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Supplementary Materials for

Molecular dissection of cobra venom highlights heparinoids as an effective snakebite antidote

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The PDF file includes:

Other Supplementary Materials for this manuscript include the following:

Data S1 to S12

Materials and Methods

Study Design

The objective of this study was to identify the human genes and pathways that Naja pallida and Naja nigricollis venoms interact with to inform the development of therapeutics. This was achieved through an unbiased whole genome CRISPR KO screen, with the lead pathway of heparan sulfate further validated genetically through single KO pools and pharmacologically through heparinoids. Heparinoid affinity to 3FTx was then determined. For in vivo studies, the WHO-recommended preclinical model for local envenoming was followed. Groups of 5 mice were randomly allocated into each treatment group based on sample sizes previously used to assess venom inhibition in this model. Experimenters were not blinded to the venom or treatment doses. A single lesion measurement from the venom-plus-PBS control group was excluded.

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.

Venoms

Venoms were sourced from wild caught or captive bred snakes maintained in the herpetarium of the Liverpool School of Tropical Medicine's (LSTM) Centre for Snakebite Research Interventions (CSRI; United Kingdom). This facility and its protocols for the husbandry of snakes are approved and inspected by the UK Home Office and the LSTM and University of Liverpool Animal Welfare and Ethical Review Boards. The venom pools were from: East African *Naja nigricollis*

(Tanzanian, TZN), West African *Naja nigricollis* (Nigerian, NGA), *Naja pallida* (Tanzanian), captive bred *Naja kaouthia*, *Naja atra*, and *Naja naja*, *Echis ocellatus* (Nigerian), and *Bitis arietans* (Nigerian). Crude venoms were lyophilized and stored at -20 °C. Prior to use, venoms were resuspended to 10 mg/ml in DPBS and stored at -80 °C in small aliquots with freeze-thaw cycles minimized to prevent degradation.

Cell culture

HAP1 cells were generously provided by Dr. 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 (*60*, *61*), were purchased from Caltag Medsystems (Buckingham, UK). 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 and PI multiplexed assays, respectively, as previously described (*35*) 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).

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 (*62*). 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 (*63*). 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 (*14*) 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 (*64*). sgRNA and PCR/sequencing primer sequences are in **data 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 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).

Animal ethics and maintenance

Liverpool, UK: All drug-plus-venom precincubation and ID-envenoming followed by SC-drug dosing animal experiments were conducted using protocols approved by the Animal Welfare and Ethical Review Boards of the Liverpool School of Tropical Medicine and the University of Liverpool and were performed in pathogen-free conditions under licensed approval (PPL #P58464F90) of the UK Home Office and in accordance with the Animal [Scientific Procedures] Act 1986 and institutional guidance on animal care. All experimental animals (18-28 g [4-6 weeks old], male, SWISS (CD-1) mice from Janvier, France or Charles River, UK) were acclimated for a minimum of one week before experimentation with their health monitored daily. Mice were grouped in cages of five, with room conditions of approximately 22 °C at 40-50% humidity, with 12/12 hour light cycles, and given ad lib access to CRM irradiated food (Special Diet Services, UK) and reverse osmosis water in an automatic water system. Mice were housed in specific-pathogen free facilities in Techniplast GM500 cages containing Lignocell bedding (JRS, Germany), Sizzlenest zigzag fibers as nesting material (RAJA), and supplied with environmental enrichment materials.

San José, Costa Rica: All ID-envenoming followed by ID-drug dosing animal experiments were carried out at Instituto Clodomiro Picado. These were conducted using protocols approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUA) of the University of Costa Rica (approval number CICUA 82-08). All experimental animals (18-20 g [4-5 weeks old], mixed sex, CD-1 mice, Instituto Clodomiro Picado, Costa Rica) were acclimatized before experimentation. Mice were grouped in cages of five, with room conditions of approximately 22-

24 °C at 60-65% humidity, with 12/12 hour light cycles, given ad lib access to food and water, and housed in Tecniplast Eurostandard Type II 1264C cages.

Preclinical anti-dermonecrosis efficacy of heparinoids via a preincubation model of envenoming

The *in vivo* experimental design was based upon 3R-refined WHO-recommended envenoming protocols (*35–37*) and the anti-dermonecrosis methods were based on the Minimum Necrotizing Dose (MND) principles originally described in Theakston and Reid (*36*). As similar experiments with heparinoids as dermonecrosis treatments have never been performed previously, *a priori* power calculations were not possible; therefore, groups of 5 mice were randomly allocated into each treatment group based on sample sizes previously used to assess venom inhibition in this model (*35*, *65*). These groups of five mice (n=5; 45 mice total) received, in random order by a treatment-preparer separate from the treatment-injectors, experimental doses per mouse that consisted of venom from TZN *N. pallida* (25 µg), TZN *N. nigricollis* (63 µg), or NGA *N. nigricollis* (57 µg) combined with saline vehicle control, tinzaparin or dalteparin (60 µg [3 mg/kg]). Albulescu, et al. previously used 60 µg per 20 g mouse (3 mg/kg) of marimastat in their preclinical ID haemotoxicity trials (*37*); therefore this same comparative mg/kg concentration was chosen for our heparinoid dermonecrosis trials. *In vivo* team members were unblinded to the treatment randomisation post-injection to allow for the appropriate observation of venom-specific systemic endpoints that would have necessitated the implementation of early euthanasia. Stock solutions of tinzaparin and dalteparin were dissolved in PBS (50 mg/mL) and stored at -20 ºC prior to use in these *in vivo* experiments. All experimental doses were prepared to a volume of 50 µL and preincubated at 37 °C for 30 minutes the morning of the experiments, then kept on ice for no more than 3 hours until the mice were injected. For dose delivery, mice were briefly anesthetized using inhalational isoflurane (4% for induction of anesthesia, 1.5-2% for maintenance) and ID-injected in the shaved rear quadrant on the dorsal side of the flank skin with the 50 µL treatments. The mice were observed at least three times daily up to 72 hours post-injection to ensure signs of systemic envenoming or excessive external lesion development did not present. At the end of the experiments (72 hours) the mice were euthanized using rising concentrations of CO_2, after which the skin surrounding the injection site was dissected and the width and height of internal skin lesions measured with calipers, from which area was calculated, and photographed. Cross-section strips down the middle of the skin lesions were cut with microtome blades, placed in tissue cassettes, and preserved in 10% neutral formalin (BAF-6000-08A; CellPath) before being prepared for downstream histopathological analysis as previously described (*35*).

Preclinical anti-dermonecrosis efficacy of drug combinations via SC and ID ‘rescue’ models of envenoming

SC-drug rescue (UK): Groups of five mice (20 mice total) were ID-injected with TZA *N. nigricollis* venom (63 µg) diluted in 25 µL of PBS, followed by an immediate 25 µL SC-injection of either: (i) vehicle control (PBS), (ii) 3 mg/kg tinzaparin, or (iii) 21.5 mg/kg of tinzaparin (calculated human equivalent dose (*66*) based on therapeutic dose of tinzaparin in humans being 175 IU/kg (*67*) [roughly 1.75 mg/kg]), directly underneath the ID-injected venom. After 72 hours mice were euthanized by CO_2 inhalation, the skin surrounding the injection site was dissected, and

the internal necrotic lesions were measured and photographed as described above. Separate (n=5) venom-plus-PBS control groups were completed for both the 3 and 21.5 mg/kg tinzaparin trials and data were combined due to being identical treatments.

ID-drug rescue (Costa Rica): Groups of five mice (20 mice total) were pre-treated with the analgesic tramadol (50 mg/kg by the subcutaneous route). Fifteen minutes later, mice were ID-injected with venom from TZA *N. nigricollis* (63 µg) diluted in 25 µL of PBS, after which they were immediately ID-injected in the same location with 25 µL of tinzaparin vehicle control (PBS) or 3 or 21.5 mg/kg of tinzaparin. After 72 hours mice were euthanized by CO_2 inhalation, the skin surrounding the injection site was dissected, and the internal necrotic lesions were measured and photographed as described above. Separate (n=5) venom-plus-PBS control groups were completed for both the 3 and 21.5 mg/kg tinzaparin trials and were combined due to being identical treatments. A single lesion measurement from the venom-plus-PBS control group was excluded as only a minor lesion developed in a mouse that we suspect of being misinjected, and thus did not receive the correct venom dose. This data point was formally identified as an outlier based on a Grubb's outlier test (Alpha = 0.2) (*68*), but to ensure transparency, images of this lesion are displayed in **fig. S8**.

Statistical Analysis

Statistical analyses of data, unless otherwise stated, were conducted using GraphPad Prism (9.3.1) software. All error bars in this manuscript report SEM. A P value below 0.05 was considered significant. Specific statistical tests used for each dataset are stated in respective figure legends. All flow cytometry data was analyzed using FlowJo Software v10.6 (BD Life Sciences).

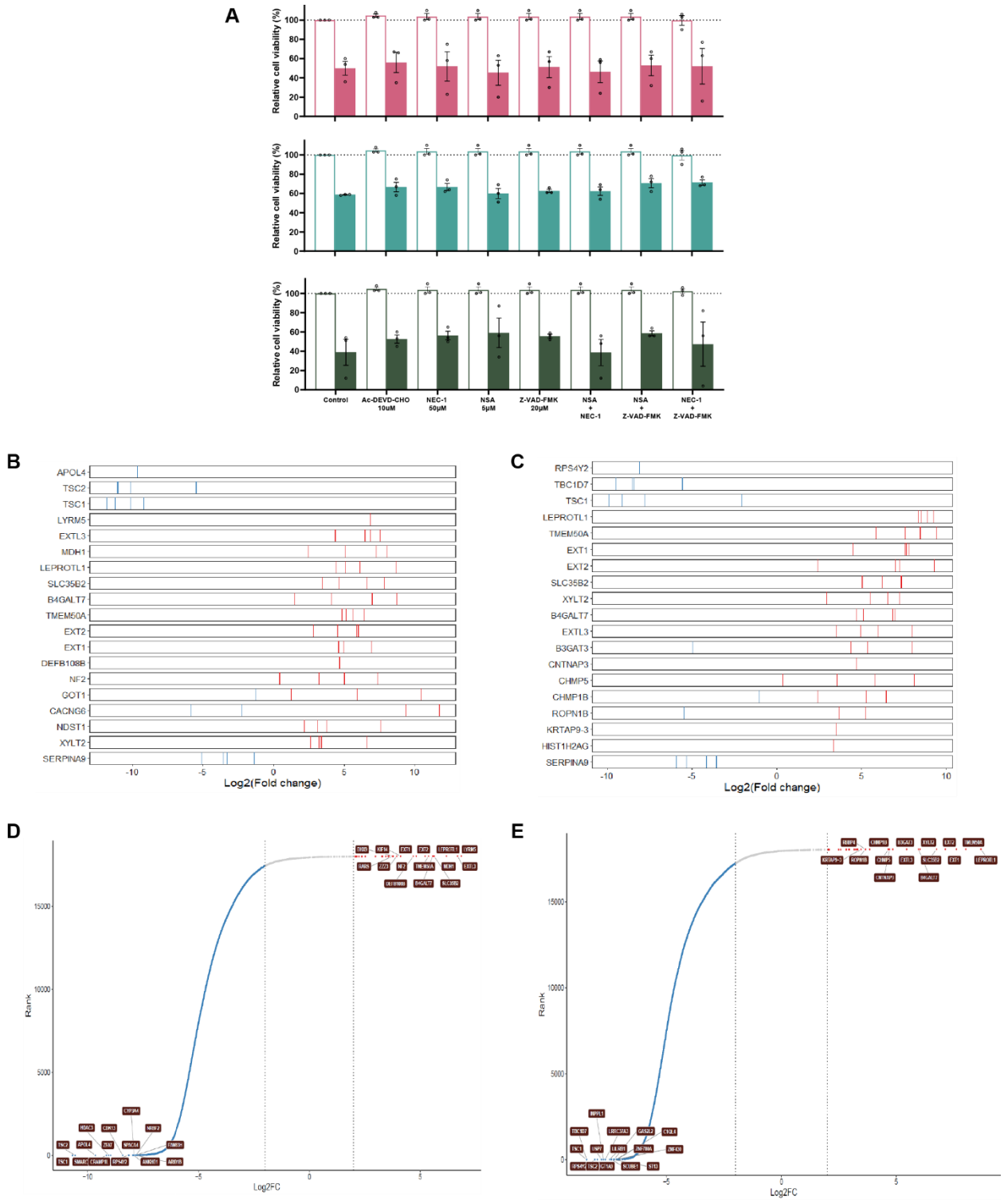

Fig. S1. CRISPR KO screen additional information.

(**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). (**B**) Performance in *N. pallida* screen of example sgRNAs. Top 3 sgRNAs targeting sensitizing genes, Top 15 sgRNAs

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

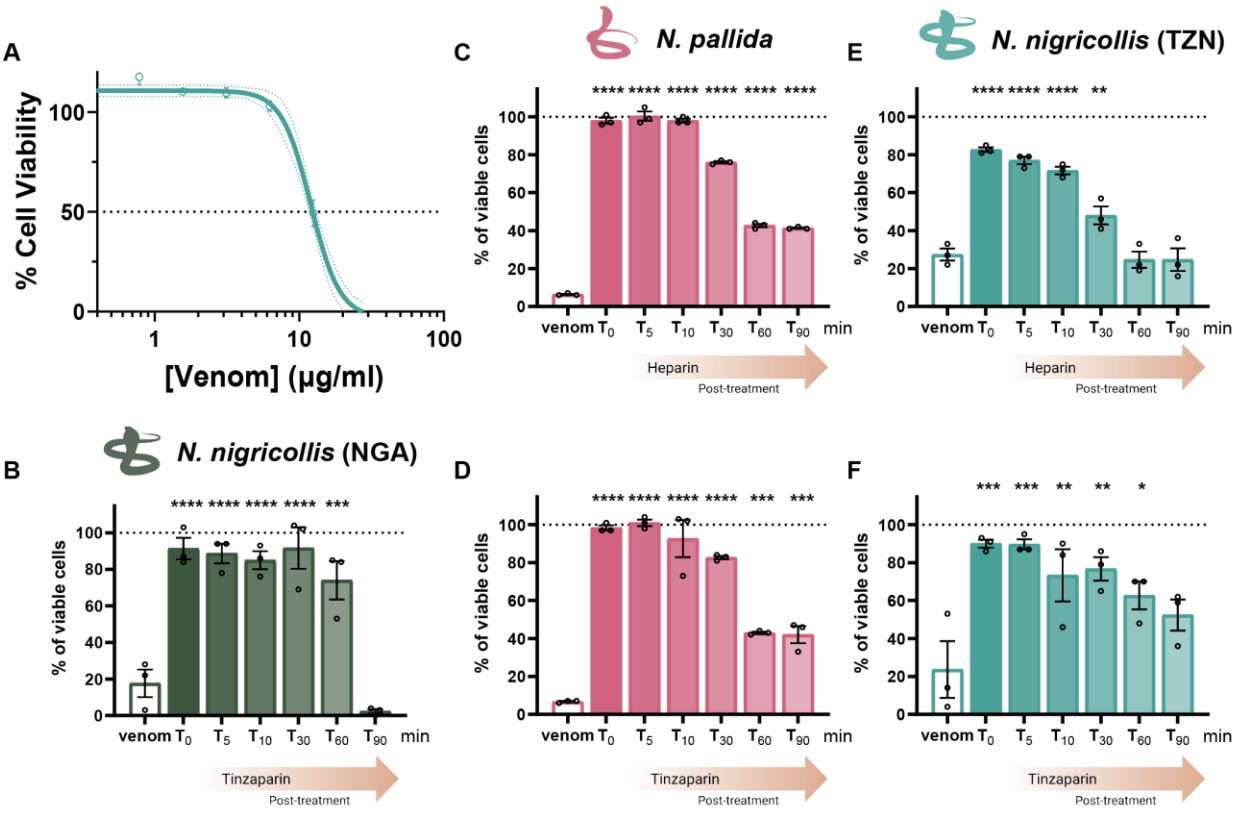

Fig. S2. Additional heparin and tinzaparin time course information

(**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. pallida* or (**C**) *N. nigricollis* (TZN) venom before addition of 20 µM heparin immediately after, or 5-, 10-, 30-, 60- or 90-min post venom application. (**D**) HAP1 cells were treated with 10 µg/mL *N. nigricollis* (NGA), (**E**) *N. pallida* or (**F**) *N. nigricollis* (TZN) venom before addition of 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)

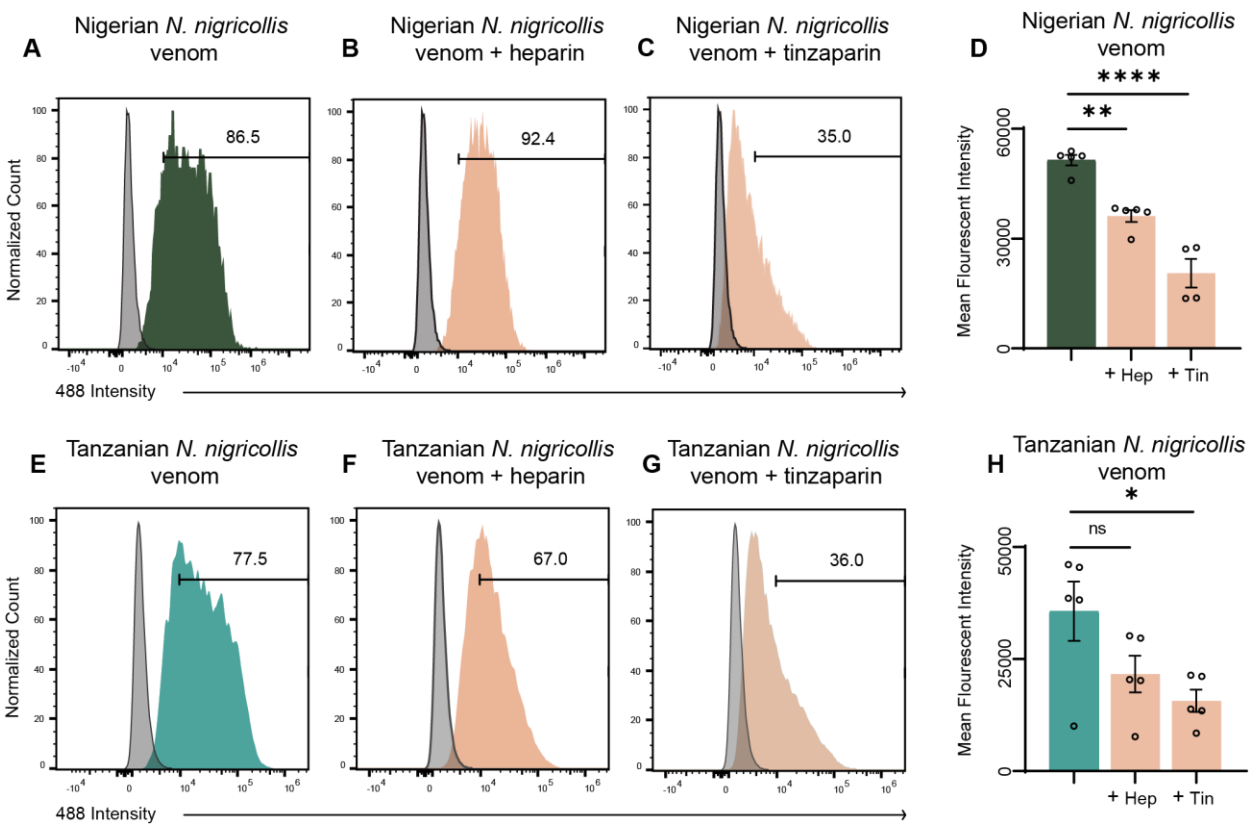

Fig. S3. Additional flow cytometry information.

(**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.

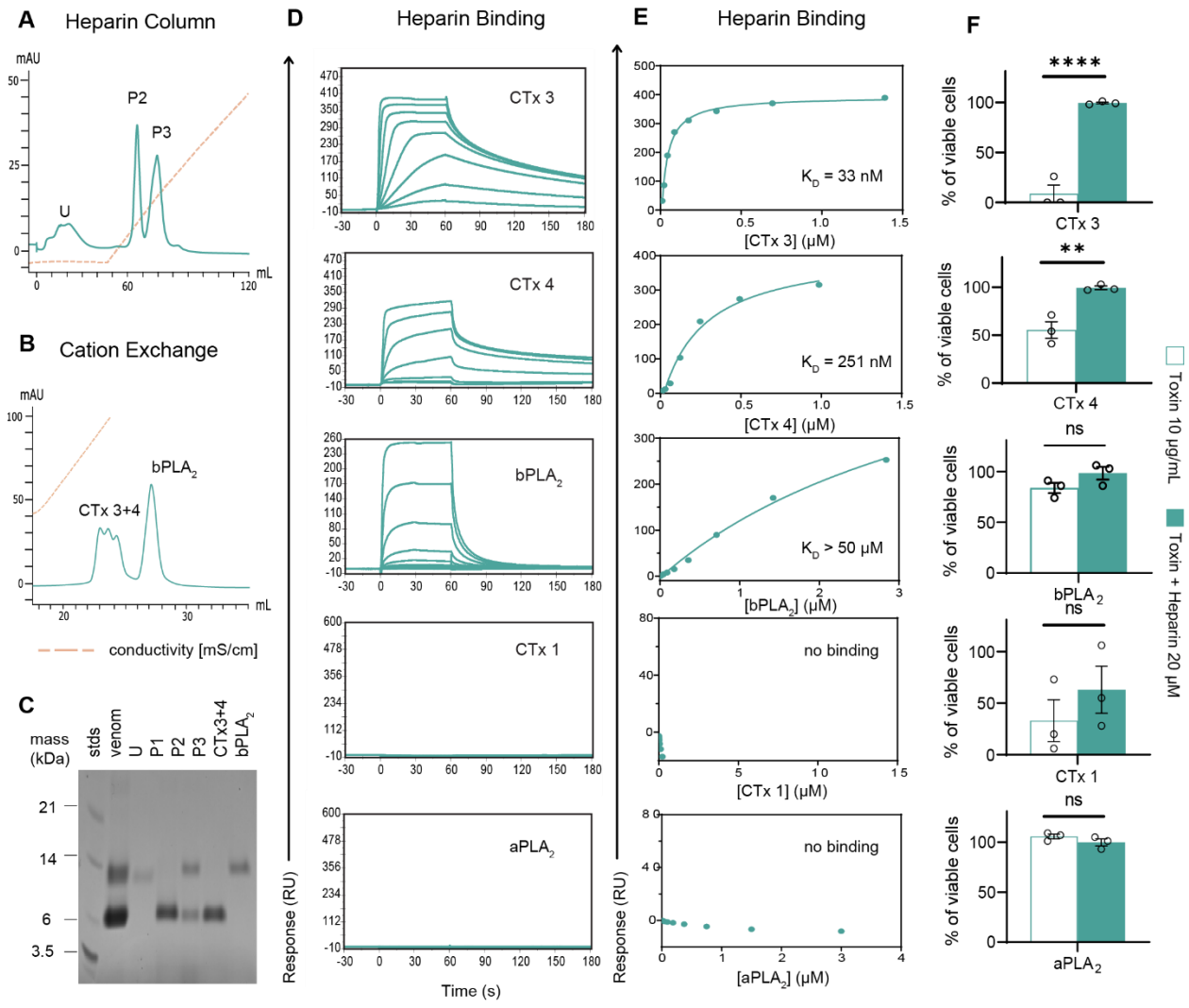

Fig. S4. Tanzanian *N. nigricollis* venom binding

(**A**) Heparin affinity chromatography of Tanzanian *N. nigricollis* venom. Unbound (U), Peak 2 (P2) and Peak 3 (P3). (**B**) Cation exchange chromatography of Peak 3. (**C**) SDS-PAGE gel of whole venom and resulting toxin fractions. (**D**) Surface plasmon resonance (SPR). Representative normalized sensorgrams of toxin binding to heparin. (**E**) Fits of the SPR data from (**D**) to a 1:1 binding model are shown. K_Ds are indicated on each plot. (**F**) Cytotoxicity of 10 μg/mL of each toxin fraction and rescue by 20 μM heparin. Significance determined by 2-way ANOVA and Sydak test, **P<0.01, ****P<0.0001 (*n* = 3).

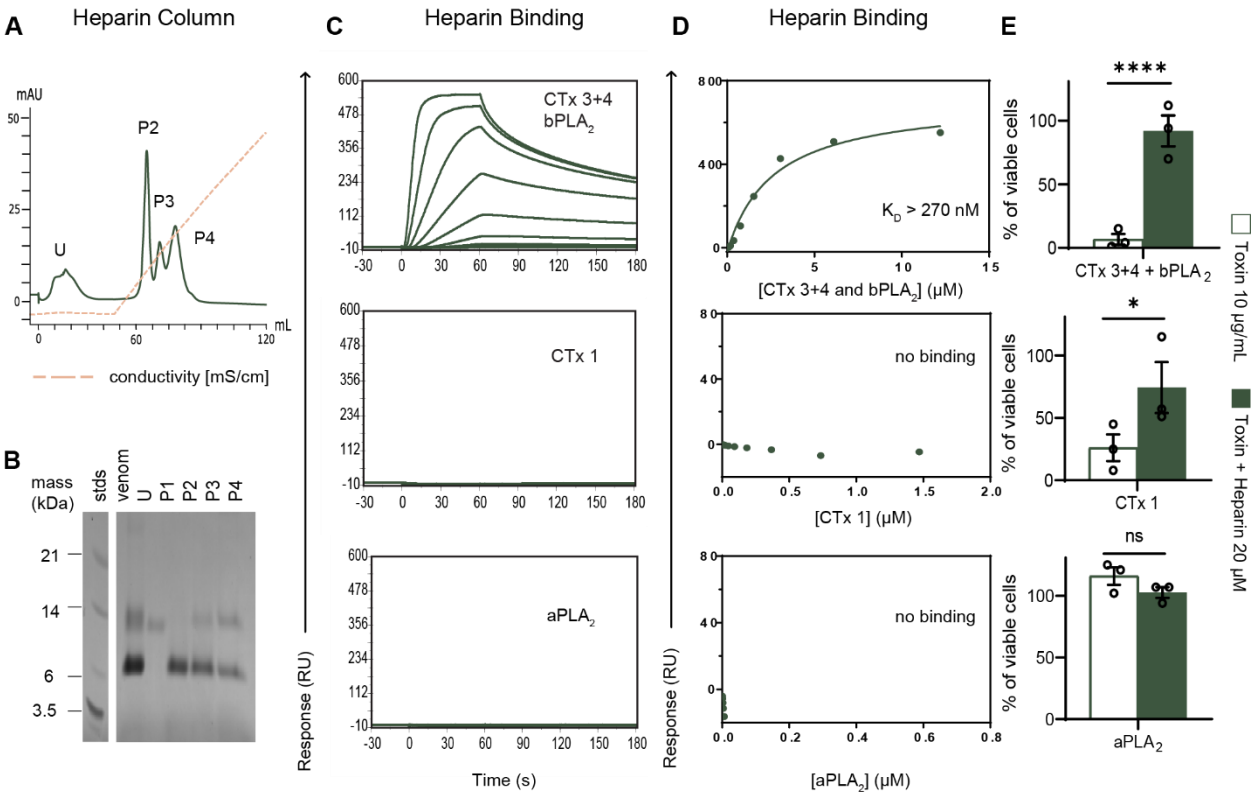

Fig. S5. Nigerian *N. nigricollis* venom binding

(**A**) Heparin affinity chromatography of Nigerian *N. nigricollis* venom. Unbound (U), Peak 2 (P2), Peak 3 (P3) and Peak 4 (P4). (**B**) SDS-PAGE gel of whole venom and resulting toxin fractions. (**C**) Surface plasmon resonance (SPR). Representative normalized sensorgrams of toxin binding to heparin. (**D**) Fits of the SPR data from (**C**) to a 1:1 binding model are shown. K_Ds are indicated on each plot. (**E**) Cytotoxicity of 10 μg/mL of each toxin fraction and rescue by 20 μM heparin. Significance determined by 2-way ANOVA and Sydak test, *P<0.05, ****P<0.0001 (*n* = 3).

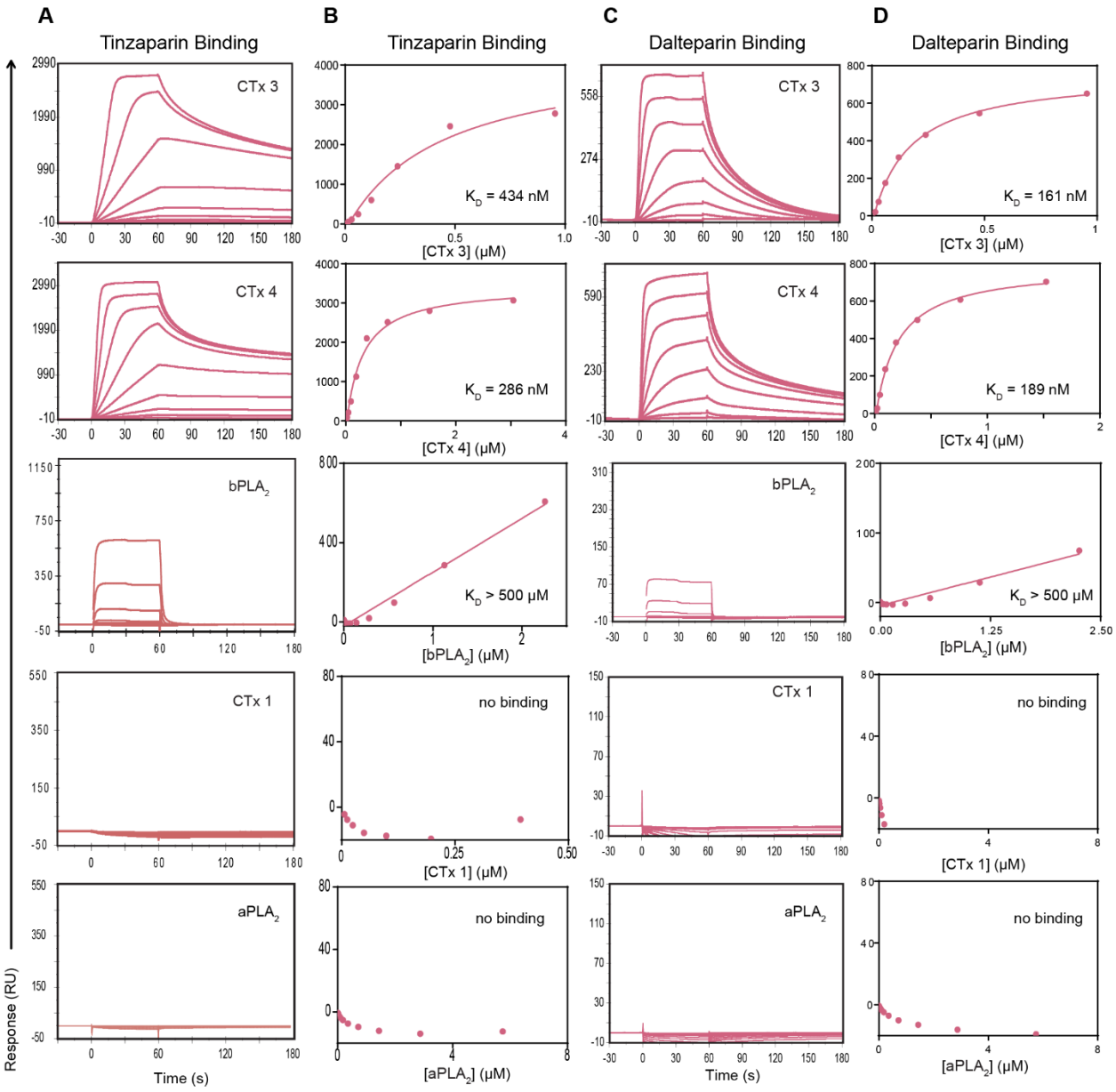

Fig. S6. Heparin and dalteparin binding to *N. pallida* toxins.

(**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.

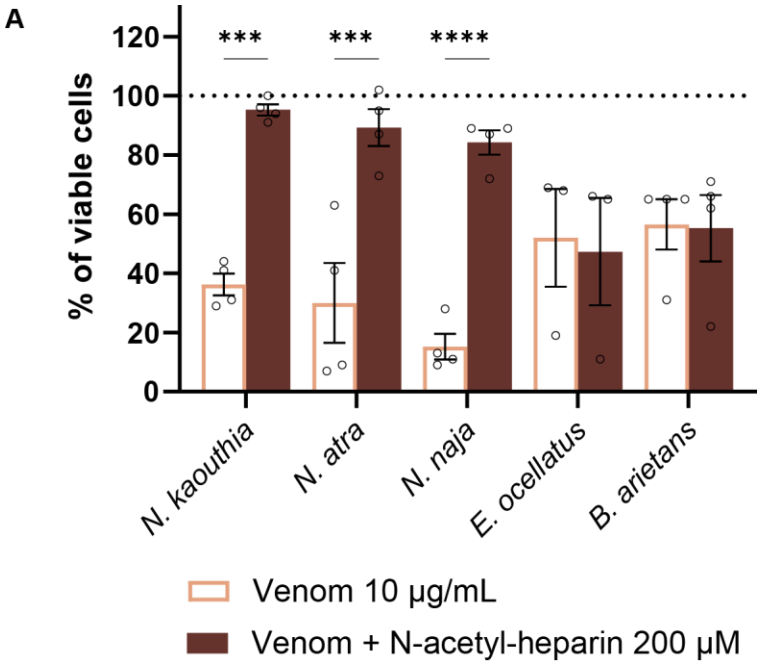

Fig. S7. N-acetyl-heparin interaction with snake venoms

(**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 2-way ANOVA and Sydak test, ***P<0.001, ****P<0.0001 (*n* = 3).

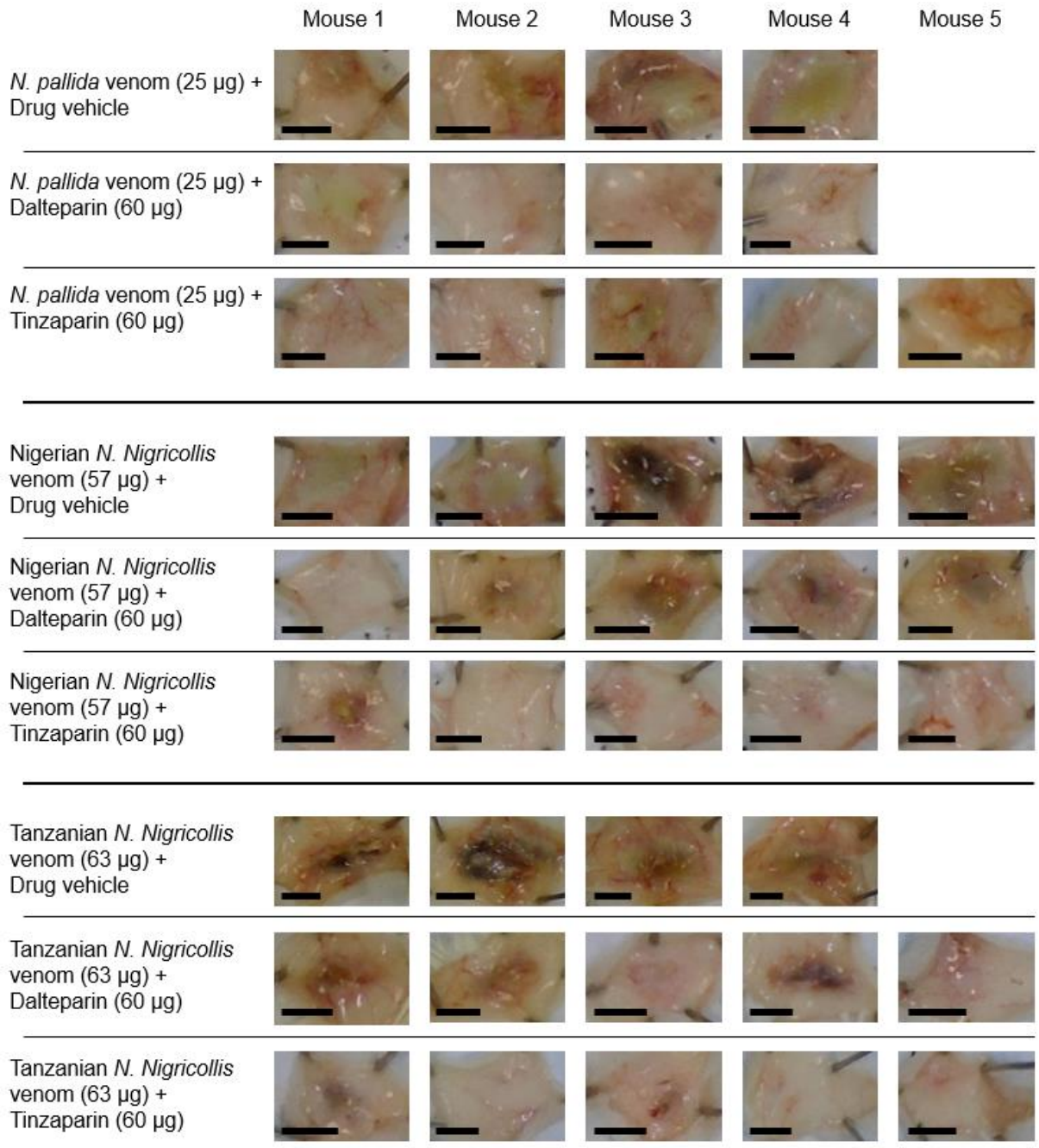

Fig. S8. All lesion images from every mouse in pre-incubation trials, minus those that were culled before the desired timepoints due to humane endpoints being reached.

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. Scale bar = 5 mm

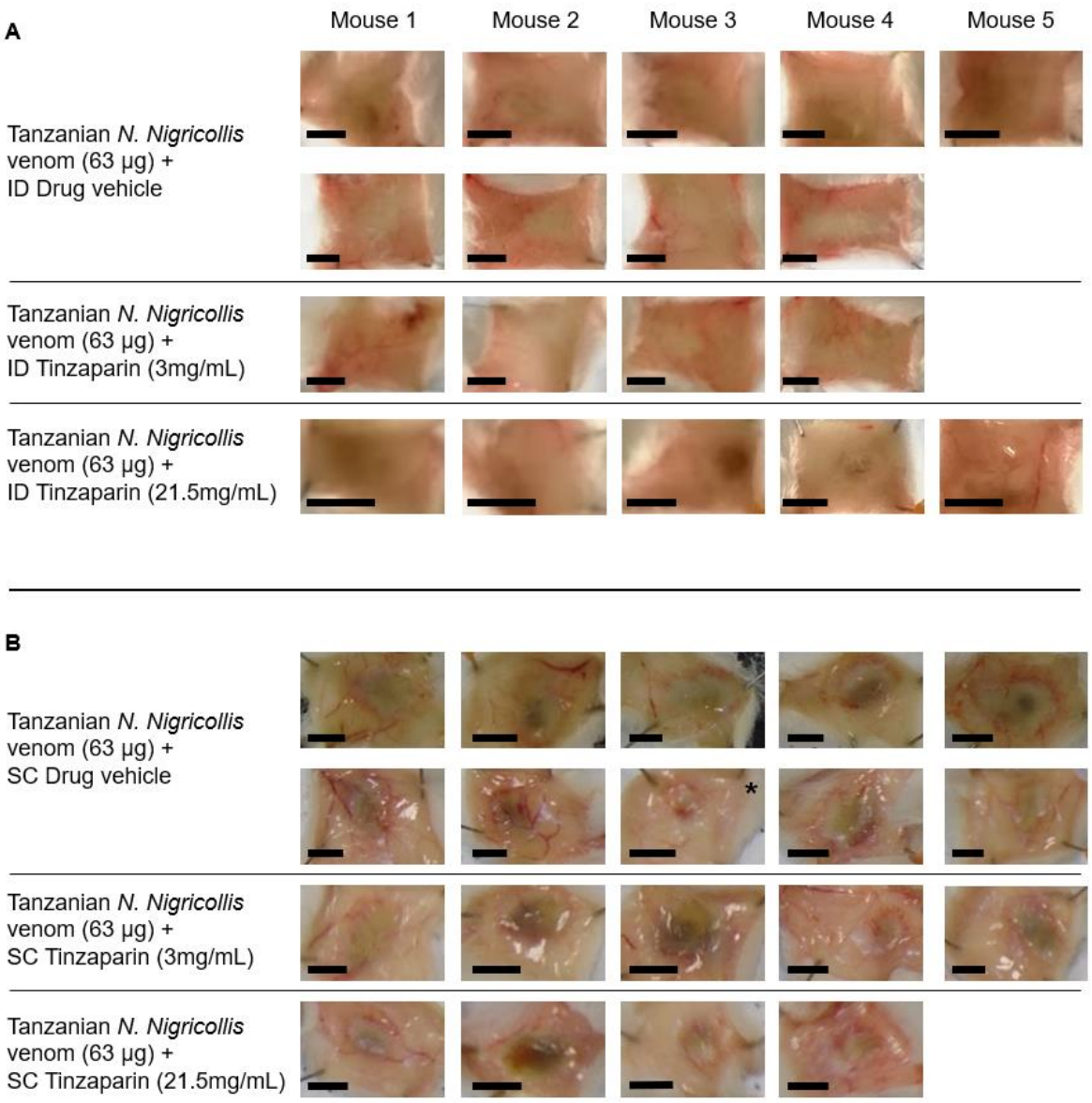

Fig. S9. All lesion images from every mouse in post-envenomation trials, minus those that were culled before the desired timepoints due to humane endpoints being reached.

(**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. 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 S1-12. (separate file)

Data S1 – full list of hits *N. pallida* screen
Data S2 – full list of hits Nigerian *N. nigricollis* screen
Data S3 – ICE analysis
Data S4 – LCMS of P1 of *Naja pallida*
Data S5 – LCMS of P2 of *Naja pallida*
Data S6 – LCMS of P3 of *Naja pallida*
Data S7 – LCMS of P1 of Tanzanian *Naja nigricollis*
Data S8 – LCMS of P2 of Tanzanian *Naja nigricollis*
Data S9 – LCMS of P3 of Tanzanian *Naja nigricollis*
Data S10 – LCMS of P1 of Nigerian *Naja nigricollis*
Data S11 – LCMS of P2 of Nigerian *Naja nigricollis*
Data S12 – LCMS of P4 of Nigerian *Naja nigricollis*