Novel haemoglobin modifying activity discovered through screening T-VDA^{CV}

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Abstract; Venoms, particularly those from snakes, have long been known to affect haemostasis in prey species and human victims. Previously discovered venom activities include; thrombosis, haemolysis, hypotension, oedema and haemorrhage. However, knowledge of the direct effects of venoms on mammalian haemoglobin are very rare in the literature and restricted to saliva from haemtophagic animals. To understand if this is due to a lack of knowledge or a true lack of biological effect, the authors set about screening a Cardiovascular – Venom Discovery Array (T-VDA^{CV}, Venomtech Ltd) for effects on haemostasis, using a simple 96 and 384 well assay with the FLUOstar Galaxy (BMG Labtech). Briefly, lysed whole blood was dosed with venom from the T-VDA^{CV} and absorbance (A_{595} and A_{492}) measured kinetically over 16.5 hours. The results presented within show discovery of a novel activity of venoms, specifically from the black necked spitting cobra (*Naja nigricollis*) on mammalian haemoglobin. Thus screening of the T-VDA^{CV} enables discovery of new activities from venoms and enhances the discovery of new biologicals.

Figure 1. Screening for Potential Haemoglobinase Activity from T-VDA^{CV}

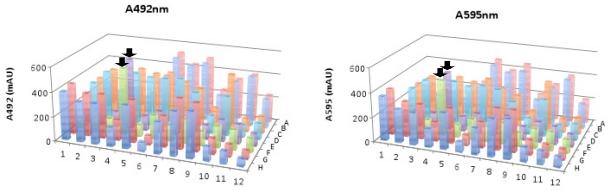
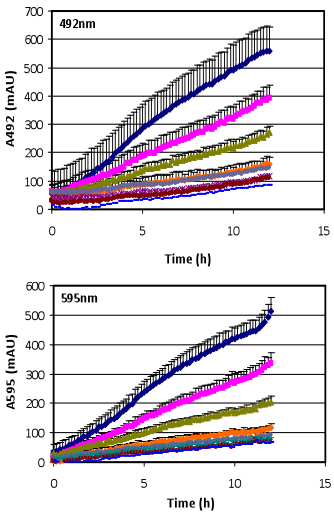


Figure 2. Concentration-dependent Effect of *Naja nigricollis* Venom on Lysed Blood

For the experiment, *Naja nigricollis* venom was diluted in PBS to give final assay concentrations of between 1/200 and 1/25600. Venom was aliquoted into a 96 well clear plate (50µl/well) and 50µl of lysed ovine blood was added to each well. Plates were incubated at 30ºC and absorbance at 492nm and 595nm read at 10 minute intervals using a BMG Labtech FLUOstar plate reader. Graphs show the increase in absorbance over time for each wavelength, error bars represent standard deviation (n=6).

There is a clear time-dependent increase in absorbance at both 492nm and 595nm and this was dependent on venom concentration. This haemoglobin-modifying activity was lost following heat-treatment of venom at 65ºC or 95ºC suggesting an enzyme-mediated effect (data not shown.) Gel-based assays did not show any evidence of proteolytic degradation of haemoglobin by *N. nigricollis* venom (data not shown).



Conclusions

- *Naja nigricollis* venom possesses potentially novel haemoglobin modifying activity first identified through screening of the T-VDA^{CV} venom array
- Further characterisation suggests that this activity is enzyme-mediated and is catalysing the oxidation of haemoglobin to methaemoglobin
- This work shows that Venomtech T-VDAs are a rich source s of novel biological activities

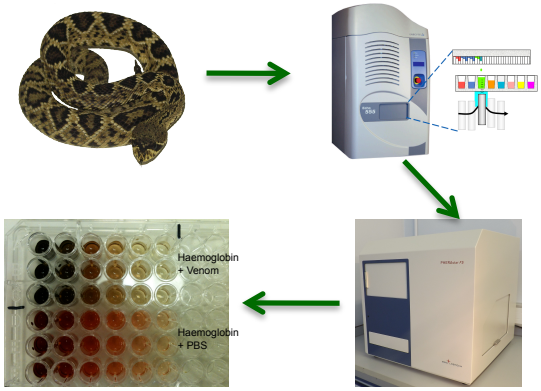
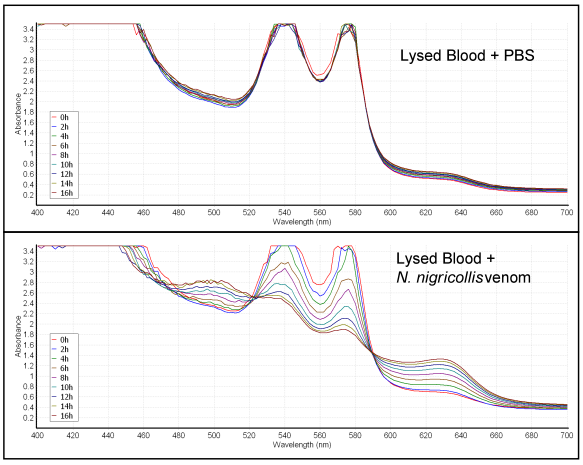


Figure 1 method, 46 duplicate venom samples from the T-VDA^{CV} venom array were diluted in 1x PBS to 1/1000 and 50µl of each venom sample was mixed with 50µl of lysed ovine blood (Harlan) in a clear-base 96 well plate (Greiner). Wells G12 and H12 are controls without venom. Plates were incubated at ambient temperature for 16.5 hours and absorbance at 492nm and 595nm was read at 10 minute intervals using a BMG Labtech FLUOstar plate reader. The above charts (left) show increase in absorbance at each wavelength over 16.5 hours indicating that while many of the Crotalinae and Viperinae species represented in the array show activity in this assay only one Elapidae species, namely *Naja nigricollis* (black-necked spitting cobra), shows activity (black arrows). Venoms were also acoustically dispensed from 384PP and 384LDV Echo qualified plates (Labcyte Inc).

Figure 3. Changes in Haemoglobin Absorbance Spectrum Following Incubation with *Naja nigricollis* venom



Lysed ovine blood (10ml) diluted 1 in 2 in phosphate-buffered saline was incubated with an equal volume of either 1x PBS or a 1 in 10 dilution of *Naja nigricollis* venom in a 384 well plate. Plates were incubated at 30ºC in a BMG Labtech PHERAStar platereader and the absorbance spectrum between 400nm and 700nm was read at 10 minute intervals over 16.5 hours. In the presence of venom, there was a time-dependent decrease in absorbance maxima at 540nm and 570nm with a corresponding time-dependent increase in absorbance maxima at 500nm and 630nm. The absorbance spectra remained unchanged in the presence of 1x PBS alone. The loss of oxygenated haemoglobin peaks at 540nm and 570nm and the increase in the peaks at 500nm and 630nm suggest that venom is promoting the oxidation of oxyhaemoglobin to methaemoglobin. The presence of dithiothreitol at 1mM or 5mM did not prevent the venom-mediated oxidation of haemoglobin, providing further evidence that the modification of haemoglobin by *N. nigricollis* venom is an enzyme-mediated effect (data not shown).

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