Supporting Information for

Original article

Neutralising effects of small molecule toxin inhibitors on nanofractionated coagulopathic Crotalinae snake venoms

Chunfang Xie[a,b], Julien Slagboom[a,b], Laura-Oana Albulescu[c,d], Govert W. Somsen[a,b], Freek J. Vonk[a,b,e], Nicholas R. Casewell[c,d], Jeroen Kool[a,b,*]

[a]*Amsterdam Institute of Molecular and Life Sciences, Division of BioAnalytical Chemistry, Department of Chemistry and Pharmaceutical Sciences, Faculty of Science, Vrije Universiteit Amsterdam, De Boelelaan 1085, Amsterdam 1081HV, The Netherlands*

[b]*Centre for Analytical Sciences Amsterdam (CASA), Amsterdam 1098 XH, The Netherlands*

[c]*Centre for Snakebite Research and Interventions, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK*

[d]*Centre for Drugs and Diagnostics, Liverpool School of Tropical Medicine, Pembroke Place, Liverpoo L3 5QA, UK*

[e]*Naturalis Biodiversity Center, Darwinweg 2, Leiden 2333 CR, The Netherlands*

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*Corresponding author. Tel.: +31 20 5987542.

E-mail address: j.kool@vu.nl (Jeroen Kool).

S1. Inhibiting effects of varespladib, marimastat and dimercaprol on venoms from *Bothrops* species

The inhibiting effects of varespladib, marimastat and dimercaprol were investigated on both nanofractionated *B. asper* and *B. jararaca* venom toxins for coagulation activity. The reconstructed bioassay chromatograms for *B. asper* venom are shown in Figs. S1–S3. In this study, each coagulation bioassay was performed at least in duplicate on nanofractionated venom toxins for reproducibility assessment. In each Figure, the right-side bioassay chromatograms which do not have superimposed correlated UV data depict the duplicate results of the left side figures. For each venom, the concentration of 1.0 mg/mL was used for nanofractionation. The bioassay chromatograms consist of three parts to fully depict all coagulation activities; the very fast coagulation activity (lower series of superimposed bioassay chromatograms), the slightly/medium increased coagulation activity (middle set of bioassay chromatograms), and the anticoagulation activity (upper series of superimposed bioassay chromatograms).

The effect of varespladib on *B. asper* venom after nanofractionation is shown in Fig. S1. For the very fast coagulation activity, a sharp positive peak (20.9 min) followed by a broad positive peak (21.2–22.4 min) was observed in the venom-only analysis (10 μL PBS solution was added before pre-incubation and is shown as "PBS" in the graphs). The sharp positive peak (20.9 min) was decreased in a concentration-dependent manner by increasing the varespladib concentration. The broad positive peak (21.2–22.4 min) changed into several sharp positive peaks after varespladib addition, and the activity of these peaks decreased further with increasing varespladib concentrations. Neither the sharp positive peak (20.9 min) nor the broad positive peak (21.2–22.4 min) could be fully neutralised by varespladib at the highest concentration of 20 μmol/L tested. For the slightly/medium increased coagulation activity, a sharp positive peak (20.9 min) followed by a broad positive peak (21.2–22.4 min), as was also observed in the very fast coagulation chromatograms, was observed in the venom-only analysis. Varespladib also showed a concentration–dependent effect on the sharp positive peak (20.9 min). Full inhibition of both peaks however was not achieved at a 20 μmol/L varespladib concentration. For the anticoagulation activity, a sharp negative peak (19.4 min) followed by a broad negative peak (19.9–20.9 min) and a tailing sharp negative peak (21.1 min) were observed in the chromatogram of the venom-only analysis. The first sharp negative peak (19.4 min) was already fully neutralised at a 0.8 μmol/L varespladib

concentration, while the broad negative peak (19.9–20.9 min) and the tailing sharp negative peak (21.1 min) were not inhibited at all by varespladib.

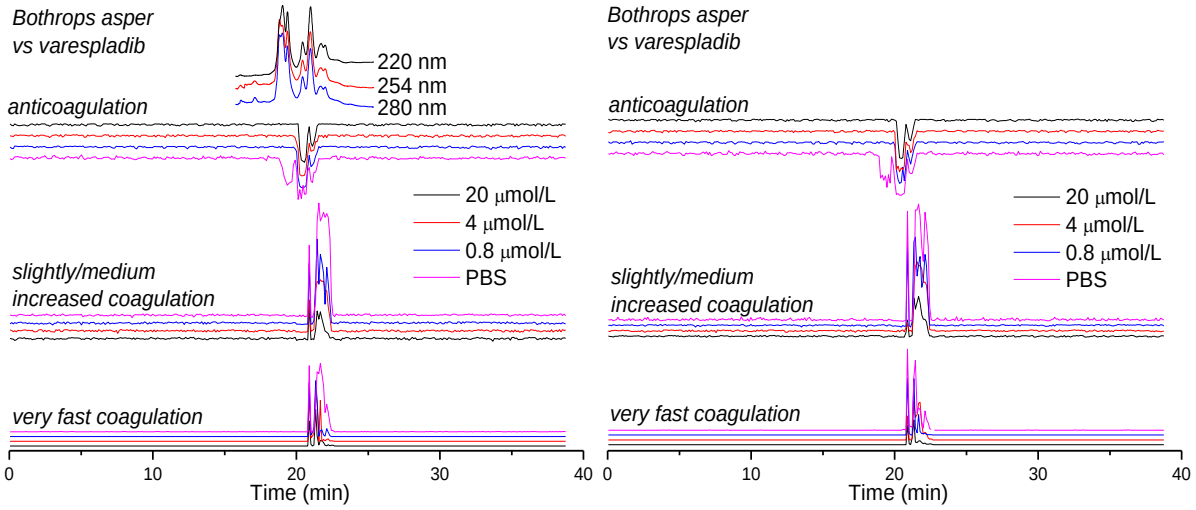

Figure S1 Reconstructed coagulation chromatograms in duplicate of nanofractionated *B. asper* venom toxins in presence of different concentrations of varespladib.

The effect of marimastat on *B. asper* venom after nanofractionation is shown in Fig. S2. In the venom-only analysis, a sharp positive peak (21.0 min) and a positive shoulder peak (21.3–22.1 min) were detected for the very fast coagulation activity; the sharp positive peak (21.0 min) decreased with increasing marimastat concentrations. The tailing part of the positive shoulder peak (21.3–22.1 min) was already neutralised by 0.8 μmol/L marimastat while the front part was shown to have less effect on the 0.8 μmol/L and also the 4 μmol/L marimastat concentrations. Only a very weak positive peak (21.0 min) was left at the marimastat concentration of 20 μmol/L tested. For the slightly/medium increased coagulation activity, three sharp positive peaks (21.0, 21.5, and 21.9 min) were observed in the venom-only chromatogram. The front two peaks (21.0 and 21.5 min) decreased with no inactivation achieved for the 20 μmol/L marimastat analysis, while the third positive peak (21.9 min) was split into two smaller peaks which were eventually neutralised at the 20 μmol/L marimastat concentration. For the anticoagulation activity, a large broad negative peak (18.9–21.0 min) followed by a small sharp negative peak (21.1 min) was observed in the venom-only analysis. The broad negative peak (18.9–21.0 min) was split into two negative peaks (18.9–20.0 and 20.1–21.0 min) but the activity of these two peaks (18.9–20.0 and 20.1–21.0 min) together with the later eluting weak negative peak (21.1 min) were not found to be inhibited at all by marimastat. To summarize, no full neutralisation was achieved by marimastat for the pro-coagulation activity as well as the anticoagulation activity.

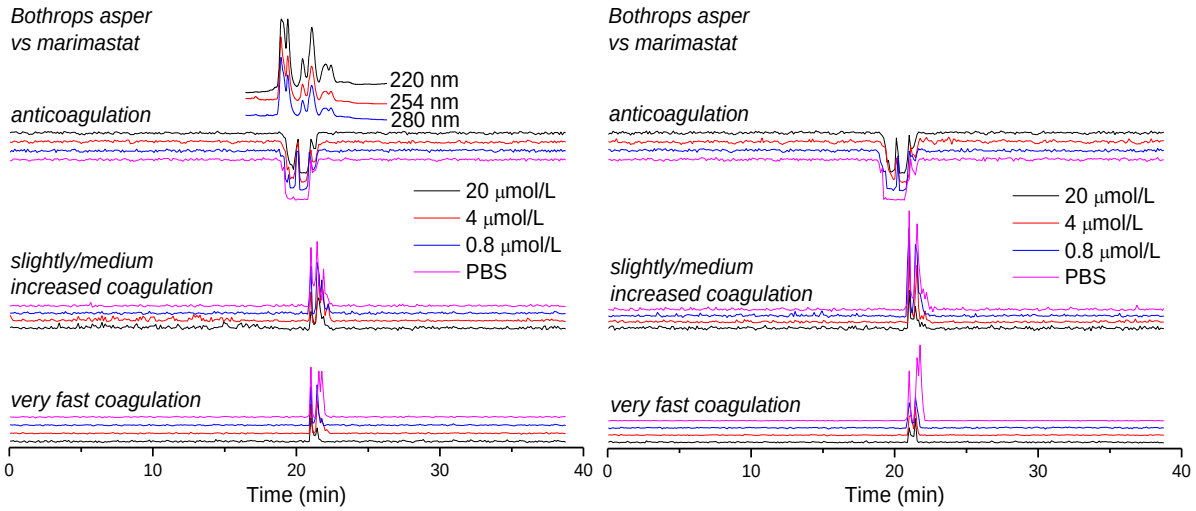

Figure S2 Reconstructed coagulation chromatograms in duplicate of nanofractionated *B. asper* venom toxins in presence of different concentrations of marimastat.

The effect of dimercaprol on inhibiting nanofractionated *B. asper* venom coagulation toxins is shown in Fig. S3. Two sharp positive peaks (21.1 and 21.5 min) were detected for the very fast coagulation activity in the venom-only analysis and both showed a limited response to dimercaprol. A sharp positive peak (20.9 min) followed by a broad positive peak (21.2–22.8 min) was observed for the slightly/medium increased coagulation activity in the venom-only analysis. The sharp positive peak (20.9 min) decreased by increasing dimercaprol concentrations. The broad positive peak (21.2–22.8 min) showed a limited response to 0.8 μmol/L dimercaprol, but was reduced significantly at the 4 μmol/L dimercaprol analysis. Only weak activities were left for both these two peaks at the 20 μmol/L dimercaprol analysis. A weak negative (19.4 min) and a broad negative peak (19.9–20.9 min) followed by one additional weak negative peak (21.2 min) for the anticoagulation activity in the venom-only analysis. No inhibition was observed at all by dimercaprol for the anticoagulation activity.

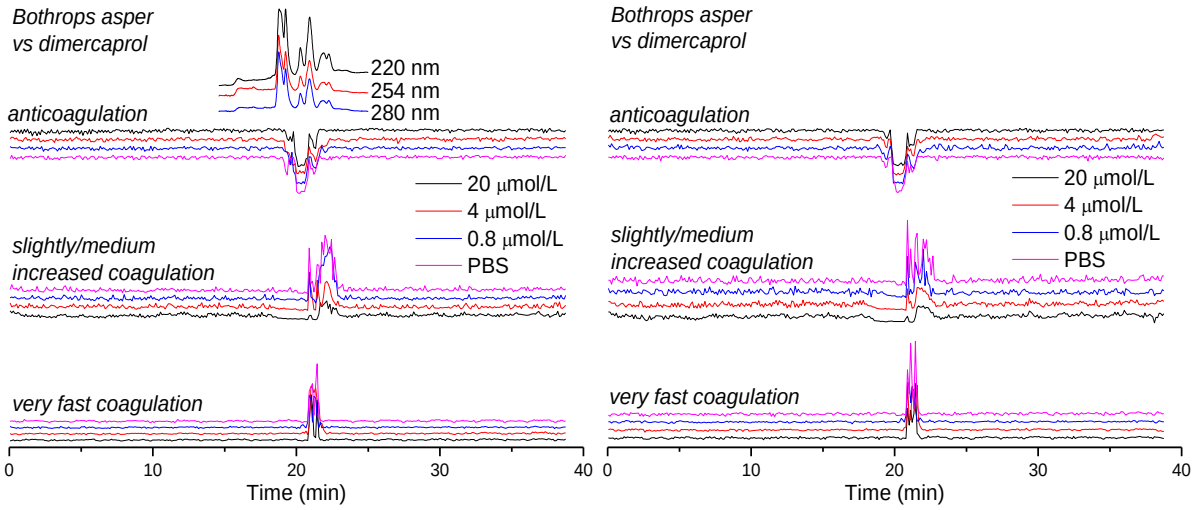

Figure S3 Reconstructed coagulation chromatograms in duplicate of nanofractionated *B. asper* venom toxins in presence of different concentrations of dimercaprol.

The reconstructed duplicate chromatograms showing inhibiting effects of varespladib, marimastat and dimercaprol on *B. jararaca* venom after nanofractionation are depicted in Figs. S4–S6.

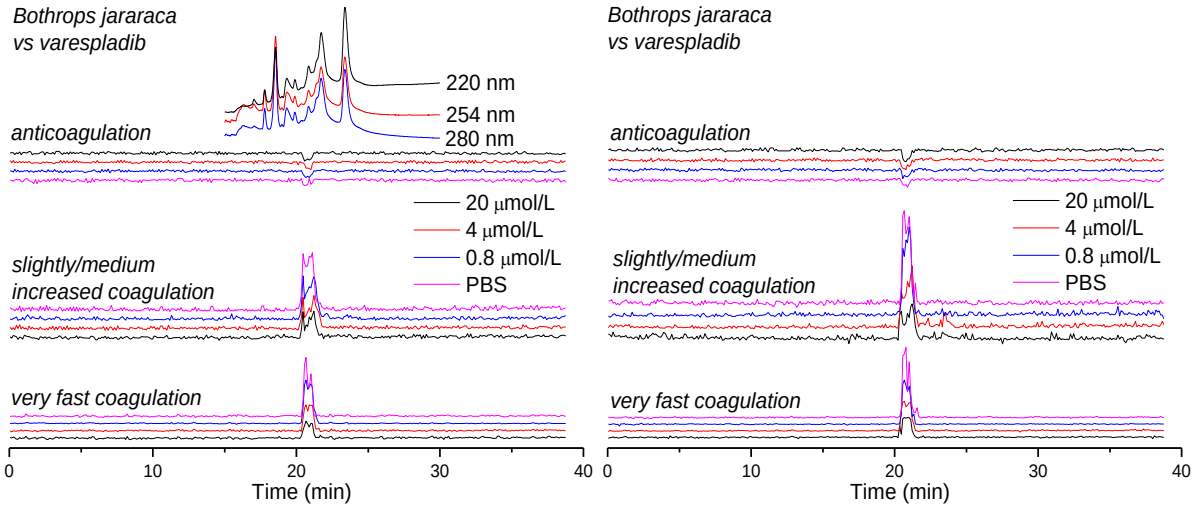

Figure S4 Reconstructed coagulation chromatograms in duplicate of nanofractionated *B. jararaca* venom toxins in presence of different concentrations of varespladib.

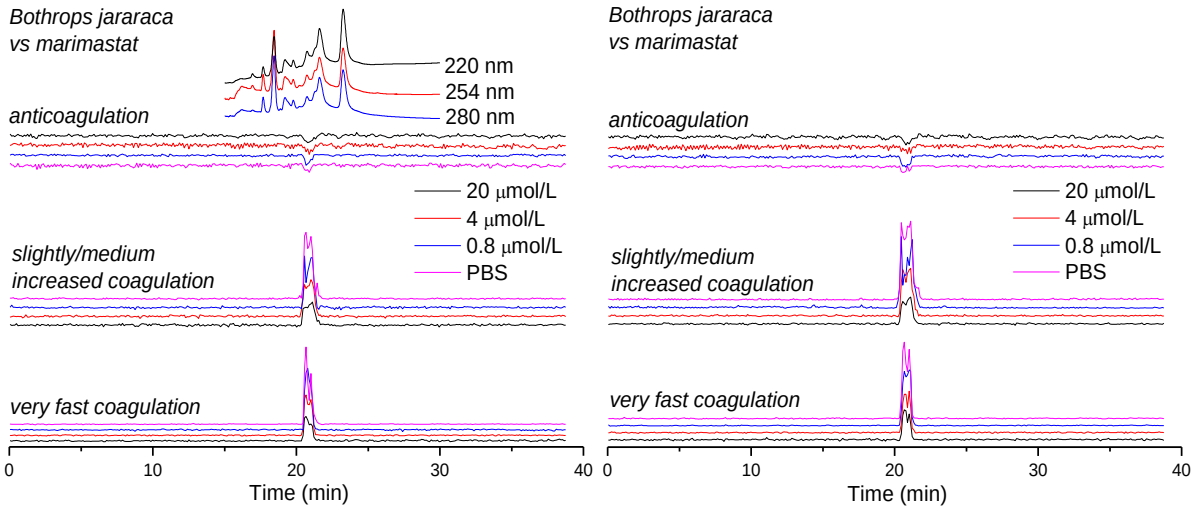

Figure S5 Reconstructed coagulation chromatograms in duplicate of nanofractionated *B. jararaca* venom toxins in presence of different concentrations of marimastat.

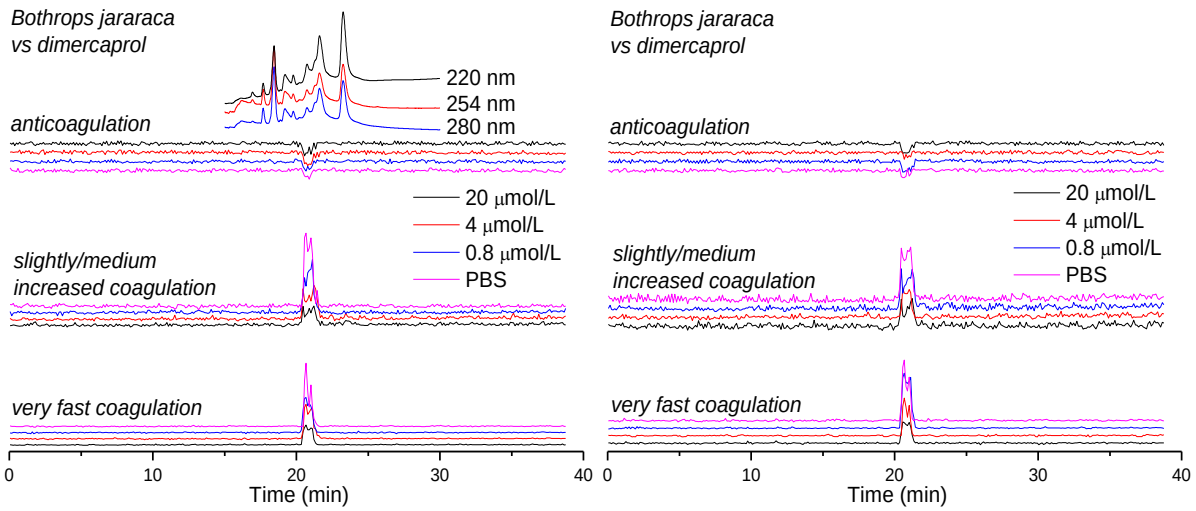

Figure S6 Reconstructed coagulation chromatograms in duplicate of nanofractionated *B. jararaca* venom toxins in presence of different concentrations of dimercaprol.

S2. Inhibiting effects of varespladib, marimastat and dimercaprol on venoms from *Calloselasma* and *Deinagkistrodon* species

The duplicate plasma coagulation bioassay results for *C. rhodostoma* and *D. acutus* venoms inhibited by varespladib, marimastat and dimercaprol are shown in Figs. S7–S12. Fig. S7 shows the bioassay chromatograms for nanofractionated *C. rhodostoma* venom toxins in presence of varespladib. In the venom-only analysis, two sharp positive peaks (19.7 and 20.5 min) are shown in the very fast coagulation chromatogram; a sharp positive peak (19.7 min) together with a broad positive peak (20.3–21.6 min) which consisted of three closely eluting peaks is shown for the slightly/medium increased coagulation activity. The activity of these positive peaks was reduced by increasing varespladib concentrations, but none of them were fully neutralised with the highest varespladib concentration tested (20 μmol/L). Two negative peaks (19.8 and 20.1 min) were observed for the anticoagulation activity in the venom-only analysis. The first negative peak (19.8 min) was fully neutralised by varespladib at the concentration of 4 μmol/L, while the activity of the second negative peak (20.1 min) was not significantly affected by varespladib.

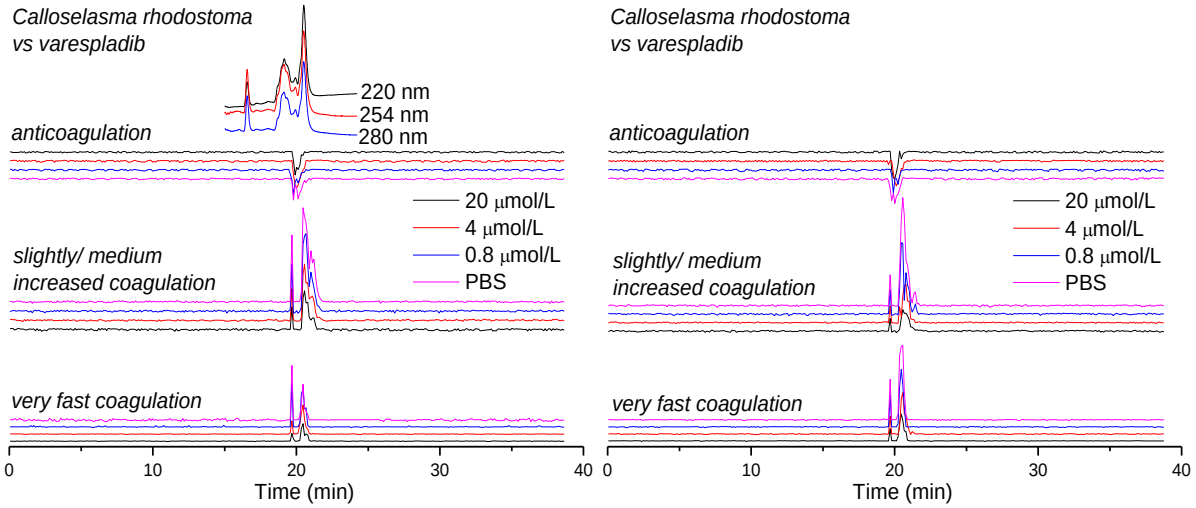

Figure S7 Reconstructed coagulation chromatograms in duplicate of nanofractionated *C. rhodostoma* venom toxins in presence of different concentrations of varespladib.

The inhibiting effect of marimastat on *C. rhodostoma* venom is shown in Fig. S8. In the venom-only analysis, two sharp positive peaks (19.7 and 20.3 min) are shown for both the very fast coagulation activity and the slightly/medium increased coagulation activity, and a moderate negative peak (19.9 min) was observed for the anticoagulation activity. Both the anticoagulation activity and the later eluting positive peak (20.3 min) in the very fast coagulation activity chromatograph were not affected significantly by marimastat. The first sharp positive peak (19.7 min) in both the very fast coagulation activity and the slightly/medium increased coagulation activity chromatogram reduced by increasing marimastat concentrations, but full inactivation was not achieved at even the highest marimastat concentration tested (20 μmol/L). The second intense positive peak (20.3 min) for the slightly/medium increased coagulation activity was not inhibited by 0.8 μmol/L marimastat. The intensity of this peak (20.3 min) decreased significantly at the 4 μmol/L marimastat analysis, although the peak (20.3 min) showed about the same intensity for the test with a marimastat concentration of 20 μmol/L. A possible explanation for this observation is that this peak (20.3 min) consisted of at least two co-eluting pro-coagulant toxins of which one could be neutralised by 4 μmol/L marimastat, while the other one was not inhibited at all.

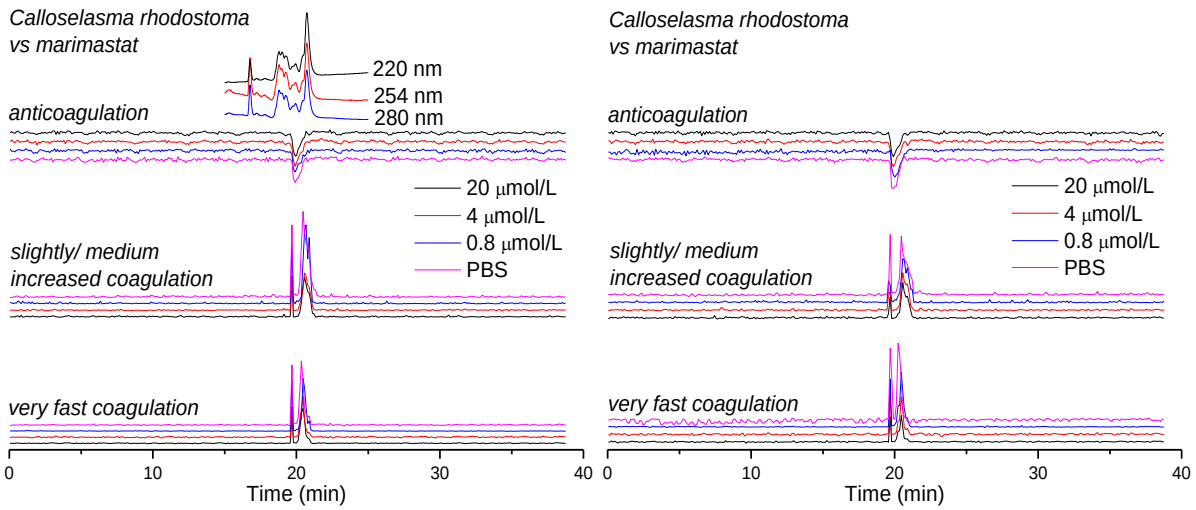

Figure S8 Reconstructed coagulation chromatograms in duplicate of nanofractionated *C. rhodostoma* venom toxins in presence of different concentrations of marimastat.

The inhibiting effect of dimercaprol on *C. rhodostoma* venom is shown in Fig. S9. In the venom-only analysis, a sharp positive peak (19.7 min) followed a relative broad positive peak (20.3–23.0 min) was observed for both the very fast coagulation activity and the slightly/medium increased coagulation activity, and a moderate negative peak (19.9 min) was observed for the anticoagulation activity. Both procoagulation activities were inhibited by dimercaprol in a very limited extent at 4 μmol/L, but no further inhibition was observed by further increasing the dimercaprol concentration to 20 μmol/L. The anticoagulation activity was not influenced by dimercaprol at any tested concentration.

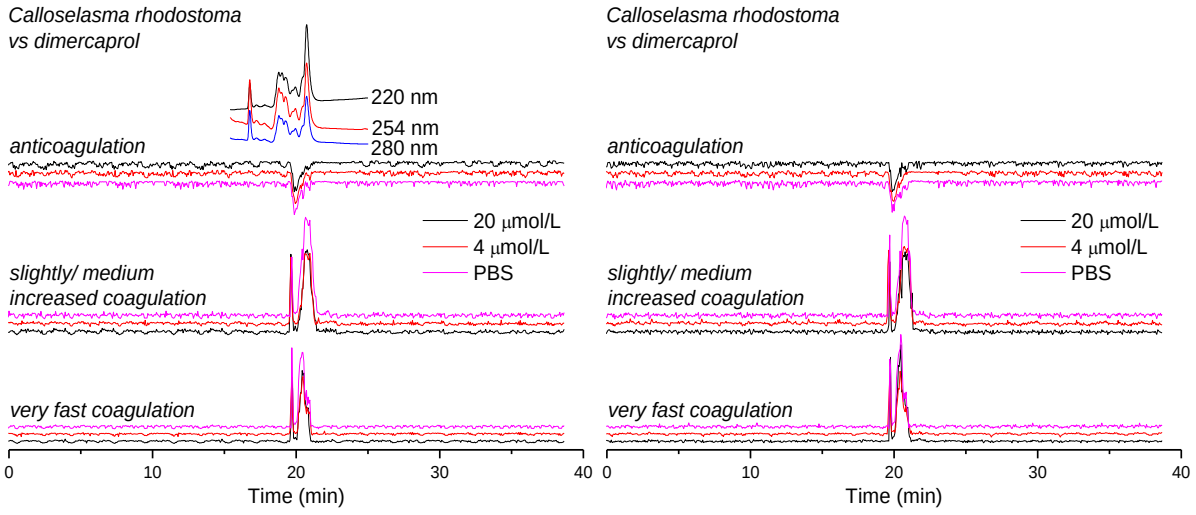

Figure S9 Reconstructed coagulation chromatograms in duplicate of nanofractionated *C. rhodostoma* venom toxins in presence of different concentrations of dimercaprol.

Figs. S10–S12 show the bioassay chromatograms for nanofractionated *D. acutus* venom toxins in presence of different concentrations of varespladib, marimastat and dimercaprol. The effect of varespladib on *D. acutus* venom is shown in Fig. S10. Two intense positive

peaks (21.2 and 21.7 min) together with a weak positive peak (22.3 min) are shown for the very fast coagulation activity in the venom-only analysis. The intensity of these peaks was decreased with increasing varespladib concentrations of which the weak peak (22.3 min) was fully inhibited at 4 µmol/L varespladib. After increasing the varespladib concentration to 20 µmol/L, only a weak positive shoulder peak (21.0–21.9 min) was left. In the slightly/medium increased coagulation activity chromatogram, a sharp positive peak (21.2 min) followed by a co-eluting broad positive peak (21.3–23.4 min, probably consisting of several closely co-eluting venom toxins) was observed. Both were reduced by increasing the varespladib concentration, the majority of the signal of the broad positive peak (21.3–23.4 min) was neutralised by 20 µmol/L varespladib. Only a small positive peak (21.0–22.0 min) was left in the 20 µmol/L varespladib analysis. A sharp intensive negative peak (20.7 min) followed by a sharp weak negative peak (21.5 min) were found for the anticoagulation activity in the venom-only analysis. The intensive negative peak (20.7 min) was reduced by increasing the varespladib concentration and was fully neutralised at 4 µmol/L varespladib. In contrast, the weak negative peak (21.5 min) could not be neutralised at all by varespladib.

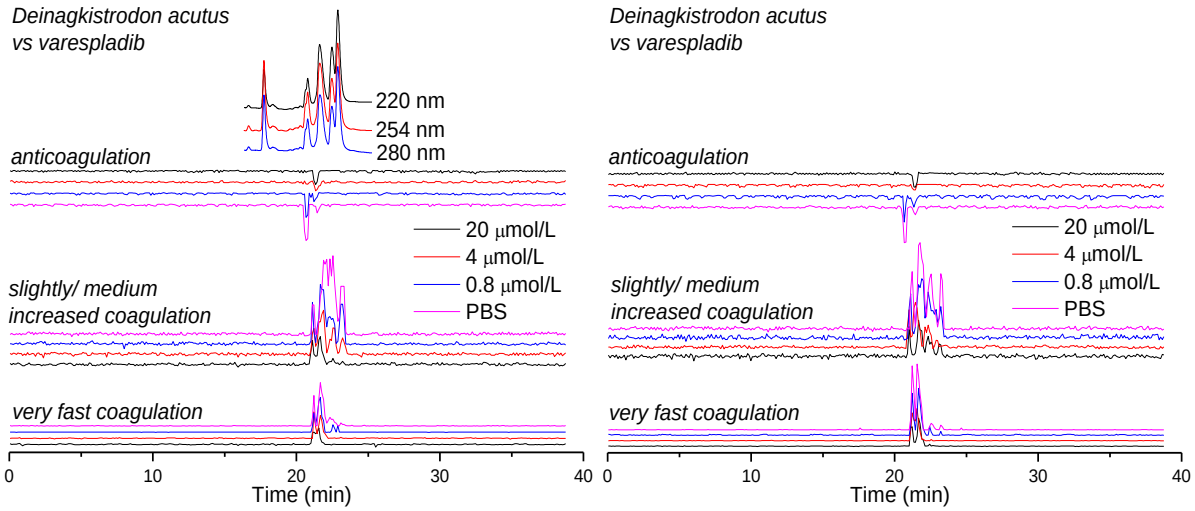

Figure S10 Reconstructed coagulation chromatograms in duplicate of nanofractionated *D. acutus* venom toxins in presence of different concentrations of varespladib.

The effect of marimastat on *D. acutus* venom is shown in Fig. S11. An intense positive peak (21.1–22.1 min) followed by a very weak positive peak (22.9 min) is shown in the very fast coagulation activity chromatogram of the venom-only analysis. The intensity of both positive peaks did not change with 0.8 µmol/L marimastat, while increasing the marimastat concentration to 4 µmol/L resulted in a slightly sharper for the intense positive peak (21.1–22.1 min) and inactivation of the weak positive peak (22.9 min). By further increasing the marimastat concentration to 20 µmol/L, no significant difference was observed compared

to the chromatogram from the 4 μmol/L marimastat analysis. Probably in this case, several toxins co-eluted of which some could be neutralised while others could not. For the slightly/medium increased coagulation activity, several moderately positive peaks (20.9–240.3 min) eluting close together and were observed as non–baseline separated peaks in the venom-only analysis. These peaks had a limited influence of marimastat and only the marimastat concentration of 20 μmol/L partly neutralised the later eluting peaks. For the anticoagulation activity, there was a sharp and intense negative peak (20.5 min) in the chromatogram which was not inhibited at all by marimastat.

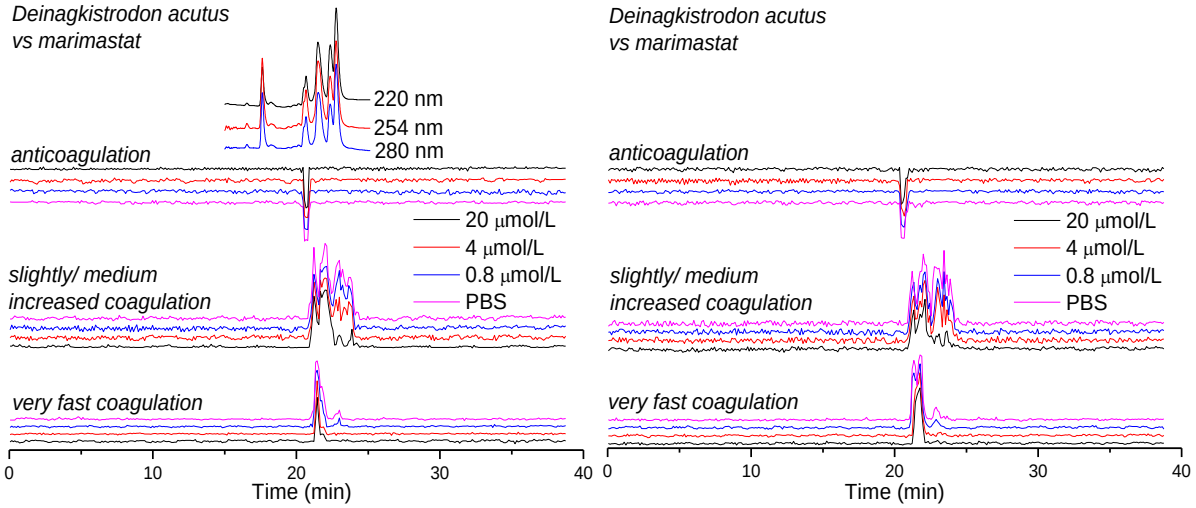

Figure S11 Reconstructed coagulation chromatograms in duplicate of nanofractionated *D. acutus* venom toxins in presence of different concentrations of marimastat.

The effect of dimercaprol on *D. acutus* venom is shown in Fig. S12. A positive peak (21.6–23.3 min) was observed in both the very fast coagulation chromatogram and the slightly/medium increased coagulation chromatogram. They we concentration-dependently inhibited by dimercaprol, however, no full inactivation was achieved at the highest concentration tested (20 μmol/L). A potent sharp negative peak (20.7 min) was shown in the anticoagulation activity, which was not influenced at all by dimercaprol.

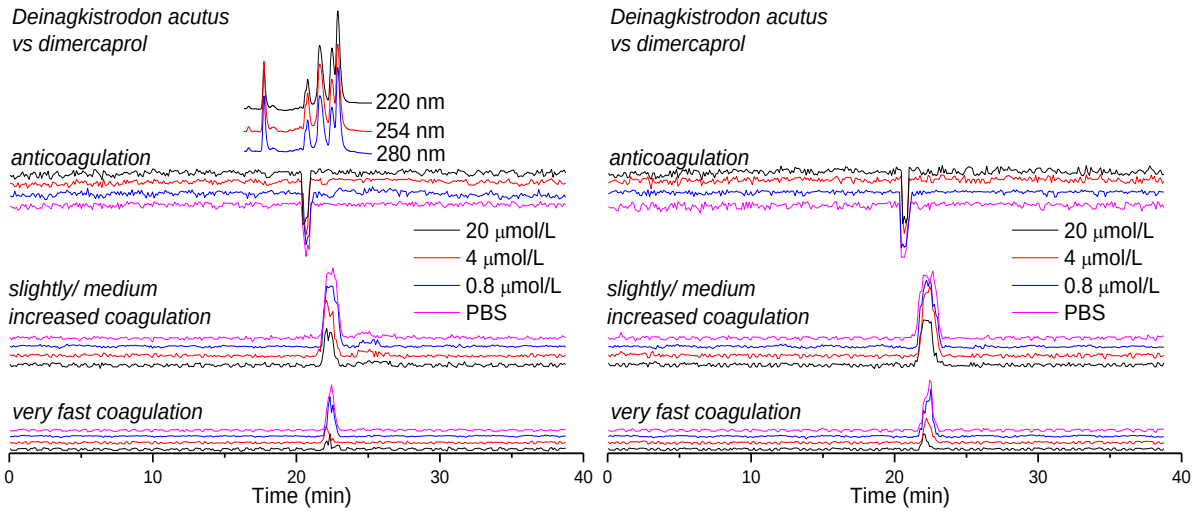

Figure S12 Reconstructed coagulation chromatograms in duplicate of nanofractionated *D. acutus* venom toxins in presence of different concentrations of dimercaprol.

S3. Inhibitory efficacy of small molecule inhibitors and metal chelators on venom toxins that identified and given in Table 1.

In *B. asper* venom, proteomics results indicated the toxins PA2H2_BOTAS, PA2HA_BOTAS, PA2H3_BOTAS, PA2B3_BOTAS, PA2A2_BOTAS and VM2_BOTAS as tentative anticoagulant toxins, while VSPL_BOTAS, VM1B1_BOTAS and SLA_BOTAS were identified as procoagulant toxins. Of these toxins, PA2H2_BOTAS was neutralised by 0.8 μmol/L varespladib, while VM2_BOTAS, a SVMP co-eluting with PLA_2s, was not neutralised by the inhibitors tested. The toxins PA2HA_BOTAS, PA2H3_BOTAS, PA2B3_BOTAS and PA2A2_BOTAS were assigned to anticoagulation activities that were partly inhibited by 20 μmol/L varespladib. Most procoagulant toxins partly co-eluted in the activity peaks and could only partly be inhibited by varespladib, marimastat and dimercaprol. As such, it could not be determined which ones were the active ones and from these which ones were (partly) inhibited. The LC–MS data this way provided clear additional confirmation, next to the proteomics data, of the bioactive PLA_2s involved in the anticoagulation toxicities measured.

In *B. jararaca* venom, the procoagulation activity was inhibited in a dose–response manner by varespladib, marimastat and dimercaprol, but full neutralisation was not achieved by any of the tested inhibitors. The toxins VSPA_BOTJA, VSP1_BOTJA, VSP2_BOTJA, VSP12_BOTJA, VSP14_BOTJA and VSP20_BOTJA were identified as procoagulants. These procoagulant toxins partly co-eluted in the activity peaks and could only partly be

inhibited by varespladib, marimastat and dimercaprol. As such, it could not be determined which ones were the active ones and from these which ones were (partly) inhibited. No toxins were identified from Mascot searches for the weak anticoagulation activity observed.

In *C. rhodostoma* venom, the toxins PA2BD_CALRH, PA2AB_CALRH, VSPF1_CALRH, SLEA_CALRH and SLEB_CALRH were assigned as candidates responsible for the observed anticoagulation activity. The toxins VSPF2_CALRH, SLYA_CALRH and SLYB_CALRH were tentatively assigned to cause the procoagulation activity. The toxin PA2BD_CALRH has been reported to display calcium independent myotoxicity by Tsai et al.[1] in the UniprotKB. The anticoagulation activities observed were neither inhibited by varespladib nor by marimastat. The procoagulation activities were partly inhibited both by varespladib and by marimastat, however no full inhibition was observed.

In *D. acutus* venom, the toxins PA2A_DEIAC and SL_DEIAC were tentatively identified as being responsible for the anticoagulation activity observed, which could be neutralised by 4 μmol/L varespladib but could not be neutralised by marimastat. The toxins VSP1_DEIAC, VSPA_DEIAC, SLCB_DEIAC, VM1AC_DEIAC, VM11_DEIAC, VM1H5_DEIAC and VM3AK_DEIAC were tentatively assigned to the procoagulation activity observed, which was only partly inhibited by varespladib, marimastat or dimercaprol. The procoagulant toxins VM3A2_DEIAC and VM3AH_DEIAC were both fully inhibited by 4 μmol/L marimastat and by 20 μmol/L varespladib.

Reference

1. Tsai IH, Wang YM, Au LC, Ko TP, Chen YH, Chu YF. Phospholipases A_2 from *Callosellasma rhodostoma* venom gland: cloning and sequencing of 10 of the cDNAs, three-dimensional modelling and chemical modification of the major isozyme. *Eur J Biochem* 2000;**267**:6684–91.