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Toxin synergism in snake venoms

Synergism between venom toxins exists for a range of snake species. Synergism can be derived from both intermolecular interactions and supramolecular interactions between venom components and can be the result of toxins targeting the same protein, biochemical pathway, or physiological process. Few simple systematic tools and methods for determining the presence of synergism exist, but include co-administration of venom components and assessment of Accumulated Toxicity Scores. A better understanding of how to investigate synergism in snake venoms may help unravel strategies for developing novel therapies against snakebite envenoming by elucidating mechanisms for toxicity and interactions between venom components.

Keywords: Toxin synergism, intermolecular synergism, supramolecular synergism, Toxicity Score, snake venom, snake toxins, Accumulated Toxicity Score.

1. Introduction

Producing and replenishing venom comes with a metabolic cost for snakes (Morgenstern and King, 2013). This metabolic cost can, however, be lowered through toxin synergism, which may allow a venom to reach high potency with less toxins. According to the Oxford Dictionaries, synergism is *"the interaction or cooperation of two or more organizations, substances, or other agents to produce a combined effect greater than the sum of their separate effects"* (http://www.oxforddictionaries.com/definition/english/synergy). Within toxinology, synergism arises when a venom displays a much higher toxicity than the sum of the toxicities for the individual venom components, which may possibly provide an evolutionary advantage. Toxin synergism has been studied and reported for snakes, spiders, and scorpions (Lauridsen et al., 2016, Strydom, 1976, Wullschleger et al., 1998, Chan et al., 1975, Lazarovici et al., 1984), whose venoms have evolved to subdue prey and deter predators and competitors (Barlow et al., 2009, King et al., 2011). Due to its

inherent complexity, synergism is difficult to study, and to the best of my knowledge, no common framework or method exists for determining the presence of synergism in a venom. Also, limited agreement on how to classify types of synergism and distinguish between synergism and auxiliary effects exists in the scientific literature. In this review, a distinction between synergism and auxiliary effects will be discussed, the different types of synergism that have been reported for snake venoms will be reviewed, and existing tools and methods for determining the presence of synergism in venoms and toxin mixtures will be presented. This review will thus help provide an overview of toxin synergism in snake venoms and provide guidance on how to study the phenomenon.

2. Synergism vs. auxiliary effects

The difference between toxin synergism and auxiliary effects could be viewed as the difference between potentiating and facilitating toxic effects in an envenomed victim. Auxiliary effects could thus be defined as effects of venom components that do not contribute directly to venom toxicity, but instead have other functions for supporting toxicity, such as modifying venom delivery, half-life, and distribution (Figure 1). As an example of auxiliary effects, adenosine is often found in snake venoms (Aird, 2002), where its vasodilatory effects may increase blood perfusion at the site of envenoming. This leads to an increased rate of systemic venom distribution in the victim, but not necessarily to an increase in venom toxicity. Also, hyaluronidases in snake venoms may primarily have a supportive function by increasing the distribution rate of venom components through degradation of hyaluronic acid in the extracellular matrix (Kemparaju and Girish, 2006). As an example, the toxicity of the venom of *Crotalus durissus terrificus* (South American rattlesnake) has been shown to be dependent on

facilitated distribution of crotoxin and other phospholipases A_2 (PLA_2s) by hyaluronidases with no toxicity themselves (Bordon et al., 2012). This shows that auxiliary effects may in some cases be crucial for the ability of a venom to exert its toxic functions, although the proteins responsible for the auxiliary effects do not increase the toxicity of any of the venom toxins *per se*. In contrast to these examples, synergistic effects directly contribute to increased toxicity, and the venom components that act synergistically usually have a toxic effect on the same or related physiological targets or pathways or interact with and potentiate the effects of a key toxin.

3. The different types of synergism occurring in snake venoms

Following the above, synergism is the phenomenon where two or more venom components interact directly or indirectly to potentiate toxicity to levels above the sum of their individual toxicities. From a molecular perspective, synergism may exist in two general forms:

(1) Intermolecular synergism, when two or more toxins interact with two or more targets on one or more (related) biological pathways, causing synergistically increased toxicity (Figure 2A, B).

(2) Supramolecular synergism, when two or more toxins interact with the same target in a synergistic manner (Figure 2C) or when two or more toxins associate and create a complex with increased toxicity (Figure 2D).

3.1 Intermolecular synergism

Intermolecular synergism may exist in several forms, where toxins may either act on different targets of a physiological process causing a combined synergistic effect

(Figure 2A) or different physiological processes, which subsequently affect another process further downstream in a synergistically enhanced manner (Figure 2B). An example of the first case is illustrated by the venom of *Dendroaspis polylepis* (black mamba), which has been shown to display synergism (Strydom, 1976). This synergism is possibly created by dendrotoxins exerting an excitatory effect on the neuromuscular system, combined with a rapid abrogation of neuromuscular function by α-neurotoxins (Laustsen et al., 2015A). Combined, these toxins cause a synergistic potent toxic effect leading to flaccid paralysis and respiratory failure in victims and prey. In contrast to these examples, synergistic behaviour is not observed for the neurotoxic venom of *Naja kaouthia* (monocled cobra), which may be due to the fact that the α-neurotoxins dominating the venom all target the same receptor (Tan et al., 2015, Laustsen et al., 2015B). Instead of causing synergistic action, the α-neurotoxins compete with each other for the target, causing additive, but not synergistic effects (Figure 2E). Similarly, additive effects have been reported for venom toxins from the elapids *hemachatus haemachatus, Naja nigricollis, N. melanoleuca, N. haje, N. nivea, D. polylepis,* and *D. jamesoni* (Viljoen and Botes, 1973).

An example of synergism arising through a combined effect of toxins targeting different pathways (Figure 2B) is reported by Chen and co-workers. Chen et al. suggest that the actions of L-amino acid oxidases combined with the actions of other venom components of *Daboia russelii* (Russell's viper) venom cause prolonged and enhanced bleeding in envenoming cases by this viper species (Chen et al., 2012). Other synergistically acting toxins affecting blood coagulation are known to exist. Prominent examples include the prothrombin activators from group B (Ca^{2+}-dependent metalloproteinases found in viper venoms) and the prothrombin activators group C (serine proteinases found exclusively in Australian elapids) (Kini et al., 2001). These

prothrombin activators exert their function via proteolytic activation of prothrombin to mature thrombin (Kini, 2005), which may combine with other toxin-induced coagulopathies causing synergistically enhanced toxicity.

Another example of toxin synergism is provided by the 'Asp49' and 'Lys49' myotoxins of *Bothrops asper* (Fer-de-lance), which combined cause synergistic myonecrotic effects *in vitro* and *in vivo* (Mora-Obando et al., 2014). These types of myotoxins have been shown to increase plasma membrane Ca^{2+} permeability, causing rapid cell death for myotubes (Cintra-Francischinelli et al., 2009), which is a scenario that is conceptually similar to Figure 2C. Synergism may also appear when toxins from different venoms are mixed. This is exemplified by the venoms of *Dienagkistrodon acutus* (sharp-nosed viper) and *Agkistrodon halys* (Siberian pit viper), which in combination produce synergistic procoagulation effects on dog plasma (Wei et al., 1996).

3.2 Supramolecular synergism

Supramolecular synergism may arise when toxins form complexes with synergistic effects via a mechanism conceptually similar to Figure 2D, in which several venom components combine to create a hyper-potentiated toxin. An example of such synergistic enhancement includes cytotoxins from different cobra species. Cobra cytotoxins have been shown to enhance PLA_2 activity through complex formation and deformation of cell membranes, causing cellular lysis due to hydrolysis of phospholipids (Gasanov et al., 1997, Gasanov et al., 2014). On a more toxin-specific level, Chaim-Matyas et al. (1995) showed that cytotoxin P4 from *Naja nigricollis* (black-necked spitting cobra) venom acted synergistically with various PLA_2s when cancer cell lines were subjected to these toxins *in vitro*, causing cell lysis. Similar

synergistic effects on cellular and subcellular membranes have been observed for a range of other cell types, including erythrocytes, liver mitochondria, and platelets (Condrea, 1974).

In contrast to these examples of synergism, some snake venoms have evolved to have different potent effects, which do not necessarily interact in a synergistic manner. Herrera et al. (2012) showed that the venom of *Oxyuranus scutellatus* (coastal taipan) displays strong presynaptic neurotoxicity at low doses, while the clinical manifestations at high doses include intravascular thrombosis due to the presence of prothrombin activators. Instead of displaying synergism, these two venom effects seem to constitute a dual strategy, where the two effects complement each other rather than enhance the toxicity of the counterpart (conceptually similar to Figure 2F). However, in addition to having non-synergistic toxic functions working in parallel, the venom of *O. scutellatus* also displays supramolecular synergism. This supramolecular synergism is observed for the presynaptic neurotoxin, taipoxin (Fohlman et al., 1976), which is assembled by three homologous phospholipase A_2 subunits (Montecucco and Rossetto, 2008), and the calcium channel inhibitor, taicatoxin, which is assembled by an 8 kDa α-neurotoxin-like peptide, a 16 kDa neurotoxic PLA_2, and four 7 kDa serine protease inhibitors through non-covalent bonding (Possani et al., 1992). Similar to taipoxin and taicatoxin, the presynaptic neurotoxin textilotoxin from another Australian elapid (*Pseudonaja textilis*, eastern brown snake) displays supramolecular synergism via oligomerization (see Figure 2D). Textilotoxin is assembled by five homologous PLA_2 subunits, whereby the toxicity of subunit A (Pearson et al., 1991) is hyper-potentiated (Montecucco and Rossetto, 2008). It has been suggested that the synergistic effects for such presynaptic neurotoxin complexes from elapids derive from their enhanced ability to localize in the synapse facilitated by non-toxic PLA_2 subunits (Rigoni et al., 2005). An equivalent

example is also present in viper venom. Crotoxin from the venom of the South American rattlesnake (*Crotalus durissus terrificus*) exists in different structural isoforms and is a heterodimeric toxin complex formed by an acidic PLA_2 (subunit A) devoid of toxicity and a basic PLA_2 (subunit B) of low toxicity (Bon et al., 1979, Faure and Bon, 1988). Following complexation, subunit A synergistically enhances the potency of subunit B (Choumet et al., 1999), causing its lethal neurotoxic effects (Bon et al., 1988) (see Figure 2D).

Supramolecular synergism has also been reported for the two three-finger toxins, hemextin A and hemextin B, from the African Ringhals cobra (*Hemachatus haemachatus*). These two toxins combine to form a hemextin AB complex, which inhibits the TF.VIIa complex formed between freshly exposed tissue factor and factor VIIa, thereby synergistically abrogating the formation of blood clots (Banerjee et al., 2005, Kini, 2006, Kini, 2011) (conceptually similar to Figure 2D). Opposite procoagulant effects are observed for the prothrombin activator, Pseutarin C, from *P. textilis* venom, which is formed by dimerization of an enzymatic and a non-enzymatic subunit (Rao and Kini, 2002), highlighting the diverse range of synergistic effects that can be achieved through supramolecular interactions.

Another example of supramolecular synergism is likely to exist for the venom of *D. jamesoni* (Jameson's mamba). In this venom, the component S2C4 with relatively low toxicity interacts with angusticeps-type toxins in a synergistic manner when both toxins are co-administered *in vivo* (Joubert and Taljaard, 1979). In comparison, Strydom and Botes (1970) showed that separated venom fractions from the venom of the related *D. angusticeps* (Eastern green mamba) were devoid of lethality after 48 hours when administered alone, whereas the whole venom was lethal within 10 minutes when administered at a similar dose. Finally, non-toxic Kunitz-type inhibitors have been

shown to interact with ammodytoxins in the venom of *Vipera ammodytes ammodytes* (Horned viper) (Brgles et al., 2014) and PLA_2s in the venom of *Micrurus tener* (Texas coral snake) in order to create complexes of increased toxicity (Bohlen et al., 2011, Olivera and Teichert, 2011). A wealth of other examples of how venom components combine to create toxins with potent effects occur in Nature, and a comprehensive review on protein complexes in snake venoms can be found here (Doley and Kini, 2009).

4. Determining the presence of synergism

Few standardized methods are used to determine the presence of synergism between toxins in venoms. However, in the scientific literature, two fundamentally different approaches have been reported. One focuses on the observed toxicity when selected components are co-administered *in vitro* or *in vivo*, whereas the other is based on a toxicovenomic approach (Calvete and Lomonte, 2015) involving an assessment of toxicities and abundancies of all venom components.

4.1 Co-administration of venom components

An example provided by Strydom involves the co-administration of two venom fractions in different doses and comparing the observed toxicities with the toxicity observed when each component is administered individually. In his work, he demonstrated that fractions F and I from the venom of *D. polylepis* combined showed significantly increased toxicity, despite only having low toxicities on an individual basis (Strydom, 1976). The author (2015A) employed a similar method for detection of synergism between toxins from *D. polylepis*. In this method, a constant (sub-lethal) dose

of a single venom component is co-administered, while the LD_{50} of another venom component is determined. If the LD_{50} of the second component is significantly lowered by the presence of the sub-lethal dose of the first component, synergism may be present. We were however not able to detect synergism between short neurotoxin 1 and dendrotoxins in the venom of *D. polylepis* following this method (Laustsen et al., 2015A). However, in later work performed on the venom of *D. angusticeps*, synergism was detected by this method between components present in fractions 4-12 of the venom (Lauridsen et al., 2016), supporting the previous observation by Joubert and Taljaard (1979).

Co-administration experiments have the benefit that they can elucidate direct synergistic interactions between selected components. However, the drawback of such experiments is that they require laborious experimentation and optimization in order to yield useful results. When synergism is investigated using an *in vivo* test, this disadvantage also comes with a concern of how many experimental animals it is ethical to employ (and thus sacrifice) in such studies.

4.2 Accumulated Toxicity Score

Another approach for establishing the presence of synergism in venoms involves a more holistic approach based on the Toxicity Score. The Toxicity Score for a given toxin is calculated by dividing the lethality (LD_{50}) of a toxin with its relative abundance in the venom from which the toxin is derived (Laustsen et al., 2015C). The Toxicity Scores of different toxins in a venom can then be used to index the toxins according to their relative importance in an envenoming case, which may be useful for determining which toxins are essential to neutralize with antivenom, and which are not. In addition to its usefulness in determining which toxins are the medically most important in a venom,

the use of the Toxicity Score for establishing whether a venom displays synergism has recently been reported (Lauridsen et al., 2016, Laustsen et al., 2015C). In these cases, it was shown that the Toxicity Scores of the whole venoms of *Dendroaspis angusticeps* and *Aipysurus laevis* were much larger than the sum of the Toxicity Scores of the individual toxins, indicating that the toxins present in these venoms act in a synergistic manner. The presence of synergism can thus be determined, when the Toxicity Score of the whole venom is significantly larger than the sum of the Toxicity Scores for individual toxins (or venom fractions), termed the Accumulated Toxicity Score:

$$Toxicity\ Score_{Whole\ venom} > \sum_{Toxin\ 1}^{Toxin\ n} Toxicity\ Score_{Toxin\ X} \qquad \text{(I)}$$

Examples of how the Accumulated Toxicity Score can be employed to determine the presence or absence of synergism are provided in Table 1.

Due to limitations on what type of toxins that can be assessed using the Toxicity Score, this approach can only be readily employed for determining the presence of synergism in venoms from elapid snake species and where toxic effects culminate in lethality. The approach applies well to these venoms, since they are lethal, and since their toxins are generally not denatured during isolation and purification (Laustsen et al., 2015C). For other species (such as viperid snakes, spiders, centipedes, jellyfish etc.) the approach is compromised by the fact that the severity of envenoming cannot purely be judged by lethality, but also has to take account of other toxic effects, such as necrosis, coagulopathy, and systemic hemorrhage (Warrell, 2010), and since many of the medically relevant toxins (such as metalloproteinases) are easily denatured during their isolation (Laustsen et al., 2015C). Additionally, this simple approach may potentially also be useful for scorpion venoms, which are generally characterized by

being neurotoxic (Goyffon and Tournier, 2014), but it is unlikely to be useful for venoms from various other species, whose toxins are either easily denatured (jellyfish) (Endean, 1987) or whose venoms are devoid of lethal effects in relevant model animals (centipedes and most spiders) (Undheim et al., 2014, Sannaningaiah et al., 2014).

A drawback of using the Accumulated Toxicity Score for determining the presence of synergism compared to the approach involving co-administration of individual venom components is that it does not specify between which components synergism exists. Combined, the initial use of the Accumulated Toxicity Score may, however, unveil if a venom displays synergism, which can then be followed up with co-administration approaches in the relevant cases to determine the exact components responsible for the observed synergism.

5. Conclusion

Toxin synergism is a fascinating phenomenon, which has been shown to occur in many different snake venoms. The phenomenon is still poorly investigated and understood due to its inherent complexity. The importance of being able to determine the presence of synergism and having a mechanistic model for how venoms exert their toxic effects is, however, becoming increasingly more important driven by a trend in antivenom development where biotechnological approaches are being employed to identify specific antitoxins targeting individual toxins of medical importance (Roncolato et al., 2015, Richard et al., 2013, Chavanayarn et al., 2012, Laustsen et al., 2016A, Laustsen et al., 2016B, Carmo et al., 2015). Therefore, when selecting which toxin targets to focus on, it is important to focus both on toxins that are medically relevant on an individual basis (Laustsen et al., 2015C) and on potential non-toxic venom components, which may enhance the effect of other components through synergism (Lauridsen et al., 2016). The

current tools and methods used for assessing toxin synergism in snake venoms are few, but may be utilized to obtain a better understanding of how to develop novel snakebite envenoming therapies targeting key toxins involved in synergistically enhanced toxicity.

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Conflict of interest

The author declares that he has no conflicts of interest concerning this manuscript.

Table 1. Overview of Toxicity Scores for toxins (venom fractions) and whole venoms from all reported toxicovenomic studies.

Species	**Toxicity Score (whole venom)**		**Accumulated Toxicity Score***	**Synergism**	**Reference**
Dendroaspis polylepis	147.1	>	127.8	Yes	Laustsen et al., 2015A
Dendroaspis angusticeps	117.6	>	62.6	Yes	Lauridsen et al., 2016
Naja kaouthia	423.2	≈	419.7	No	Laustsen et al., 2015B
Aipysurus laevis	676	>	355.8	Yes	Laustsen et al., 2015D

*In the determination of this value, only calculated Toxicity Scores have been included. Toxins with Toxicity Scores below the chosen threshold for relevant toxicity in the toxicovenomic study have thus been excluded. For *D. polylepis*, *N. kaouthia*, and *A. laevis* this threshold was generally when the Toxicity Score was < 1. For *D. angusticeps*, the threshold was when the Toxicity Score was < 7.

Figure 1: *Schematic representation of the differences between auxiliary effects and toxin synergism.* ***A)*** *Envenoming without auxiliary effects and without toxin synergism: All mice survive envenoming after 10 minutes, whereas some mice die after 24 hours.* ***B)*** *Envenoming with auxiliary effects but without toxin synergism: The onset of the toxic effects of envenoming is advanced, but toxicity remains similar to A).* ***C)*** *Envenoming without auxiliary effects but with toxin synergism: Onset of envenoming is similar to A), however, toxicity is higher, leading to an increased number of deaths after 24 hour. The time intervals (10 min and 24 hours) are chosen solely for illustrative purposes.*

Figure 2*: Schematic representation of theoretical scenarios in which synergism between toxins occur (A, B, C, and D), and do not occur (E and F).* ***A)*** *When toxins target different targets on the same physiological pathway, this may create a synergistic effect due to amplification.* ***B)*** *When toxins target different pathways, which all regulate or interact with another physiological pathway, this may create a synergistic effect due to amplification.* ***C)*** *When toxins target the same target in a cooperative manner, synergism may occur.* ***D)*** *When toxins combine to form an oligomeric toxin that has a toxicity, which is higher than the combined toxicities of the individual components, synergism occurs.* ***E)*** *When toxins target the same target in a competitive manner, synergism does not occur.* ***F)*** *When toxins target different pathways, which are unrelated, synergism does not occur. The colors do not specify any particular class of toxins or targets, but they are only provided to illustrate that the toxins and targets are different from each other.*

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