Exploration of the Effect of Protease Inhibitor Activity on Snake Venoms

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Abstract

Envenomation by snakes is a worldwide health crisis. Anti-venom, the current treatment standard, is a costly and imperfect treatment. Without proper ID of the snake species, the treatment provider is guessing at which anti-venom to use. Many snakes are also able to control venom release as they age, so not every bite by a venomous snake is envenoming. Treatment by anti-venom has the potential of severe side effects so treating a snake bite that hasn't resulted in envenoming could cause more problems than withholding treatment.

Can protease inhibitors be used to block or decrease the gelatinase activity of snake venom?

Established baseline venom activity and dose dependence of inhibition.

Activity was measured with a fluorescein-labeled gelatin. N-Isobutyl-N-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid, NNGH, is the enzyme inhibitor that was used.

In dose-response experiments, there is significant gelatinase activity and over 50% inhibition by NNGH in *Agkistrodon contortrix* and complete inhibition in *Crotalus atrox*. We also see 50% inhibition of *Cerastes cerastes* venom by NNGH but the species has near half the initial gelatinase activity of *C. atrox.*

These results provide substantial support that venoms of *Crotalinae* species, pit vipers, are inhibited by the protease inhibitor NNGH, supporting future research endeavors.

Introduction

There are an estimated 5 million snakebites each year worldwide, with nearly 3 million of them involving venomous snakes. Currently, the treatment for envenomation is species-specific anti-venom and supportive care. Anti-venom treatment comes with several intricacies, including species identification, cost, and occupational hazards for manufacturers (Snakebite envenoming). For these reasons, the development of an alternative treatment is imperative. Pathology of some toxins can be treated strictly by way of supportive care, such as neurotoxins, while others cannot, i.e. proteolytic toxins (Price, 2015). Protease inhibitors have been suggested as a potential treatment that could allow for a more generalized treatment to be used in conjunction with or as an alternative to anti-venom. Previous studies have suggested that the venom matrix metallopeptidases, or MMPs, do not follow the classical models of inhibition, and we are seeking to verify and extend those findings.

Methods

The methods of these experiments have been published previously (Price 2015). Venoms and the inhibitor were prepared in bulk as follows and stored at -80ºF for consistency. The crystalline venoms were dissolved in saline at 2 mg/mL and the NNGH was dissolved in DMSO at 10 mM. Venom from three species of snake was used.

The assays were performed by adding any experimental reactant to a 96 well low-binding microplate, followed by a fluorescein-labeled gelatin substrate. The assay reaction was then activated by adding the venom solution.

Various experiments were run to determine both assay optimization and protease inhibitor efficacy in inhibiting snake venom's MMPs. Analysis and figures were done with GraphPad Prism version 8.0.0

Data

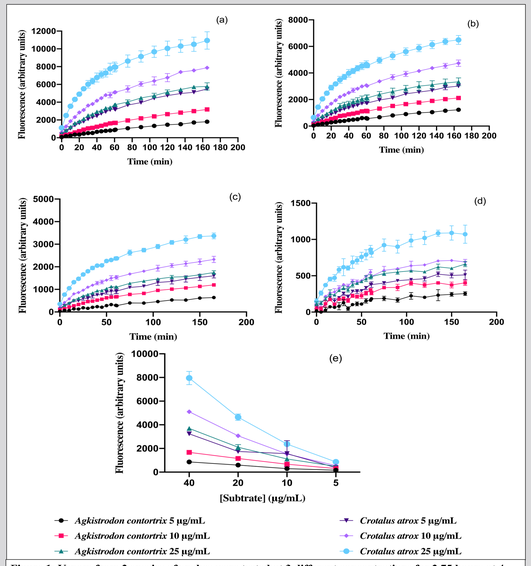

Figure 1: Venom from 2 species of snakes were tested at 3 different concentrations for 2.75 hours at 4 different substrate concentrations: a) 40 µg/mL, b) 20 µg/mL, c) 10 µg/mL, and d) 5 µg/mL. e) A 60 minutes plot comparing the activity of each venom concentration within each substrate concentration.

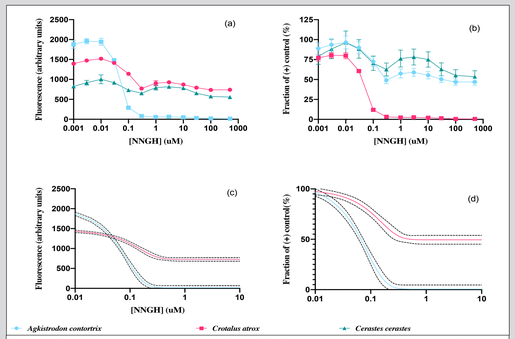

Figure 2: Dose responses to the protease inhibitor NNGH at 60 minutes, a) The response is directly shown with venom from 3 species. b) the transformation of the response seen in 1a to express the ratio of activity after inhibition to the uninhibited control. c) The response shown as interpolation with 95% confidence interval with *C. atrox* and *A. contortrix*, d) the transformation of the response seen in 1c to express the ratio of activity after inhibition to the uninhibited control.

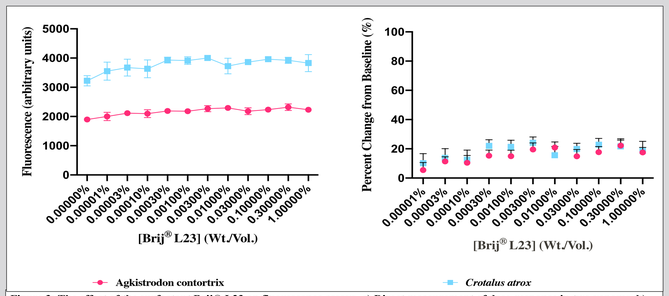

Figure 3: The effect of the surfactant Brij® L23 on fluorescence assays. a) Direct measurement of dose-response in two venoms. b) Transformation of data to show the percent change relative to the baseline of no surfactant.

Results

C. atrox and *A. contortrix* both reveal a degree of gelatinase activity albeit *C. atrox* being more active than *A. contortrix* under each comparable experimental condition (Figure 1). When incubated in the presence of NNGH, there was a decrease in protease activity within all venoms tested (Figure 2). *C. atrox* had 0.5% of the activity of the uninhibited venom, while *A. contortrix* and *C. cerastes* had 47% and 53.5% respectively of their uninhibited activity (Figure 2b, 2d).

In the surfactant experiment, there was a statistically significant increase in activity with most concentrations of the surfactant (Figure 3). Two-way Anova of the data in Figure 3 provides a p-value of <0.0001.

Discussion

Figure 1 shows a multivariable assay that allowed for the optimization of future assays. To maintain optimal baseline activity between the two venoms it was decided to move forward with a *C. atrox* concentration of 10 µg/mL and an *A. contortrix* concentration of 20 µg/mL with a substrate concentration of 20 µg/mL.

In Figure 2, there is gelatinase activity and inhibition seen in all venoms. The gelatinase activity of *C. atrox* has a 99.5% inhibition, while *A. contortrix* has only a 53% inhibition. The activity and inhibition of *C. cerastes* were significantly lower than that of *C. atrox* but had a comparable NNGH inhibition to that of *A. contortrix*. So although *C. cerastes* venom had a nearly 50% inhibition, the use of NNGH alone appears questionable for clinical use due to low initial activity suggesting the *C. cerastes* venom uses alternate MMPs in conjunction with gelatinase.

The surfactant used in Figure 3 is suggestive that there is some microplate adhesion occurring, however, due to the inconsistency in the percent change, it was decided that the potential negative effects outweighed the binding effect that we experienced until further experimenting is completed.

This is supportive of protease inhibition as a plausible mechanism for the treatment of envenomation by some species either independently or in conjunction with anti-venom.

References

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Snakebite envenoming. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/snakebite-envenoming. Accessed July 9, 2019.

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