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

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Abstract: Snakebite affects ~1.8 million people annually. The current standard of care are antibody-based antivenoms, which are difficult to access and are not effective against local tissue injury, the primary cause of morbidity. Here we use a functional genomics approach to define human genes that genetically interact with spitting cobra venoms. Most genes that confer resistance to venom cytotoxicity control proteoglycan biosynthesis, suggesting heparinoids as possible inhibitors. We show that heparinoids prevent venom cytotoxicity by inhibiting three-finger cytotoxins. Critically, the FDA-approved heparinoid tinzaparin was found to reduce tissue damage *in vivo* when given via a medically relevant route and dose. Overall, our systematic molecular dissection of cobra venom mechanisms provides insight into how we can treat cobra bites, information that can help improve the lives of millions of people worldwide.

One-Sentence Summary: Spitting cobra venom cytotoxins use a common heparin-sensitive mechanism to cause tissue damage

Main Text:

Snakebites kill an estimated ~138,000 people each year, with another ~400,000 people experiencing devastating long-term morbidity (*1*). Most of these envenomings occur in Sub-Saharan Africa and South/Southeast Asia, with young adults and children disproportionately impacted (*2, 3*). This makes snakebite envenoming the deadliest of the neglected tropical diseases (NTDs) with its burden landing mainly on impoverished rural communities (*4*). Current antibody-based antivenom treatments are species-specific, rely on a cold-chain, and require intravenous administration in hospital settings. Moreover, antivenoms can induce adverse reactions, and are often prohibitively expensive (*5, 6*). Crucially, antivenoms are ineffective against severe local envenoming, which involves painful progressive swelling, blistering and/or tissue necrosis around the bite site and can lead to loss of limb function, amputation, and lifelong disability (*7*). The resulting annual disease burden from snakebite in West Africa and Southeast Asia alone amounts to ~319,000 and ~392,000 disability adjusted life years (DALYs), respectively, with associated costs for the latter (2.5 billion USD) representing ~0.1% of the region's GDP (*8*). Consequently, the World Health Organization (WHO) recently elevated snakebite to a 'priority category A NTD' and announced the ambitious goal of reducing the global burden of snakebite in half by 2030 (*9*).

To meet this target, we require a basic molecular understanding of how diverse snake venoms interact with human physiology to inform the development of new therapeutics (*10*). Here we use a functional genomics approach to define venom/target genetic interactions that modify cytotoxicity and use this information to develop a local acting venom antidote.

Results

Whole genome CRISPR knockout screens for spitting cobra venom cytotoxicity

Based on severe morbidity-causing pathology, such as local dermonecrosis and permanent disability *(7, 11-13),* the WHO lists many cobras (*Naja* spp.) as "Category 1" species of highest medical importance. Envenoming from the African red (*N. pallida*, Tanzania) and black-necked (*N. nigricollis*, Nigeria) spitting cobra (**Fig. 1A**) cause extensive local tissue damage *(12)* and these venoms showed potent cytotoxicity in the human haploid cell line, HAP1 (**Fig. 1B**). Pharmacological inhibition of apoptosis (Ac-DEVD-CHO; Z-VAD-FMK) did not suppress venom cytotoxicity, however the necroptotic inhibitor necrosulfonamide (NSA) limited some cell death, suggesting cobra venom cytotoxicity may partially trigger necroptotic death (**fig. S1A**). To guide the development of therapeutics, we defined the molecular mechanisms involved in venom-induced cell death using whole genome CRISPR KnockOut (KO) screening (**Fig. 1C**). Cells were transduced with the TKOv3 library which targets most human protein-coding genes with ~4 guides/gene (*14*). This pool of CRISPR KO cells was then selected with 5 µg/mL of *N. pallida* or *N. nigricollis* venom three times. After recovery, guide sgRNA in surviving cells was isolated, amplified by PCR, and quantified by next generation sequencing. Guide enrichment was compared to a control unselected population using the MaGeCK pipeline (*15*). Guide RNAs associated with venom sensitization (Log_2<-2, FDR<0.1) or resistance (Log_2>2, FDR<0.1) were identified, and substantial overlap was observed between the two snake species (**Fig. 1D** and **E,** and **data S1** and **S2**).

For *N. pallida* venom, the top significant genes that, when targeted, promoted venom sensitization include the chromatin remodelling SWI/SNF component SMARCD1 (*16*), the cyclin dependent

kinase CDK13 (*17*), the histone deacetylase HDAC3 (*18*), the anti-apoptotic protein ZFAT (*19*), and CRAMP1L, an uncharacterised gene linked with susceptibility to skin rash (*20*) (**data S1**). For *N. nigricollis* venom, the top sensitizers included the cell growth/tumour suppressors TSC1/TSC2 (*21*), the TSC subunit TBC1D7 (*22*), the SWI/SNF component SMARCC1 (*23*), the lipid phosphatase Inositol Polyphosphate Phosphatase Like 1/SHIP2 (*24*), and the microtubule interaction protein APPBP2 (*25*) (**data S2**).

For *N. pallida* venom, the top significant genes that, when targeted, promoted venom resistance included the uncharacterized transmembrane protein TMEM50A, the suppressor of growth hormone tetraspanin membrane protein LEPROTL1 (*26*), and components of proteoglycan biosynthesis NDST1, XYLT2, EXT1, EXTL3, and SLC35B2 (*27*). For *N. nigricollis* venom, the top promoters again included LEPROTL1 and TMEM50A, as well as multiple components of the proteoglycan biosynthesis machinery including EXT1, B4GALT7, EXT2, EXTL3, XYLT2, NDST1 and SLC35B2. Further, pathway analysis of these data highlighted heparin sulfate, chondroitin sulfate, and dermatan sulfate biosynthesis as critical pathways required for cytotoxicity of both *N. pallida* and *N. nigricollis* venoms (**Fig. 1F** and **G**).

Heparin biosynthesis is required for venom cytotoxicity

The top pathway required for venom cytotoxicity from *N. pallida* or *N. nigricollis* was heparan/heparin sulfate biosynthesis (*N. pallida*: $p<10^{-10}$, *N. nigricollis*: $p<10^{-8}$) and our screening data showed that targeting of most of the heparan/heparin sulfate biosynthesis pathway components individually was sufficient to block venom activity (*N. pallida* 7/11, and *N. nigricollis* 8/11 components of the pathway were hit, see **Fig. 2A**). To validate these results, we targeted each resistance gene individually (**data S3**) and tested cytotoxicity. Targeting each component of the heparan/heparin biosynthesis pathway conferred some resistance to each venom (**Fig. 2B and 2C**), confirming a role for heparan in cobra venom cytotoxicity. To test the generalizability of this requirement, we also treated gene targeted cells with an additional spitting cobra venom (**Fig. 2D, fig. S2A**; Tanzanian *N. nigricollis*), and again components of heparan/heparin sulfate biosynthesis were required for cytotoxicity.

Heparan and heparin sulfate share a sugar backbone synthesized by a common pathway (**Fig. 2A**). While heparan sulfate is a ubiquitous component of the extracellular matrix, heparin is primarily produced by tissue mast cells. Heparin is a highly sulfated, polyanionic polysaccharide used clinically for its potent anticoagulant activity. Heparin is on the WHO Model List of Essential Medicines (EML), however, multiple low molecular weight (LMW) medical variants of heparin (tinzaparin, T; dalteparin, D) termed “heparinoids" are also available and approved for antithrombotic use (*28*, *29*) (**Fig. 3A**). Since heparan/heparin sulfate biosynthesis was necessary for venom to cause cytotoxicity, we hypothesized that adding excess free heparin or LMW heparinoids may be sufficient to block venom cytotoxicity. Indeed, immediate treatment with heparin, tinzaparin, or dalteparin, all block cytotoxicity in response to *N. pallida* (**Fig. 3B**, quantified in **C**), Nigerian *N. nigricollis* (**Fig. 3B**, quantified in **D**), or Tanzanian *N. nigricollis* (**Fig. 3E**) venom, and the related non-anticoagulant heparinoid N-acetyl-heparin *(30)* showed similar effects (**Fig. 3F**).

To test if heparinoids can block venom cytotoxicity therapeutically, we first treated cells with *N. nigricollis* venom and then added heparin over time. Addition of heparin or tinzaparin up to 60 minutes after venom could still block Nigerian *N. nigricollis* venom cytotoxicity (**Fig. 3G, fig.**

S2B) and heparin or tinzaparin treatment after *N. pallida* and Tanzanian *N. nigricollis* venom could also block cytotoxicity (**fig. S2C-F).**

Heparinoids prevent venom interaction with the cell surface

Since heparan sulfate and related molecules bind soluble effectors including growth factors and proteases *(31, 32),* we hypothesized that in the context of its venom antidote activity, heparin may act as a "decoy" venom receptor and block venom/cell interactions. To test this hypothesis, we labelled each cobra venom with an Alexa-488 fluorophore and then evaluated venom/cell interactions by flow cytometry. While labelled cobra venom showed a strong interaction with untreated cells (*N. pallida* venom shown in **Fig. 4A-C**), adding heparin (**Fig. 4B**) or tinzaparin (**Fig. 4C**) blocked venom/host cell interactions. These data are quantified in **Fig. 4D**, and similar results were observed for venom from the two geographical variants of *N. nigricollis* (**fig. S3A-H**). Thus, by flooding the system with free heparin, we can suppress venom/target interactions, and this is sufficient to block cytotoxicity.

Heparin interacts with three-finger cytotoxins to block venom/host interactions

Snake venoms are variable mixtures of different toxins, and cobra venoms consist predominantly of multiple isoforms of phospholipases A2 (PLA_2) and three-finger toxins (3FTx) (*33*). To identify which specific venom components interact with heparinoids, we separated *N. pallida* (**Fig. 4E** and **F**) and *N. nigricollis* (**fig. S4A** and **B,** and **fig. S5A**) venom using heparin affinity chromatography. Interestingly, most of the venom material bound to the column and was eluted in 3-4 main peaks (**Fig. 4E, fig. S4A** and **fig. S5A**), suggesting heparin has a broad capacity to interact with multiple venom components. The main proteins comprising each peak were identified by liquid chromatography mass spectrometry (LC-MS): P1 (weak heparin interaction) contained mainly acidic PLA_2, P2 (moderate heparin interaction) contained the 3FTx cytotoxin 1 (CTx1), and P3 (strong heparin interaction) contained both basic PLA_2 ($bPLA_2$) and the 3FTx cytotoxins CTx3 and 4 (**data S4-10**). We then further fractionated P3 using cation exchange to separate basic PLA_2 ($bPLA_2$) from the 3FTx cytotoxins CTx3 and 4 (**Fig. 4F** and **fig. S4B)** and assessed the purity of each fraction on SDS-PAGE (**Fig. 4G**, **fig. S4C**, and **fig S5B**).

Isolated *N. pallida* toxins were then subjected to surface plasmon resonance (SPR) to assess binding affinity with heparin, dalteparin and tinzaparin. **Fig. 4H-I** shows that heparin binds with high affinity to CTx3 (K_D = 37 nM) and CTx4 (K_D = 36 nM), binds weakly to $bPLA_2$ (K_D >100 nM) and exhibits no specific binding to CTx1 or PLA_2. The same pattern of binding is observed for tinzaparin and dalteparin (**fig. S6**). Functionally, the 3FTxs CTx3 and 4 were highly cytotoxic and, in line with their binding profile, their activity was inhibited by heparin (**Fig. 4J**). While CTx1 also showed strong cytotoxicity, this activity was not heparin sensitive (**Fig. 4J**). Similar binding, cytotoxicity and inhibition data were obtained for the two *N. nigricollis* venoms (**fig. S4D-F** and **fig. S5C-E**), with CTx3 and 4 demonstrating the most potent heparinoid binding properties. Collectively, these data demonstrate that heparin and related compounds block African spitting cobra venom cytotoxicity by acting directly on the cytotoxic 3FTxs CTx3 and 4.

To assess the breadth of this anti-venom activity, we tested the ability of heparin to block other cytotoxic snake venoms (**Fig. 4K-O**). We found both heparin and N-acetyl-heparin (**fig. S7A**) could also suppress cytotoxicity caused by venom from the monocled cobra (**Fig. 4K**; *Naja kaouthia*), the Chinese cobra (**Fig. 4L**; *Naja atra*), and the Indian spectacled cobra (**Fig. 4M**; *Naja*

naja). However, neither had the ability to block cytotoxicity caused by West African saw-scaled viper (**Fig. 4N**; *Echis ocellatus*) or African puff adder (**Fig. 4O**; *Bitis arietans*) venom (**fig. S7A**). Importantly, while cobra venoms contain cytotoxic 3FTxs (*33*), the viper venoms do not (*34*). Overall, these data show that heparin and LMW heparinoid drugs can inhibit cytotoxic 3FTxs and may constitute a new and potent antidote for morbidity-causing cobra venoms.

Heparinoids protect against spitting cobra venom-induced skin damage

We investigated if heparin or heparinoids could protect human epidermal keratinocytes from *N. pallida* and *N. nigricollis* (Nigerian and Tanzanian) venom-induced cytotoxicity. Venom from each snake species induced cell death in a concentration-dependent manner (**Fig. 5A**), and treatment with heparinoids promoted cell survival (**Fig. 5B**) and inhibited this cell death (**Fig. 5C**). We next tested the ability of heparinoids to block venom-induced dermonecrosis *in vivo* using a WHO-recommended preclinical model of local envenoming (*35–37*). Mice were intradermally (ID) dosed with venom from *N. pallida*, Nigerian *N. nigricollis*, or Tanzanian *N. nigricollis* (25, 57, and 63 µg, respectively), preincubated with saline vehicle or the heparinoids dalteparin or tinzaparin (60 µg [3 mg/mL]) (**Fig. 5D**). While animals injected with venom-plus-vehicle exhibited large dermonecrotic lesions, animals that received venom-plus-dalteparin or -tinzaparin showed significant ($P<0.05$) reductions in lesion sizes, irrespective of the venom or drug tested (**Fig. 5E, fig. S8**, quantified in **Fig. 5F-H**). Tinzaparin outperformed dalteparin by providing the greatest reduction in dermonecrosis across the three venoms (mean lesion size reduction of 94% versus 63%, respectively). For these reasons, we progressed tinzaparin into more challenging rescue studies that better reflect envenoming by delivering treatment after venom dosing (**Fig. 5I**).

We used Tanzanian *N. nigricollis* venom for rescue studies as it was the most dermonecrotic of the three venoms tested (**Fig. 5H**) and we evaluated the efficacy of ID tinzaparin delivered immediately after venom injection. Both a low dose (3 mg/kg) and moderate 'human-equivalent' dose (21.5 mg/kg) of tinzaparin significantly ($P<0.05$) reduced the resulting mean sizes of venom-induced dermonecrotic lesions by 66 and 60%, respectively (**Fig. 5J, fig. S9**, quantified in **K**). Tinzaparin is an FDA-approved anti-thrombotic that is self-administered subcutaneously (SC) daily. Accordingly, we next challenged mice with the same ID venom dose before immediately delivering tinzaparin SC to a site underneath where venom was injected. The low (3 mg/kg) tinzaparin dose reduced the mean size of resulting dermonecrotic lesions by 32% though this trend did not meet statistical significance. However, the moderate (21.5 mg/kg) dose of SC tinzaparin significantly ($P<0.01$) reduced the size of venom-induced dermonecrotic lesions by 50% (**Fig. 5L, fig. S9**, quantified in **M**) demonstrating that the approved route of administration for this licensed drug is effective at reducing the severity of local envenoming caused by cobra bites *in vivo*. This inhibitory effect was further evidenced histopathologically, as skin tissue samples collected from mice injected with Tanzanian *N. nigricollis* venom exhibited prominent damage to all layers, with ulceration of the epidermis and necrosis of the underlying dermis, hypodermis and panniculus carnosus, while mice injected with venom and tinzaparin, either preincubated or delivered SC, showed substantial reduction in epidermal ulceration and underlying necrosis (**Fig. 5N**). Overall, these data show that heparinoid drugs are an effective new class of snake venom treatment that act to prevent severe local spitting cobra envenoming by blocking the cytotoxic actions of the 3FTx cytotoxins CTx3 and 4.

DISCUSSION

Defining the essential molecular interactions between cytotoxic venoms and target cells provides fundamental understanding of how these venoms act and, importantly, how we can treat them medically. Here we describe multiple critical genes and pathways required for cobra venom cytotoxicity, and for one pathway, heparan/heparin biosynthesis, we characterise this interaction in detail. Our unbiased approach led to the surprising discovery of heparinoids as a pre-clinically effective, and broad acting cobra envenoming antidote. Further, we show heparinoids act by binding to and blocking 3FTx cytotoxicity, and 3FTx inhibitors represent a much-needed class of therapeutic. Overall, the results of our study provide valuable insight into cobra venom mechanisms, information that can help reduce morbidity caused by snakebite envenomings.

Although antibody-based antivenoms are lifesaving therapies, there are issues with safety, specificity, and administration that impact their effectiveness. While progress has been made to generate broader acting recombinant antivenoms (*38*), systemic administration of antibody-based therapies are ineffective at preventing severe local envenoming (*1*, *7*, *39*). This is likely because of the rapid onset of snake venom-mediated cytotoxicity, delays in reaching a clinical environment, and the difficulty for centrally delivered antibodies to rapidly penetrate peripherally injured tissue. 3FTxs are highly abundant components of elapid venoms characterised by three loops that connect to a central core. These low molecular mass proteins (~6-9 kDa) have diverse neurotoxic, cardiotoxic and cytotoxic effects (*34*). In the context of spitting cobras, 3FTxs are highly cytotoxic and cause local tissue necrosis in snakebite victims (*40*). Thus, there is a strong need for the development of cheap, stable anti-3FTx therapeutics that can be rapidly administered on-site soon after a snakebite (*39*).

Our study demonstrates that heparinoids may have utility in treating cobra bites from diverse regions of Africa and Asia. While some snakebites cause venom-induced consumption coagulopathy (VICC), and use of heparin may be dangerous in these cases, spitting cobras are rarely, if ever, responsible for VICC *(41)*. Further, several clinical trials have been conducted where snakebite patients presenting with a coagulopathy received anticoagulant heparinoids, and no concerning safety signals relating to worsening coagulopathy or increased bleeding events were observed in any of these trials *(42–45)*. Moreover, we found that both anticoagulant and non-anticoagulant heparinoids block cobra venom cytotoxicity, and thus the activity we describe is independent of anticoagulation. Tinzaparin shows particularly strong translational promise because it is already an FDA-approved therapeutic (*28*), which should lead to reduced costs and time associated with its downstream clinical development. The potential for rapid community-level heparinoid administration holds much long-term promise for preventing morbidity caused by cobra bites.

Overall, our findings align with studies investigating the Chinese cobra (*N. atra*) 3FTx cardiotoxin (*47*, *48*), as well as several studies using compositionally distinct viper venoms that suggest a protective effect by heparin. For example, preincubation with heparin reduced local skin lesions caused by Russell's viper (*Daboia russelii*) venom (*49*), and also blocked jararacussu (*Bothrops jararacussu*) PLA_2 myotoxin II damage to muscle or endothelial cells (*50–53*). Our work reaffirms the protective action of heparin and related compounds, extending it to cytotoxic 3FTxs and provides a molecular mechanism for this activity. Critically, we find the low molecular weight heparinoid, tinzaparin, is suitable to prevent dermonecrosis clinically in a post-envenoming context. Beyond venoms, cellular heparan sulfate has also recently been found essential for SARS-

CoV-2 infection (*54*). Here as well, flooding the system with free heparin or related compounds was sufficient to block infection, and similar observations have been made with other viral and bacterial pathogens (*55–58*). Heparan sulfate proteoglycans are conserved and widely expressed on the cell surface (*59*). Thus, targeting these molecules may be an optimal evolutionary strategy to interact with a broad range of species. Conversely, by providing this structure in excess, free heparin/heparinoids may act as a decoy target for multiple unrelated environmental hazards. Overall, the emerging molecular evidence suggests that heparan sulfate is a common cellular entry point for diverse human threats, and heparinoids may have broad untapped activity to protect us.

Our study does have limitations. While effective, in this study tinzaparin when given therapeutically could not completely block dermonecrosis. Therefore more preclinical development, including dosing, delivery route, and combinations with other toxin-targeting drugs *(35, 46)* may be required to generate a fully effective local antidote. Moreover, our screening was performed in a cell line derived from blood and further insight into cobra venom cellular targets could be achieved by additional CRISPR screening using a human skin cell line. While we focused on heparan/heparin biosynthesis, our screening identified multiple other genes that may modify venom cytotoxicity that we have yet to investigate. Finally, cytotoxicity is only one physiologically relevant impact of snake envenoming, and further CRISPR screening using more functional cellular readouts beyond death may provide a more comprehensive understanding of venom mechanisms of action.

To date, much of modern molecular medicine has focused on health challenges facing high income countries; however, here we apply these same approaches to understand snakebite envenoming, a significant neglected tropical disease. From these efforts, we identify multiple new genes and pathways essential for snake venom cytotoxicity including heparan/heparin sulfate biosynthesis. Using this knowledge, we uncover affordable, safe, and effective drugs that can prevent cobra venom associated morbidity, paving the way for important future therapies.

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Competing interests: A provisional patent application ("A new broad acting antidote for venom-induced injury including local tissue damage and/or skin irritation", application number: 2024900779) has been submitted by GGN, NRC, FC and TYD based on these results. The remaining authors declare that they have no competing interests.

Data and materials availability: All data are available in the main text or the supplementary materials. Whole genome CRISPR KO sequencing datasets have been deposited on GEO with the identifier GSE262798.

Supplementary Materials

Materials and Methods

Figs. S1 to S9

References (*60* - *68*)

Data S1 to S12

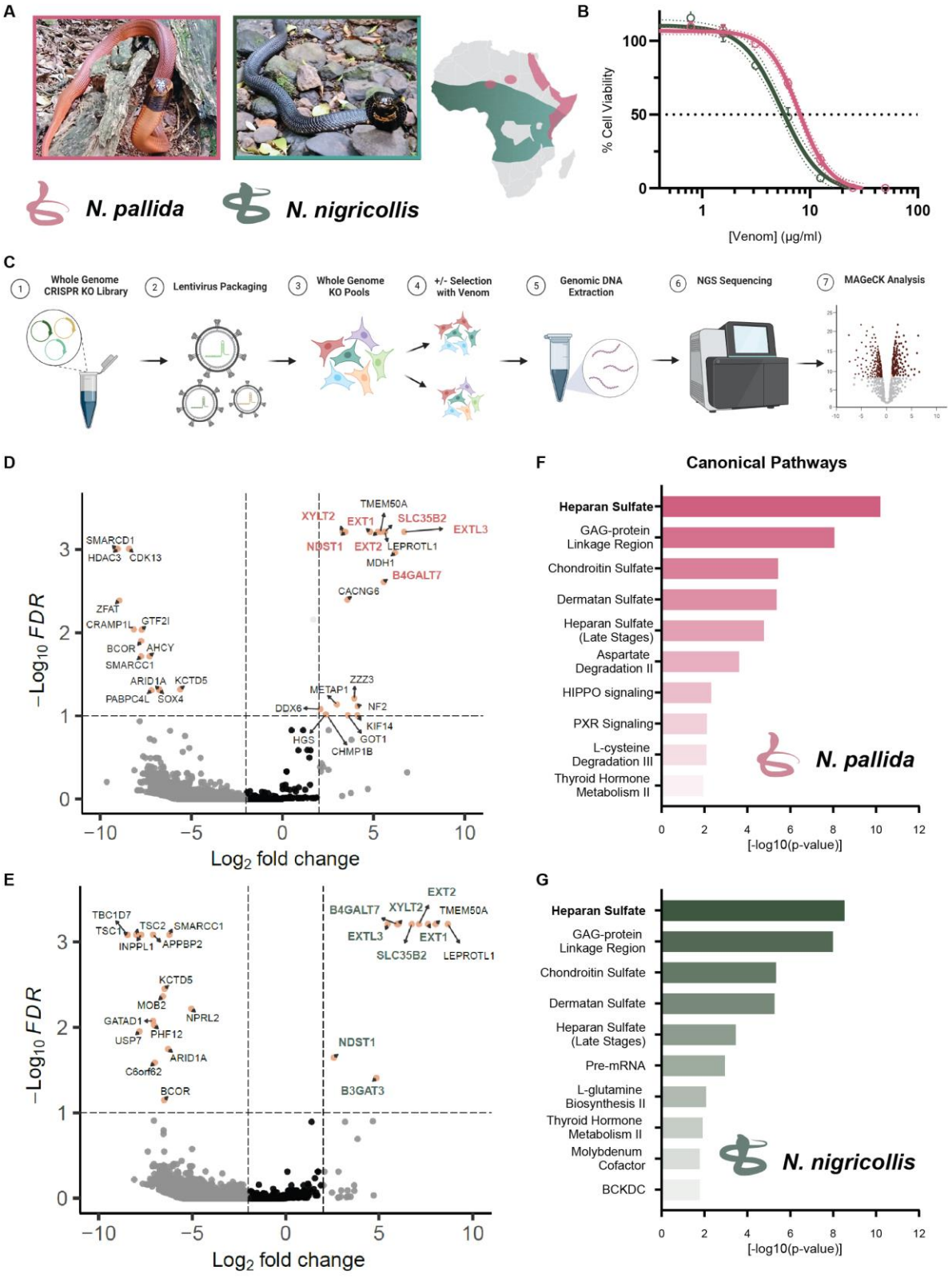

Fig. 1. A whole genome CRISPR-Cas9 knockout screen identified genes required for African spitting cobra venom cytotoxicity. (**A**) Red spitting cobra (*Naja pallida*) and black-

necked spitting cobra (*Naja nigricollis*) and their distributions. (**B**) HAP1 cell viability as determined by resazurin assays after 24 h treatment with serial dilutions of spitting cobra venoms (n = 3). (**C**) Schematic of pooled CRISPR knockout library screens. HAP1 cells were transduced with a whole genome knockout library at MOI = 0.3. Venom was added to library cells and genomic DNA extracted from selected and unselected control populations before undergoing next generation sequencing. Analysis was calculated using the MAGeCK pipeline. (**D-E**) Gene enrichment analysis of screens performed using MAGeCK (*14*). Horizontal dotted line indicates $-\log_{10}$(false discovery rate) (FDR) = 1 and vertical dotted lines indicate $\log_2$(fold changes) (LFCs) of -2 and 2. Plots generated using EnhancedVolcano (v1.10.0) R package. (**F-G**) Top canonical pathways identified through Ingenuity Pathway Analysis (IPA). Photographs in panel A are by Geoffrey Maranga.

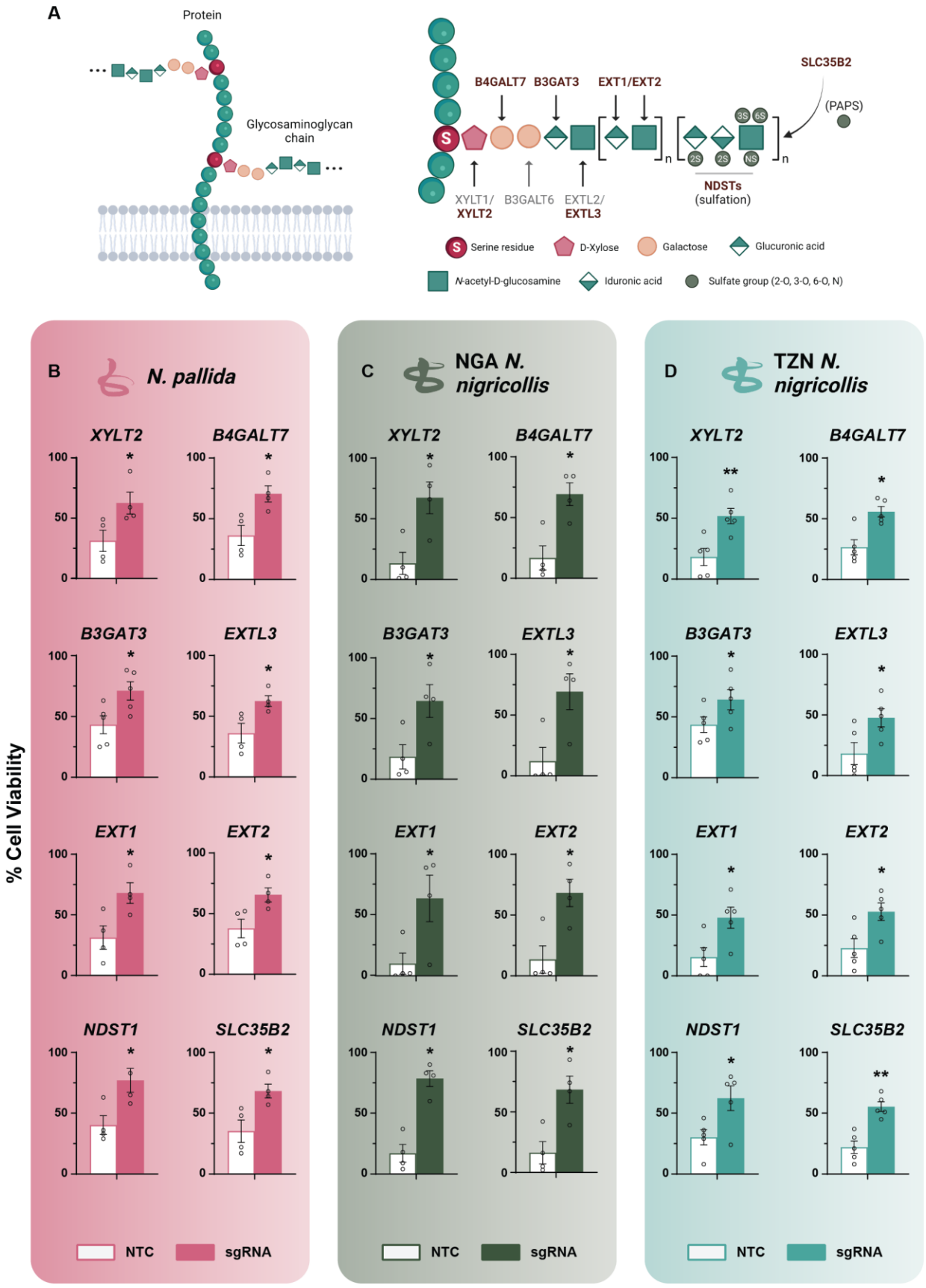
A
Protein
Glycosaminoglycan chain
B4GALT7
B3GAT3
EXT1/EXT2
SLC35B2
(PAPS)
XYLT1/ XYLT2
B3GALT6
EXTL2/ EXTL3
NDSTs (sulfation)
Serine residue
D-Xylose
Galactose
Glucuronic acid
N-acetyl-D-glucosamine
Iduronic acid
Sulfate group (2-O, 3-O, 6-O, N)
B
N. pallida
C
NGA N. nigricollis
D
TZN N. nigricollis
% Cell Viability
XYLT2
B4GALT7
B3GAT3
EXTL3
EXT1
EXT2
NDST1
SLC35B2
NTC
sgRNA

Fig. 2. Heparan sulfate biosynthesis is required for spitting cobra venom cytotoxicity. (**A**) Schematic representation of the heparan sulfate biosynthesis pathway. (**B-D**) Pools of single sgRNA knockout cells for heparan sulfate biosynthesis hits (*XYLT2, B4GALT7, B3GAT3, EXTL3, EXT1, EXT2, NDST1* and *SLC35B2*) and a non-targeting control sgRNA (NTC) were generated via lentiviral transduction in HAP1 cells. Pooled knockout cells were treated with 10 μg/mL *N. pallida* (**B**), Nigerian (NGA) *N. nigricollis* (**C**), or Tanzanian (TZN) *N. nigricollis* (**D**) venom for 24 h and viability ascertained through resazurin. Significance determined by one-tailed Mann-Whitney test, *P<0.05, **P<0.01 (n = 4-5).

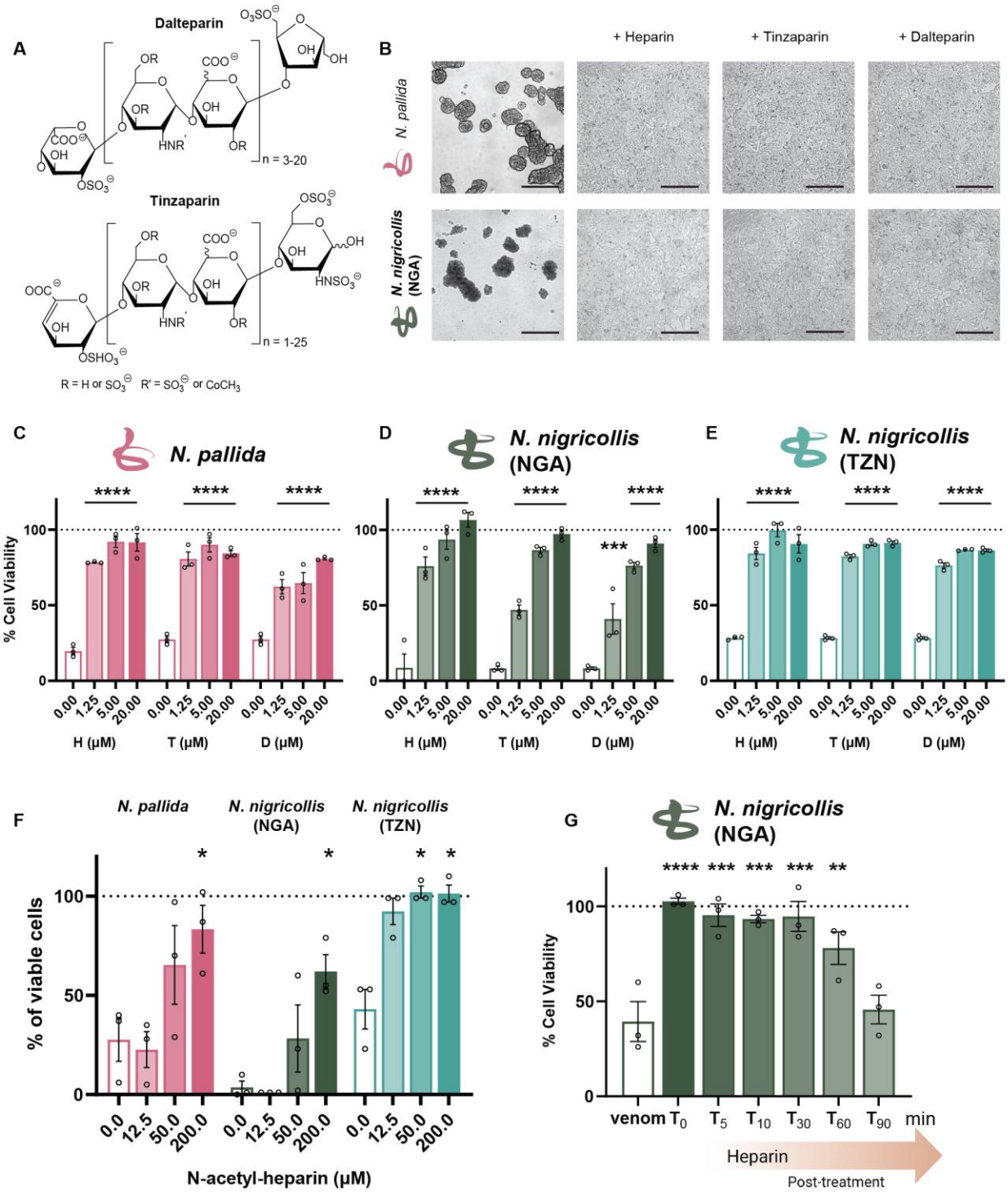

Fig. 3. Heparin and LMW variants block *Naja* venom action *in vitro*. (**A**) Chemical structures of low molecular weight heparinoids, dalteparin and tinzaparin. (**B**) Representative brightfield microscopy of HAP1 cells after 24 h exposure to 10 μg/mL *N. pallida* or Nigerian (NGA) *N. nigricollis* venom, simultaneously treated with 20 μM heparin, tinzaparin or dalteparin. Scale bar = 200 μm (**C**-**E**) Venoms (10 μg/mL) and serial dilutions of heparin (H), tinzaparin (T) and dalteparin (D) (1.25-20 μM) were added simultaneously to HAP1 cells.

Resazurin cell viability assays were performed after 24 h of treatment. Significance determined by 2-way ANOVA and Dunnett test, ****P<0.0001 (*n* = 3). (**F**) Venoms (10 μg/mL) and serial dilutions of N-acetyl-heparin (12.5-200 μM) added simultaneously to HAP1 cells. Resazurin cell viability assays were performed after 24 h of treatment. Significance determined by 2-way ANOVA and Dunnett test, *P<0.05 (*n* = 3). (**G**) HAP1 cells were treated with 10 μg/mL *N. nigricollis* (NGA) venom before addition of 20 μM heparin immediately after, or 5-, 10-, 30-, 60- or 90-min post venom application. Significance determined by Ordinary one-way ANOVA and Dunnett test, **P<0.01, ***P<0.001, ****P<0.0001 (*n* = 3).

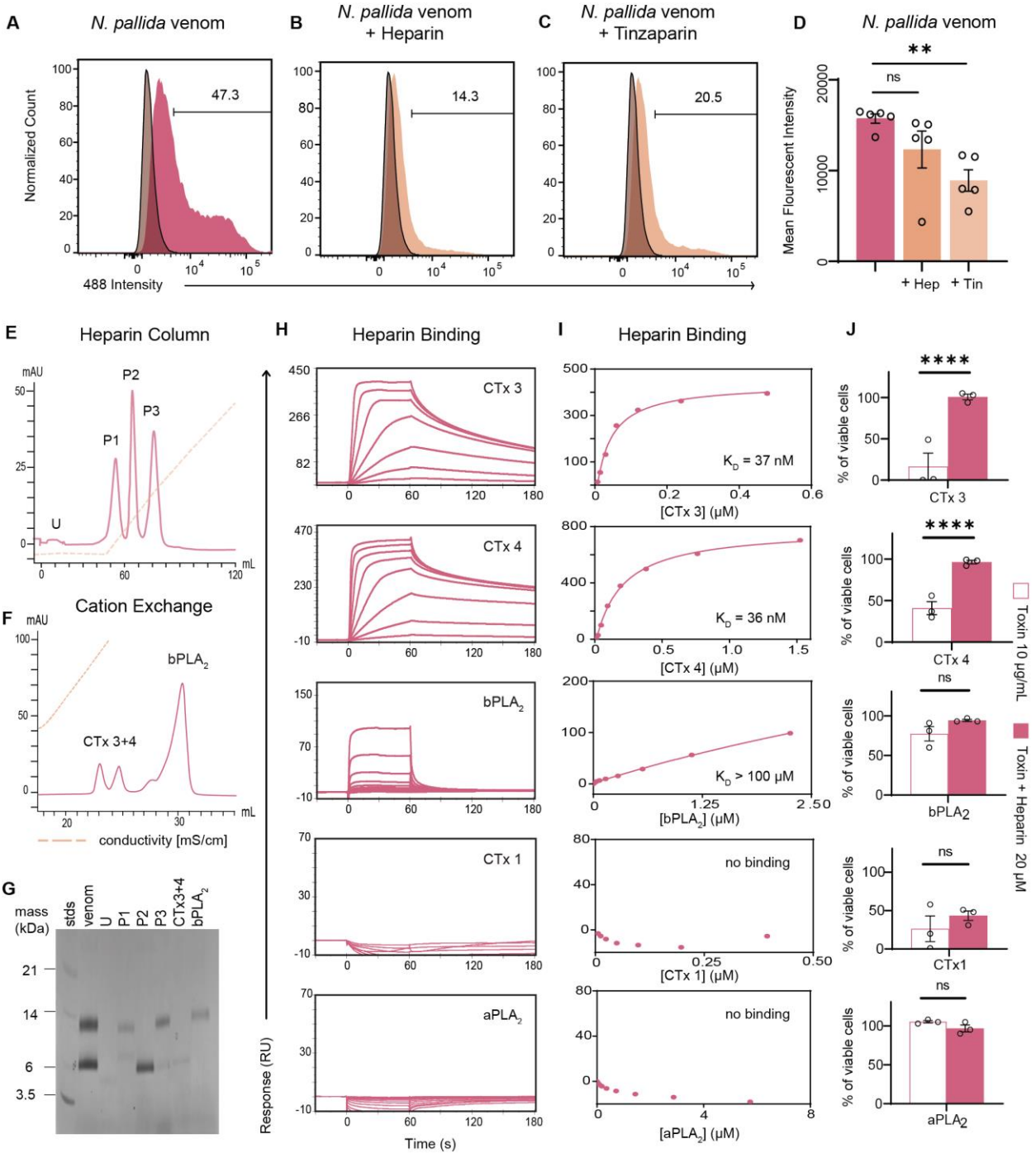
A
N. pallida venom
47.3
Normalized Count
488 Intensity
B
N. pallida venom
+ Heparin
14.3
C
N. pallida venom
+ Tinzaparin
20.5
D
N. pallida venom
**
ns
Mean Flourescent Intensity
+ Hep
+ Tin
E
Heparin Column
mAU
P1
P2
P3
U
mL
F
Cation Exchange
bPLA2
CTx 3+4
conductivity [mS/cm]
G
mass (kDa)
stds
venom
U
P1
P2
P3
CTx3+4
bPLA2
21
14
6
3.5
H
Heparin Binding
CTx 3
CTx 4
bPLA2
CTx 1
aPLA2
Response (RU)
Time (s)
I
Heparin Binding
KD = 37 nM
[CTx 3] (μM)
KD = 36 nM
[CTx 4] (μM)
KD > 100 μM
[bPLA2] (μM)
no binding
[CTx 1] (μM)
no binding
[aPLA2] (μM)
J
****
% of viable cells
CTx 3
CTx 4
bPLA2
CTx1
aPLA2
Toxin 10 μg/mL
Toxin + Heparin 20 μM
K
Naja kaouthia
L
Naja atra
M
Naja naja
N
Echis ocellatus
O
Bitis arietans
% Cell Viability
p<0.0001
p<0.001
p<0.002
Venom conc. (μg/mL)
venom
venom + heparin 20 μM

Fig. 4. Heparin binds 3FTxs and prevents their cytotoxicity. (**A**) Representative flow cytometry histograms of WT HAP1 cells in gray and cells exposed to Alexa488-tagged *N. pallida* 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. (**E**) Heparin affinity chromatography of *N. pallida* venom. Unbound (U), Peak 1 (P1), Peak 2 (P2) and Peak 3 (P3). (**F**) Cation exchange chromatography of Peak 3. (**G**) SDS-PAGE gel of whole venom and resulting toxin fractions. (**H**) Surface plasmon resonance (SPR). Representative normalized sensorgrams of toxin binding to heparin. (**I**) Fits of the SPR data from (**H**) to a 1:1 binding model are shown and K_Ds are indicated on each plot. (**J**) Cytotoxicity of 10 μg/mL of each toxin fractions and addition of 20 μM heparin. Significance determined by 2-way ANOVA and Sydak test, ****P<0.0001 (n = 3). (**K-M**) Cytotoxicity of venoms containing 3FTxs (**K-** *Naja kaouthia*, **L-** *Naja atra*, **M-** *Naja naja*) and addition of heparin. (**N-O**) Cytotoxicity of venoms from more distantly related snakes and addition of heparin (**N-** *Echis ocellatus* and **O-** *Bitis arietans*). Significance determined by simple linear regression.

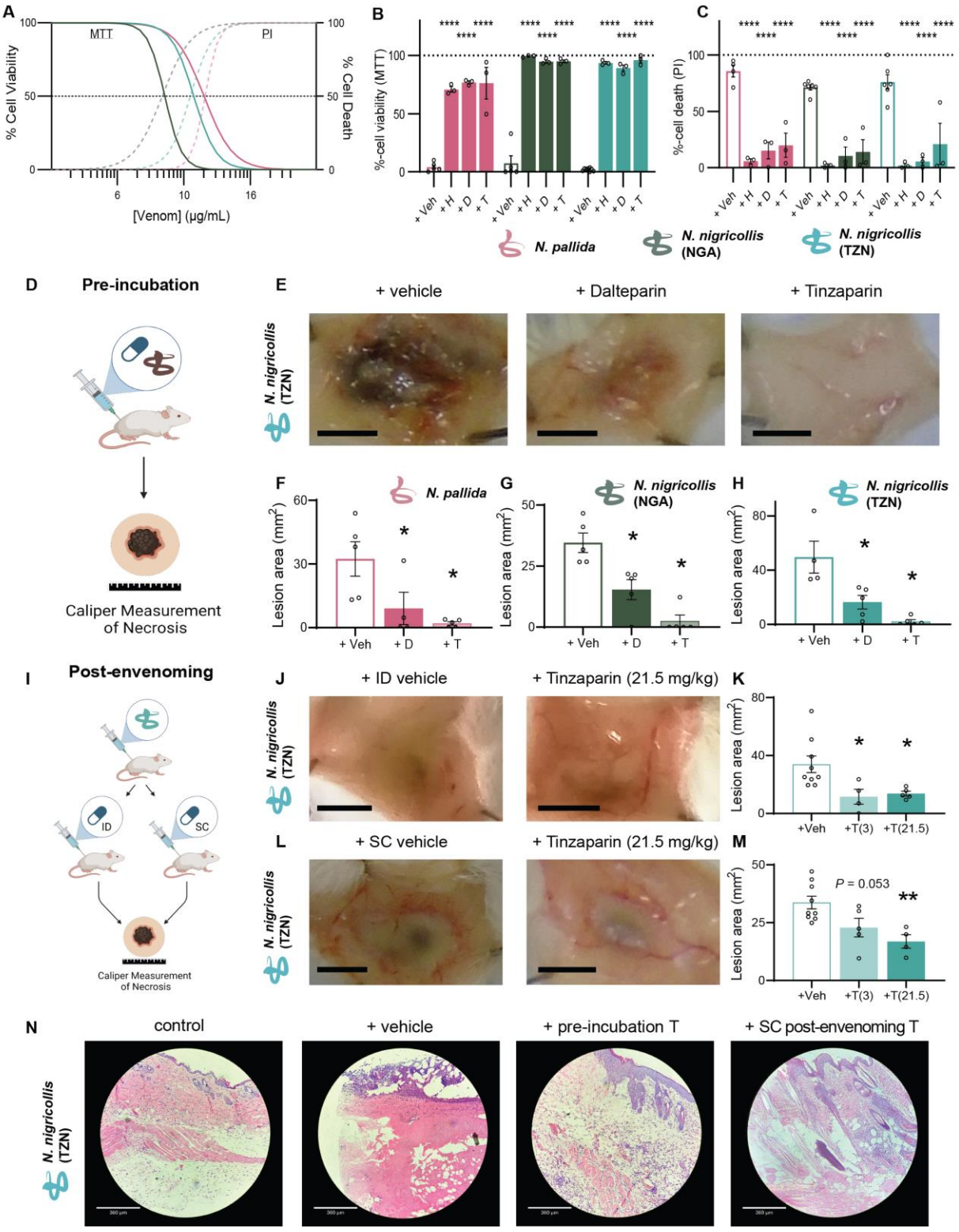

Fig. 5. Snake venom induced dermonecrosis is inhibited by heparinoids *in vivo*. (**A**) MTT cell viability and PI cell death assays on HaCaT epidermal keratinocytes exposed to serial dilutions (4.74-47.4 µg/mL) of spitting cobra venoms. (**B**) MTT-quantified %-cell viability and (**C**) PI-quantified %-cell death of HaCaT keratinocytes treated with venoms (*N. pallida* 15

μg/mL, *NGA N. nigricollis* 10 μg/mL, and *TZN N. nigricollis* 15 μg/mL) preincubated with saline vehicle control or heparin, dalteparin or tinzaparin (1000 μg/mL). (**D**) Mice ID-injection with venom that had been pre-incubated with saline vehicle control, dalteparin or tinzaparin (60 μg [3 mg/mL]). After 72 h mice were euthanized and the internal skin lesions excised for photographs and height and width measurements with calipers, from which area was calculated (bar graphs represent the mean lesion area for each treatment group and error bars represent SEM). (**E**) Representative images of necrosis, scale bar = 5 mm. Calculated areas for (**F**) 25 μg *N. pallida* (**G**) 57 μg Nigerian *N. nigricollis* (**H**) or 63 μg Tanzanian *N. nigricollis* venom, n≥4 (significance was determined by One-way ANOVA and Dunnett test, *P<0.05, **P<0.01). (**I**) Post-envenoming treatment. (**J, K**) ID-injected with Tanzanian *N. nigricollis* venom (63 μg) immediately followed by ID-injection at the venom injection site with saline vehicle control, low dose (3 mg/kg) or moderate 'human-equivalent' dose (21.5 mg/kg) tinzaparin, and (**L, M**) ID-injected with Tanzanian *N. nigricollis* venom (63 μg) immediately followed by SC-injection underneath the venom injection site with saline vehicle control, low dose (3 mg/kg) or moderate 'human-equivalent' dose (21.5 mg/kg) tinzaparin. (**N**) Light micrograph images (100X) of hematoxylin & eosin (H&E)-stained skin lesion cross-sections from mice injected with Tanzanian *N. nigricollis* venom (63 μg) and pre-incubated saline control show severe damage, with complete loss of the epidermis and necrosis of the underlying dermis, hypodermis, and panniculus carnosus. Sections from mice injected with pre-incubated tinzaparin (3 mg/kg) or receiving SC-injected tinzaparin (21.5 mg/kg) immediately post-envenoming show only minimal damage, with mild reactive changes including epidermal hyperplasia and dermal inflammation.