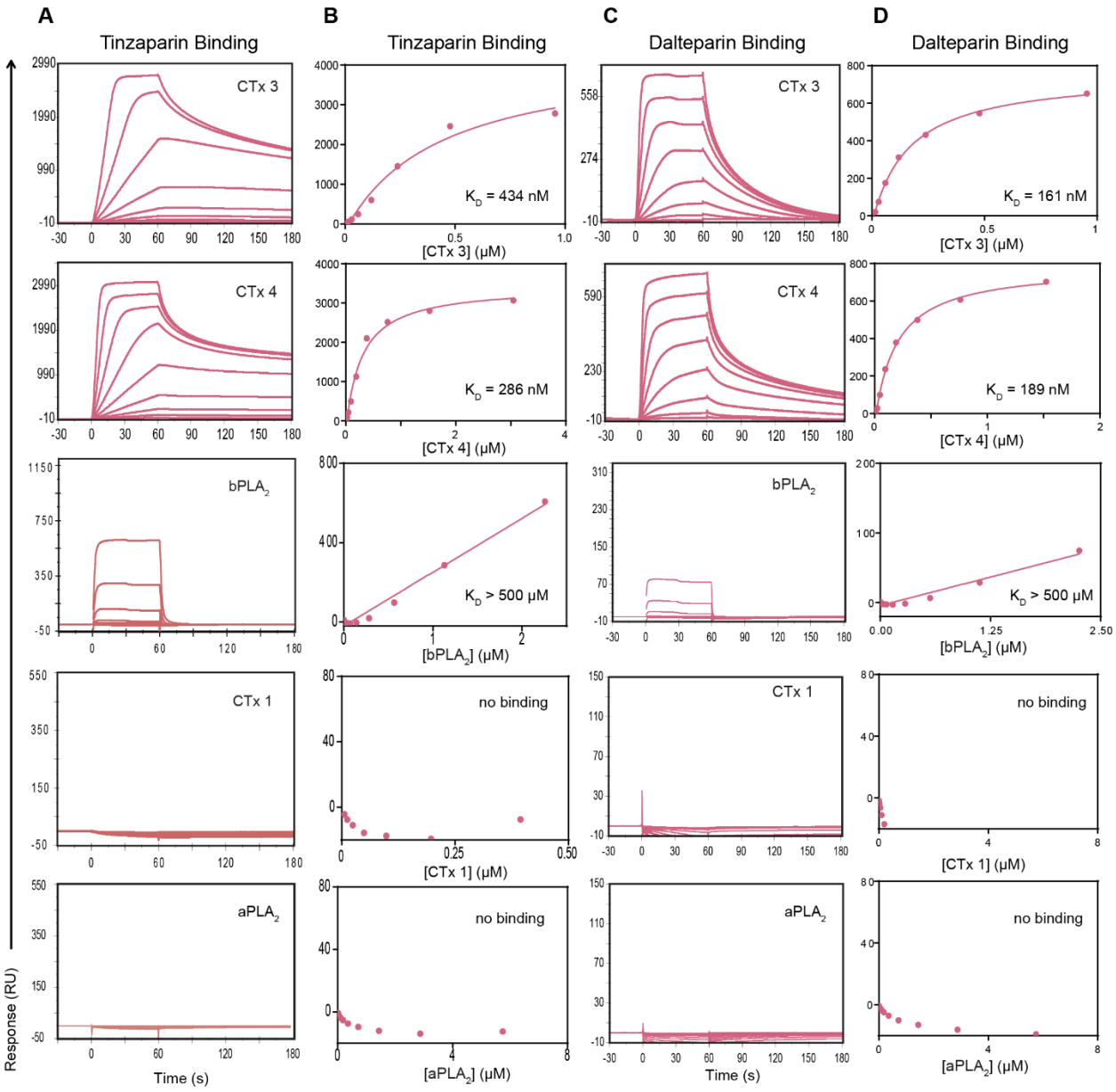

Fig. S6. Tinzaparin and dalteparin binding to *N. pallida* toxins.

(**A**) Surface plasmon resonance (SPR). Representative normalized sensorgrams of *N. pallida* toxin binding to tinzaparin. (**B**) Fits of the SPR data from (**A**) to a 1:1 binding model are shown. K_Ds are indicated on each plot. (**C**) Representative normalized sensorgrams of *N. pallida* toxin binding to dalteparin. (**D**) Fits of the SPR data from (**C**) to a 1:1 binding model are shown. K_Ds are indicated on each plot.

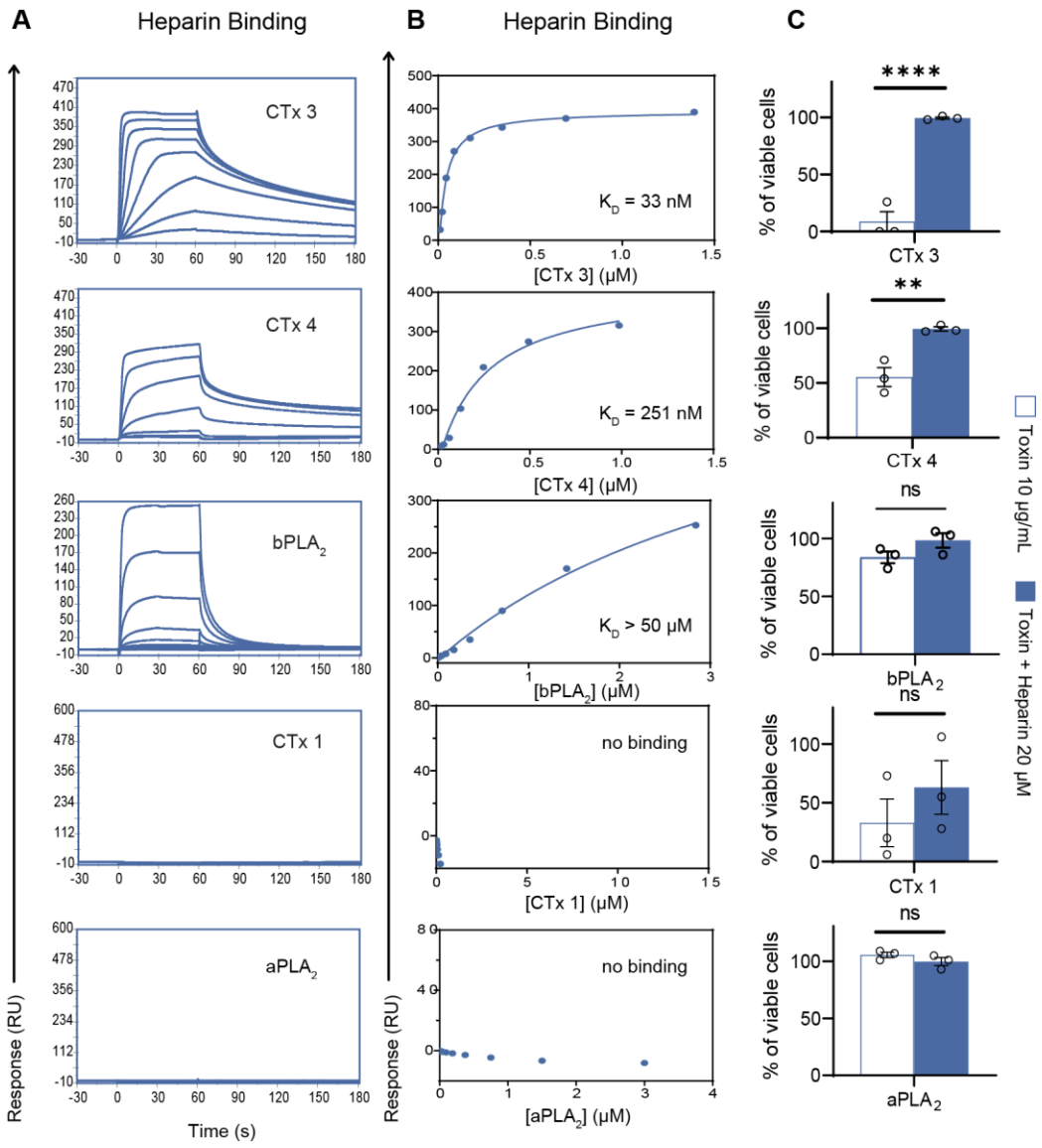

Fig. S7. Tanzanian *N. nigricollis* venom binding.

(**A**) Surface plasmon resonance (SPR). Representative normalized sensorgrams of toxin binding to heparin. (**B**) Fits of the SPR data from (**A**) to a 1:1 binding model are shown. K_Ds are indicated on each plot. (**C**) Cytotoxicity of 10 μg/mL of each toxin fraction and rescue by 20 μM heparin. Significance determined by two-way ANOVA and Sidak test, **P<0.01, ****P<0.0001 (*n* = 3).

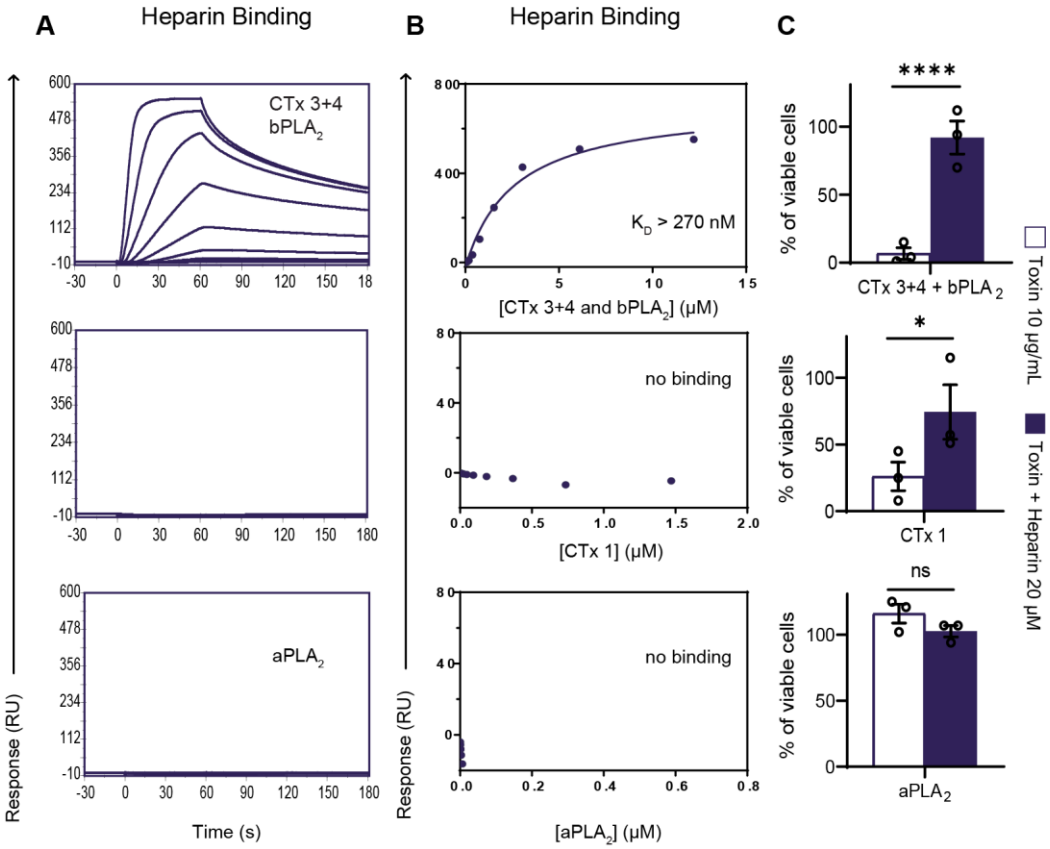

Fig. S8. Nigerian *N. nigricollis* venom binding.

(**A**) Surface plasmon resonance (SPR). Representative normalized sensorgrams of toxin binding to heparin. (**B**) Fits of the SPR data from (**A**) to a 1:1 binding model are shown. K_Ds are indicated on each plot. (**C**) Cytotoxicity of 10 μg/mL of each toxin fraction and rescue by 20 μM heparin. Significance determined by two-way ANOVA and Sidak test, *P<0.05, ****P<0.0001 (*n* = 3).

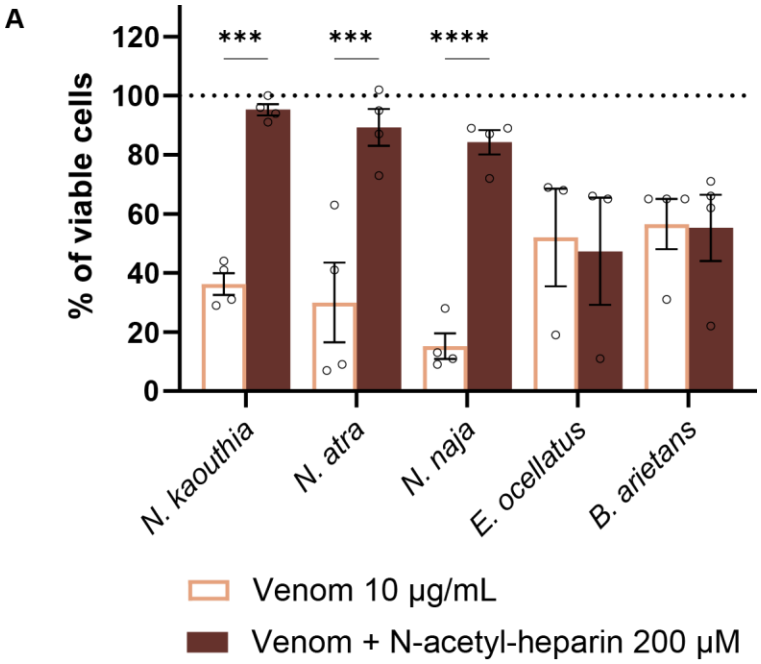

Fig. S9. N-acetyl-heparin interaction with snake venoms.

(**A**) Venoms (10 µg/mL) and N-acetyl-heparin (200 µM) added simultaneously to HAP1 cells. Resazurin cell viability assays were performed after 24 h of treatment. Cytotoxicity of venoms containing 3FTxs *Naja kaouthia, Naja atra, Naja naja* and more distantly related snakes *Echis ocellatus* and *Bitis arietans*, and the addition of N-acetyl-heparin. Significance determined by two-way ANOVA and Sidak test, ***P<0.001, ****P<0.0001 (*n* = 3).

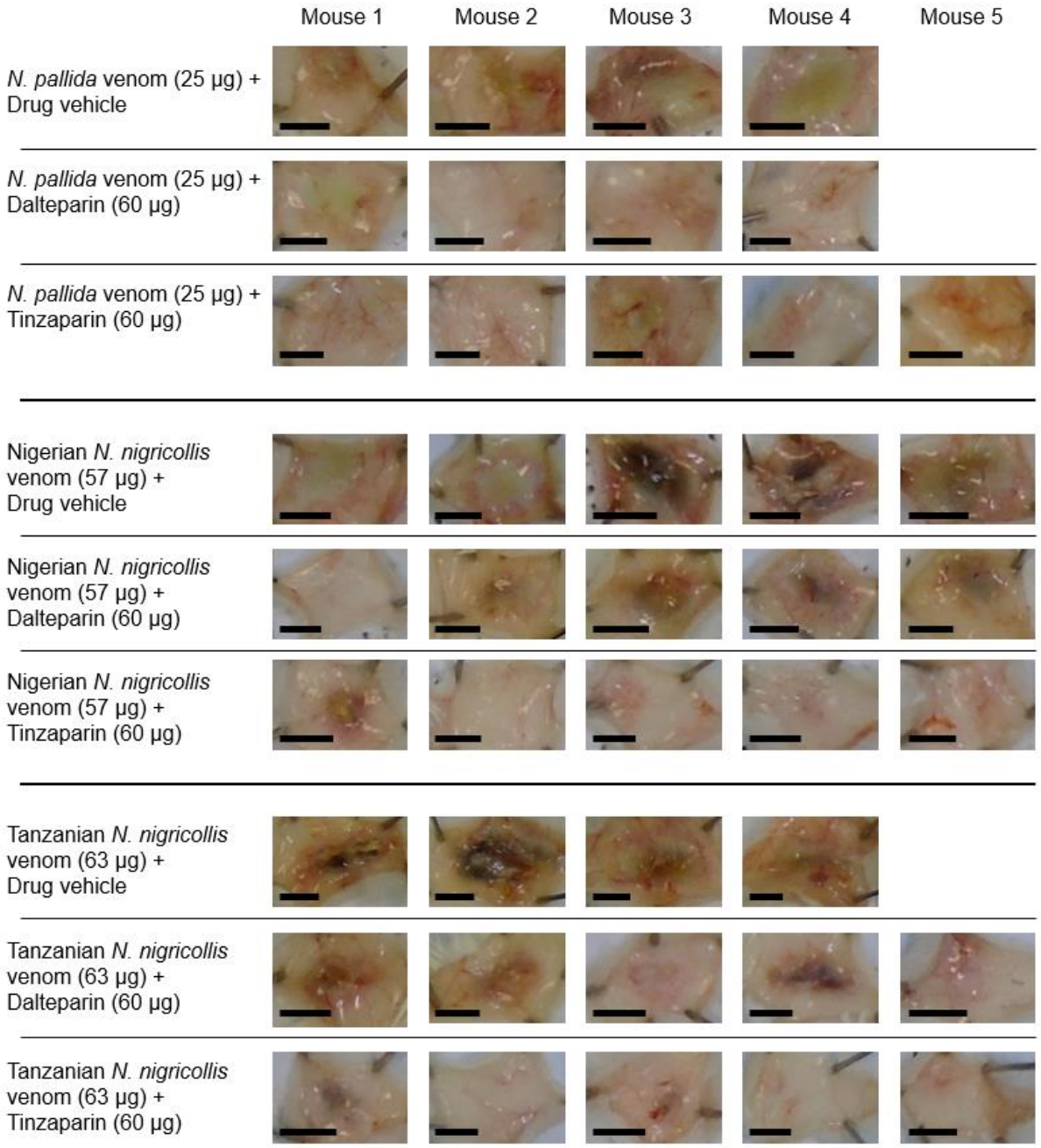

Fig. S10. Lesion images from each mouse in pre-incubation experiments, minus those that were culled before the desired timepoints due to humane endpoints being reached.

Mice were ID injected in the shaved rear quadrant on the dorsal side of the flank skin with *N. pallida* (25 µg), Nigerian *N. nigricollis* (57 µg), or Tanzanian *N. nigricollis* (63 µg) venom that had been pre-incubated with drug vehicle control, dalteparin or tinzaparin (60 µg [3 mg/mL]). After 72 hours the mice were euthanized and their lesions excised, measured with calipers, and photographed. One mouse in *N.* pallida venom + drug vehicle, *N.* pallida venom + Dalteparin,

and Tanzanian *N. nigricollis* venom + drug vehicle groups was culled before desired timepoints due to humane endpoints being reached. Scale bar = 5 mm

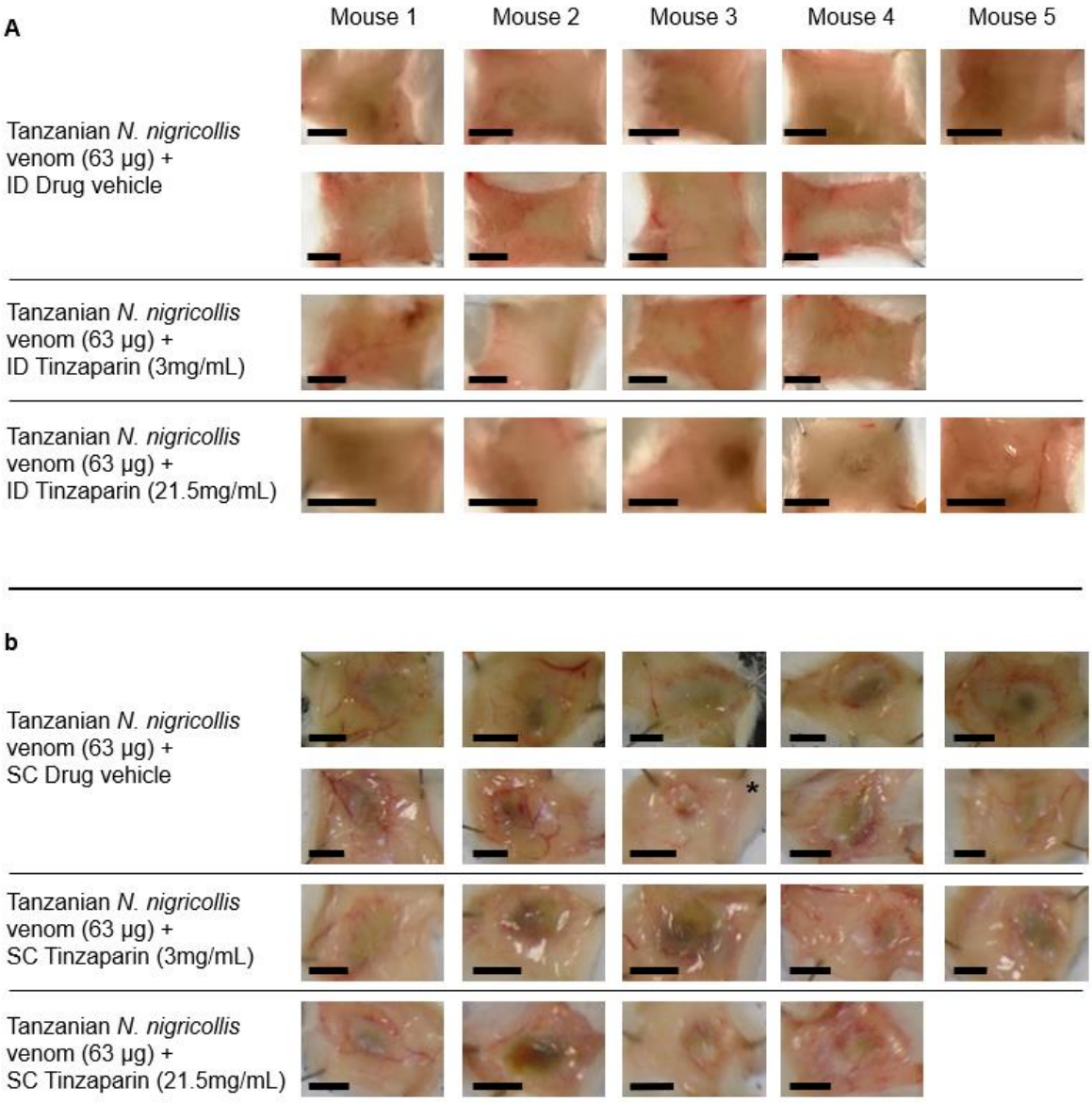

Fig. S11. Lesion images from each mouse in post-envenomation trials, minus those that were culled before the desired timepoints due to humane endpoints being reached.

(**A**) Mice were ID injected in the shaved rear quadrant on the dorsal side of the flank skin with Tanzanian *N. nigricollis* (110 µg) venom immediately followed by ID injection of drug vehicle, low dose (3 mg/kg) or moderate 'human-equivalent' dose (21.5 mg/kg) tinzaparin. (**B**) Mice were ID injected in the shaved rear quadrant on the dorsal side of the flank skin with Tanzanian *N. nigricollis* (110 µg) venom immediately followed by SC injection of drug vehicle, low dose (3

mg/kg) or moderate ‘human-equivalent’ dose (21.5 mg/kg) tinzaparin. After 72 hours the mice were euthanized and their lesions excised, measured with calipers, and photographed. One mouse in Tanzanian *N. nigricollis* venom + ID drug vehicle, Tanzanian *N. nigricollis* venom + ID tinzaparin, and Tanzanian *N. nigricollis* venom + SC tinzaparin (21.5 mg/mL) groups was culled before desired timepoints due to humane endpoints being reached. Scale bar = 5 mm. * This data point was formally identified as an outlier based on a Grubb’s outlier test (Alpha = 0.2) (*60*)

Data files S1 to 12. (separate excel file)

Data file S1. The full list of genes in the *N. pallida* CRISPR screen.
Data file S2. The full list of genes in the Nigerian *N. nigricollis* CRISPR screen.
Data file S3. ICE analysis
Data file S4. LCMS of P1 of *Naja pallida* venom.
Data file S5. LCMS of P2 of *Naja pallida* venom.
Data file S6. LCMS of P3 of *Naja pallida* venom.
Data file S7. LCMS of P1 of Tanzanian *Naja nigricollis* venom.
Data file S8. LCMS of P2 of Tanzanian *Naja nigricollis* venom.
Data file S9. LCMS of P3 of Tanzanian *Naja nigricollis* venom.
Data file S10. LCMS of P1 of Nigerian *Naja nigricollis* venom.
Data file S11. LCMS of P2 of Nigerian *Naja nigricollis* venom.
Data file S12. LCMS of P4 of Nigerian *Naja nigricollis* venom.