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Proteomic Analyses of Snake Venoms with an Examination of the Biological Roles and Anti-cancer Effects of Venom Disintegrins

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PROTEOMIC ANALYSES OF SNAKE VENOMS WITH AN EXAMINATION OF THE BIOLOGICAL ROLES AND ANTI-CANCER EFFECTS OF VENOM DISINTEGRINS

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ABSTRACT

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For decades, snakes and snake venoms have been utilized in numerous aspects of biological and biomedical research. Behaviorally, snakes have been examined for their extraordinary chemosensory capabilities, providing a detailed understanding of their foraging ecology and predatory responses. The presence of a highly complex vomeronasal organ has enabled snakes to not only respond to, but also discriminate between a high-range of heterospecific, conspecific, predatory, and prey-derived chemical odors.

Snake venom has allowed for a transition in predatory behaviors, and this often complex mixture of proteins and peptides has provided researchers with an ever growing catalog of natural compounds that may be applicable as novel therapeutics or as biomedical reagents. Research into venomous systems also provides a detailed understanding of the biological roles of venom compounds, as well as providing critical information necessary for the proper assessment and treatment of snakebite.

The current work addresses several aspects of snake behavior and snake venom toxinology and has four major objectives: i) to examine the chemosensory responses of neonate, subadult and adult Prairie Rattlesnake (*Crotalus viridis*

viridis) to various prey chemical stimuli, ii) to identify the chemical component(s) of venom which allows for prey recovery during viperid predatory episodes, iii) to examine the anti-cancer effects of a novel snake venom disintegrin towards various human derived cancer cell lines and iv) to complete in-depth proteomic analyses of the neonate and adult *C. v. viridis* and examine the efficacy of the current anti-venom treatment CroFab® against this species' venom.

Chapter I presents the objectives and aims of my dissertation work, and provides background on chemosensory systems in squamates, and the numerous studies examining prey relocation in viperid snakes. Further, this chapter addresses the importance of examining the potential medicinal values of disintegrins as anti-cancer therapeutics, and the utilization of proteomics to develop a better understanding of venom composition and anti-venom efficacy. Chapters II focuses on the chemosensory responses of wild-caught neonate, subadult, and adult *C. v. viridis* to natural and non-natural prey-derived chemical odors. Results indicate that responses to chemical stimuli shift with snake age, correlating with ontogenetic changes in snake diet. Chapter III examines this phenomenon in more detail with a group of "stunted" *C. v. viridis* which had been in captivity since birth and had only consumed neonate lab mice (*Mus musculus*). Further, these snakes were the age of adults yet only the size of large juveniles, therefore they could not consume larger prey normally taken by adult snakes. Results suggest that ontogenetic shifts in responsiveness to natural prey chemical cues are innately programmed and are not based on body size or feeding experience. Chapter IV identifies the venom component, disintegrins, which are responsible for prey recovery during strike-induced chemosensory searching in Western Diamondback Rattlesnakes (*Crotalus atrox*). In Chapter V, a novel disintegrin protein

(named tzabcanin) was isolated from the venom of the Middle American Rattlesnake (*Crotalus simus tzabcan*) and the cytotoxic and anti-adhesion properties of this protein toward Colo-205 and MCF-7 cell lines was examined. Chapter VI also examines the anti-cancer effects of tzabcanin towards A-375 and A-549 cell lines, and by specifically binding integrin αvβ3, tzabcanin inhibits cell migration and cell adhesion to vitronectin. In Chapter VII, a detailed proteomic analysis of the venoms of four individual *C. v. viridis* is presented, showing a novel trend in ontogenetic changes in venom composition, as well as identifying which compounds are, and which are not, effectively immunocaptured by the current anti-venom therapy used in the United States, CroFab®.

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DEDICATION

My dissertation is dedicated to the memory of David Chiszar. As an advisor, collaborator, and best friend, Dave taught me an appreciation for science, snakes, and life that will always be with me. His selflessness opened the door and has allowed for this dream to come true.

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CHAPTER FORMAT AND ATTRIBUTION

My dissertation is comprised of six research chapters, each of which is a manuscript intended for publication. Because of this, each chapter is formatted for the journal in which they have been published or submitted to. In regards to experimental design, data collection, writing and revising of the manuscripts I am the main contributor for each. However, my committee chair Stephen P. Mackessy and several other research collaborators are co-authors. Chapter's II and III have been published separately in the journal *Current Zoology*. Chapter IV has been published in the journal *BMC Biology*. Chapter V is in revision in the journal *Biochimie*, and chapter VI is formatted for submission to *Toxicon*. Chapter VII has been published in the *Journal of Proteomics*.

CHAPTER I

INTRODUCTION

For decades, squamates (lizards and snakes) have been considered as non-traditional yet key organisms in numerous facets of biological and biomedical research. Snakes have been especially well studied with regards to their evolution (Castoe et al. 2008, 2013; Vonk et al. 2013) ecology (Wastell and Mackessy 2011; Shipley et al. 2013; Barbour and Clark 2012; Chiszar et al. 2014), behavior (Chiszar et al. 1977; Burghardt 1971; Clark 2004a; Saviola et al. 2010, 2011, 2012), physiology (Secor 2005, 2008; Riquelme et al. 2011) and toxinology (Mackessy 1988, 2010; Calvete et al. 2009a, 2011; Lomonte et al. 2014). To date, approximately 3496 species of extant snakes are recognized (reptile.database.org), inhabiting every major continent with the exception of Antarctica and occupying virtually all habitats globally (Greene 1997). Extreme phenotypic and molecular plasticity among such a diversity of species has made snakes ideal organisms for diverse research in the biological, biochemical, physiological and even physical sciences.

Snake behavior has also been studied in depth with regards to their complex chemosensory systems, and their ability not only to recognize but also discriminate between chemical stimuli is well established (Burghardt 1970, 1971; Chiszar et al. 1992). Chemoreception in snakes modulates a diversity of behaviors including general exploratory behaviors (Chiszar et al. 1980), mate selection (Kubie et al. 1978; Shine et al. 2003), foraging and prey location (Chiszar et al. 1992; Clark 2004a) and predator,

heterospecific and conspecific recognition (Weldon and Burghardt 1979; Burger 1989; Gutzke et al. 1993; Clark 2004b). This chemical recognition is typically mediated by rapid tongue flicking, activated by the detection of volatile chemical cues by the nasal olfactory system (Burghardt 1970; Saviola et al. 2010) or by visual or thermal stimulation (Chiszar et al. 1981; Saviola et al. 2011, 2012). Consequently, tongue flicking delivers volatile and non-volatile stimuli to the vomeronasal organ located in the roof of the upper jaw (Halpern 1992; Schwenk 1995), allowing for definitive analysis of chemical information (Cowles and Phelan 1958; Halpern 1992).

Snakes consume a diversity of invertebrate and vertebrate prey items and this prey preference may vary between species (Greene 1997) as well as between age classes of the same species (Klauber 1956; Mackessy 1988). As limbless gape-limited predators, snakes can only consume prey by swallowing it whole; therefore, head size is a critical limiting factor of prey consumption (Shine 1991). As a result, ontogenetic shifts in prey preference generally occur and are correlated with snake size. For instance, neonate rattlesnakes generally consume small ectothermic prey (lizards, invertebrates), whereas subadult and adult rattlesnakes often take larger endothermic prey (birds, mammals; Klauber 1956; Mackessy 1988; Hammerson 1999). Likewise, shifts in responsiveness to prey chemical cues (Mushinsky and Lotz 1980), as well as prey-handling behaviors, may also exist (Mackessy 1988; Hayes 1991). Non-venomous snakes utilize constriction or jaw-holding behaviors to subdue prey (Savitzky 1980; Kardong et al. 1996; Bealor and Saviola 2007), whereas venomous snakes deliver a bolus of toxins, either by a strike-and-hold method as seen in rear-fanged colubrids, neonate vipers and elapid snakes, or employing a strike-and-release behavior as documented in the majority of adult viperid

snakes (Mackessy 1988; Hayes 1991). Hence it is likely that natural selection influences specific chemosensory and predatory responses, allowing for successful capture of preferred prey (Tinbergen 1951; Cooper 2008).

Biological Roles of Venoms

Snake venoms are a complex mixture of proteins and peptides (Mackessy 2010) that have allowed for the transition from a mechanical (constriction) to a chemical (venom) means of subduing prey (Savitzky 1980; Kardong et al. 1996). This mixture of enzymatic and non-enzymatic toxins is not only critical to the foraging success of the snake, but venoms are continuously being investigated by researchers to elucidate overall venom composition and complexity and to identify novel biological roles and pharmacological activities of individual compounds. An ongoing question dealing with snake venom toxinology addresses the biochemical complexity of individual proteins and the sometimes numerous protein isoforms that are sometimes found within the venom of an individual species (Jia et al. 1996). These proteins not only act in concert with each other, but individual compounds may also demonstrate discrete biological effects (Weldon and Mackessy 2012; Peichoto et al. 2007; Heyborne and Mackessy 2013).

The venoms of viperid snakes (vipers and pit vipers) contain an abundance of proteins critical for the immobilization, killing and predigestion of prey, including many that interfere with the hemostatic system and coagulation cascade (Mackessy 2008). However, it has been shown that viper venoms also assist in the relocation of prey items during predatory episodes (Duvall et al. 1978; Chiszar et al. 1983, 1992, 1999, 2008). Rattlesnakes and other pitvipers are ambush predators which strike, envenomate and release prey (Clark 2004a; Chiszar et al. 1992). This predatory strategy allows for

minimal contact or retaliation from potentially dangerous prey items, yet requires the additional task of relocating prey that may wander several meters or more from the attack site before succumbing to the venom's effects. By using rapid tongue flicking (strike-induced chemosensory searching) to detect, and the vomeronasal organs to analyze volatile and non-volatile chemical cues (Halpern 1992; Schwenk 1995), snakes must also differentiate between the trail deposited by the prey before and after envenomation has occurred, as well as the trails left inadvertently by other potential prey and non-prey sources. Several hypotheses have addressed the source of chemical cues used to discriminate between trails of struck and unstruck prey. Cues emanating from the mouse when it is punctured during the predatory strike, as well as other potential chemical cues such as urine or volatiles from venom left on the prey's integument, have been examined, with results indicating that such cues are *not* utilized by snakes (Lavin-Murcio et al. 1993; Chiszar et al. 1991, 1992; Hayes et al. 1992). These previous results indicated that venom must be injected into tissues to initiate a release of chemical odor(s), permitting discrimination of envenomated prey and their trails.

A convenient bioassay of vomeronasal chemoreception was previously developed for evaluating preference towards envenomated (E) vs. non-envenomated (NE) mouse carcasses, with snakes showing high rates of tongue flicking directed toward E carcasses (strike-induced chemosensory searching, SICS; Chiszar et al. 1992, 1999, 2008; Greenbaum et al. 2003). This preference holds when envenomation occurs by a conspecific or by a closely related heterospecific (Chiszar et al. 2008), or when lyophilized conspecific venom is injected into previously euthanized prey (Chiszar et al. 1999). Therefore, venoms represent not only a rapid-acting chemical means of

dispatching potentially fractious prey (Kardong et al. 1996); they also greatly increase the perceptibility of the envenomated prey carcass (Chiszar et al. 1992, 1999). However, the specific component(s) of snake venom allowing for successful recovery of prey has not been identified.

Compositional Diversity of Venoms – Sources of Novel Compounds

In addition to understanding the roles of venom compounds during predation, the identification and characterization of these proteins offers significant insights for basic research, clinical diagnosis of human envenomations, and anti-venom production strategies (Gutiérrez et al. 2009; Calvete et al. 2009b). A recent proteomic approach, termed venomics (venom proteomics) (Juárez et al. 2004; Calvete et al. 2009b), provides in-depth analyses of venom composition, advancing the knowledge necessary for efficient snakebite treatment and anti-venom production and efficacy (Calvete et al. 2009a, 2009b; Lomonte et al. 2008). Human envenomation due to snakebite is a global issue, often neglected by health authorities around the world (Gutiérrez et al. 2006, 2007). Estimates as high as 2.5 million envenomations and over 125,000 deaths have been reported (Chippaux 1998); however, more recent estimations suggest approximately 1,841,000 envenomations resulting in 20,000 deaths, yearly (Kasturiratne et al. 2008). These numbers are largely based on hospital records, and with the vast majority of bites impacting agricultural workers in low income countries in Africa, Asia, and Central and South America, victims may seek traditional treatments or die before reaching appropriate health care (Gutiérrez et al. 2006). Therefore, the actual occurrence of envenomation may be significantly higher than reported. Further, victims may have permanent physical disability following snakebite, drastically impacting their overall

quality of life. Snakebite in the United States is rare, and it is estimated that there are approximately 9000 venomous snake bites reported annually (O'Neil et al. 2007), with roughly 99% of these bites from viperid snakes (Jucket and Hancox 2002). Human envenomations may pose a serious or potentially deadly emergency, and early therapeutic use of antivenom is necessary if severe envenomation is suspected. In the United States, the antivenom CroFab® (Crotaline Polyvalent Immune Fab(ovine)) is commonly administered during envenomation cases. CroFab® is produced in sheep immunized with one of the following North American snake venoms: *Agkistrodon piscivorus* (Water Moccasin), *Crotalus adamanteus* (Eastern Diamondback Rattlesnake), *C. atrox* (Western Diamondback Rattlesnake), and *C. scutulatus* (Mojave Rattlesnake) (Price and Sanny 2007). Surprisingly, the most widely distributed rattlesnake in North America, the Prairie Rattlesnake (*C. v. viridis*), is not one of the species utilized for CroFab® production. Adequate treatment of snakebite is dependent on the ability of the antivenoms to reverse the pathological symptoms induced by venom. Therefore, knowledge on venom composition and inter- and intraspecies venom variability is critical for assessment of antivenom efficacy and treatment of snakebite.

Not only can proteomic methodologies be utilized to increase our overall understanding of venom composition for efficient treatment of snakebite, but these techniques can be further applied to identify venom compounds exhibiting potential medicinal value. Toxins-to- drugs have been an emphasis of research in the last decade (Fox and Serrano 2007; Mukherjee and Mackessy 2013; Saviola et al. 2014), and several novel compounds developed from the poisons and venoms of animals are currently in clinical trials and use (Fox and Serrano 2007; Takacs and Nathan 2014). Viperid snake

venoms in particular have been a useful source of several protein drugs and additional novel protein drug leads. For example, one class of snake venom proteins, known as disintegrins, has led to the design of two current therapies on the market. Integrilin (eptifibatide), used to treat acute coronary ischemic disease, and tirofiban (aggrastat), an anti-platelet aggregation drug, were both designed based on the structure of two snake venom disintegrins, barbourin (Scarborough et al. 1993) and echistatin (Gan et al. 1988), respectively. Additionally, the dimeric disintegrin contortrostatin from the Southern Copperhead (*Agkistrodon contortrix contortrix*) has demonstrated promising anti-cancer effects towards numerous cancer cell lines (Zhou et al. 2000; Lin et al. 2010).Therefore, further analyses of snake venoms may provide additional novel sources of proteins for potentially useful therapeutics.

Integrins and Cancer Therapies

In 2014, it was estimated that there were approximately 1.6 million new cancer cases and over 580,000 cancer-related deaths in the United States alone (Siegel et al. 2014). Cancer cells, and normal cells, rely on cell-cell and cell-extracellular matrix interactions, mediated by an important class of α/β heterodimeric proteins known as integrins (Cheresh 1992; Desgrosellier and Cheresh 2010). Integrins form by the appropriate pairing of one of 18 α with one of 8 β subunits, creating 24 potential integrin receptors with varying affinities toward numerous extracellular matrix proteins (Desgrosellier and Cheresh 2010). Some integrins, such as $\alpha_v\beta_3$, have been shown to be overexpressed 50-100 fold in cancerous cells (Gehlsen et al. 1992), and specific subunits, such as β_3, may not even be expressed in normal tissues (Stallmach et al. 1992; Desgrosellier and Cheresh 2010). Integrins are essential for tumor angiogenic activity,

proliferation and metastasis (Clark and Brugge 1995; Serini et al. 2006). Tumor dependence on angiogenesis is also well documented (Folkman 2007), and formation of new blood vessels is required for delivering nutrients to as well as providing a means of removing waste from tumors. Extracellular matrix proteins such as fibronectin and vitronectin also play critical roles in tumor formation and progression, as fibronectin-integrin interactions in cancer cells may lead to resistance to numerous chemotherapeutic agents as well as increased tumorigenesis (Ruoslahti 1999; Rintoul and Sethi 2002; Knowles et al. 2013). In addition, vitronectin has also been shown to play a role in tumor progression and invasion (Marshall and Hart 1996; Hurt et al. 2010; Pola et al. 2013). Therefore, isolating compounds that have potential integrin-blocking activity and anti-angiogenic functions may be a novel approach to controlling cancer cell proliferation and metastasis (Folkman 2006).

Disintegrins are small (4-15 kDa) non-enzymatic proteins currently isolated only from the venoms of vipers (e.g., Calvete 2005). Classified by their number of disulfide bonds and amino acids, disintegrins are grouped into short (~ 41-51 residues, 4 disulfide bonds), medium (~ 70 residues, 6 disulfide bonds), long (~ 84 resides, 7 disulfide bonds) and dimeric (~ 67 resides each, with 10 disulfide bonds), with a fifth group including the disintegrin domain of P-III snake venom metalloproteinases (Calvete 2005; Calvete et al. 2010). Disintegrins exhibit an Arg-Gly-Asp (RGD) domain that is also found within many extracellular matrix (ECM) proteins such as fibronectin and vitronectin (Buckley et al. 1999). Subsequently, disintegrins have been shown to selectively block $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_2\beta_1$, and numerous other classes of integrin receptors (see Calvete et al. 2010 for a review). Previous work with disintegrins has shown promising effects at inhibiting

pathways critical for metastasis (Zhou et al. 2000; Lin et al. 2010; Sánchez et al. 2009). However, disintegrins may show significant activity at inhibiting one class of integrins, yet fail to show an effect on other classes of integrins. It has been shown that the amino acids adjacent to the integrin inhibitory tripeptide sequence are critical to integrin binding affinity (Scarborough et al. 1993). Therefore, there is a continued need to isolate and purify novel disintegrins and other useful proteins which may demonstrate different integrin-blocking activities and have potential anti-cancer effects.

The work presented herein addresses all of the topics reviewed above with the following objectives: (i) examine the ontogenetic shift in chemosensory responses of Prairie Rattlesnakes (*Crotalus viridis viridis*) to a variety of prey stimuli, (ii) identify the venom compound(s) allowing for prey relocation during viper predatory episodes, (iii) identify and characterize a new snake venom disintegrin and examine the potential anti-cancer effects of this protein against human-derived cancer cell lines, and (iv) complete in-depth proteomic analyses of neonate and adult *C. v. viridis*, allowing for identification of toxins that may pose a severe threat during human envenomation and for assessment of antivenom efficacy. The primary aims of my doctoral research are as follows:

Aim 1: Identify venom compound(s) responsible for prey relocation during viperid predatory episodes. Viper venoms allow these snakes to strike, envenomate, and release prey; however, the compound(s) responsible for prey relocation has yet to be identified. I hypothesize that venom metalloproteinases, an abundant enzyme family in viper venoms, assist in prey relocation by vomeronasal chemoreception.

Aim 2: Complete in-depth proteomic analyses of venoms of *Crotalus viridis viridis*. Full proteomic analyses of venoms will allow for me to characterize and identify compounds that constitute the entire venom composition of a single species. These results will allow me to identify species that may pose a threat to humans due to snakebite envenomation as well as effectively assessing anti-venom efficacy by determining which compounds may or may not be recognized by the currently administered anti-venom. Proteomic characterization of venom compounds will also allow for me to identify any novel toxin drugs that may exhibit potential clinical use.

Aim 3: Examine potential anti-cancer effects of a novel snake venom disintegrin towards several cancer cell lines. Toxin drugs have been an emphasis of research in the last decade, with several novel compounds derived from venoms currently in clinical trials and use. Snake venoms in particular have been a useful source of several protein drugs and additional novel protein drug leads. Venom disintegrins have demonstrated significant anti-neoplastic activity by binding to, and inhibiting integrin receptors in cancer cells, yet the specificity of which integrin(s) are recognized by disintegrins may vary drastically. I hypothesize that a novel disintegrin will recognize and bind integrin receptors over-expressed in cancer cell lines and inhibit critical pathways involved in metastasis.

Chapter II of this dissertation examines the ontogenetic shift in chemosensory responsiveness to prey derived chemical cues in three age classes of *C. v. viridis*. In Chapter III, I follow up the study of Chapter II by examining chemosensory responses in a group of *C. v. viridis* who chronologically were the age of adult rattlesnakes, but the size of large neonates. In addition, the snakes studied in this chapter had never fed on natural prey items, and only had taken neonate or juvenile lab mice (*Mus musculus*). In Chapter IV, I identify the chemical component of snake venom, a small non-enzymatic class of proteins called disintegrins, as the relocation molecule, allowing for envenomated prey discrimination and successful recovery of prey during adult viperid predatory episodes. Chapter V describes the isolation and characterization of a new disintegrin isolated and characterized from the venom of the Middle American Rattlesnake (*Crotalus simus tzabcan*), named tzabcanin. Further, I examine the ability of tzabcanin to inhibit adhesion of human breast (MCF-7) and colon (Colo-205) cancer cell lines to the extracellular matrix proteins fibronectin and vitronectin. Chapter VI examines the anti-cancer effects of tzabcanin towards human melanoma (A-375) and lung (A-549) cell lines, further identifying integrin $\alpha_v\beta_3$ as one of the primary binding sites of tzabcanin. In chapter VII, I conduct a detailed proteomic analysis to examine the venom

proteome of two neonate (male and female) and two adult (male and female) *C. v. viridis*, allowing for identification of variation in venom composition between age and sex groups; additionally, the efficacy of the current anti-venom treatment used in the United States, CroFab®, is evaluated using antivenomic and Western blot methods. Finally, I conclude with Chapter VIII by providing a summary of all six research chapters and addressing possible areas of continued research on these current topics.

CHAPTER II

ONTOGENETIC SHIFT IN RESPONSE TO PREY-DERIVED CHEMICAL CUES IN PRAIRIE RATTLESNAKES *(CROTALUS VIRIDIS VIRIDIS)*

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Abstract

Snakes often have specialized diets that undergo a shift from one prey type to another depending on the life stage of the snake. *Crotalus viridis viridis* (prairie rattlesnake) takes different prey at different life stages, and neonates typically prey on ectotherms, while adults feed almost entirely on small endotherms. We hypothesized that elevated rates of tongue flicking to chemical stimuli should correlate with particular prey consumed, and that this response shifts from one prey type to another as individuals age. To examine if an ontogenetic shift in response to chemical cues occurred, we recorded the rate of tongue flicking for 25 neonate, 20 subadult, and 20 adult (average SVL = 280.9, 552, 789.5 mm, respectively) wild-caught *C. v. viridis* to chemical stimuli presented on a cotton-tipped applicator; water-soluble cues from two ectotherms, (prairie lizard, *Sceloporus undulatus*, and house gecko, *Hemidactylus frenatus*), two endotherms, (deer mouse, *Peromyscus maniculatus* and lab mouse, *Mus musculus*), and water controls were used. Neonates tongue flicked significantly more to chemical cues of their common prey, *S. undulatus*, than to all other chemical cues; however, the response to this lizard's chemical cues decreased in adult rattlesnakes. Subadults tongue flicked with a higher rate of tongue flicking to both *S. undulatus* and *P. maniculatus* than to all other treatments, and adults tongue flicked significantly more to *P. maniculatus* than to all other chemical cues. In addition, all three sub-classes demonstrated a greater response for natural prey chemical cues over chemical stimuli of prey not encountered in the wild (*M. musculus* and *H. frenatus*). This shift in chemosensory response correlated with the previously described ontogenetic shifts in *C. v. viridis* diet. Because many vipers show a similar

ontogenetic shift in diet and venom composition, we suggest that this shift in prey cue discrimination is likely a general phenomenon among viperid snakes.

Introduction

Squamate reptile response to prey is often associated with chemical cues of prey integument, visual cues, or visual-thermal cues associated with prey movement (Burghardt, 1970; Ford and Burghardt, 1993; Cooper, 1995). Reliance on chemical cues by lizards and snakes is also critical in mate selection, exploratory behavior, predator identification, prey choice and location, and kin selection (Kubie et al., 1978; Chiszar and Scudder, 1980; Weldon and Burghardt, 1979; Chiszar et al., 2008; Clark, 2004; Pernetta et al., 2009), and many studies have examined chemical cue discrimination and chemosensory responses to multiple chemical cue sources. Chemical cue discrimination between extracts of multiple prey types is often correlated with evolutionary changes in diet, such that snake response to prey cues is greatest to that of most commonly taken prey (Cooper and Burghardt, 1990; Cooper, 1994, 1997, 2008; Clark, 2004). Since snakes are gape-limited predators, swallowing prey whole, head size is a limiting factor in what can be consumed. Therefore, shifts in prey taken as well as response to specific prey cues may also change as snakes increase in age and size (Mushinsky and Lotz, 1980).

Neonate rattlesnakes primarily take smaller ectothermic prey such as lizards and anurans, and adults often specialize on larger endotherms such as rodents and birds (Klauber, 1972; Mackessy, 1988; Mackessy et al., 2003). Rattlesnakes are ambush predators, and the use of chemical cues in ambush site selection has been examined, and likewise selection of ambush sites is correlated with the presence of chemical cues of the most commonly consumed prey (Clark, 2004; LaBonte, 2008). During predatory events,

adult rattlesnakes use visual-thermal cues to strike, envenomate, and release endotherm prey (Hayes and Duvall, 1991; Kardong, 1992), inducing strike-induced chemosensory searching (SICS) and further use of chemical stimuli to relocate the envenomated carcass (Chiszar et al., 1977; 1992). However, neonate rattlesnakes demonstrate different prey handling behaviors. Mackessy (1988) noted that neonate pacific rattlesnakes (*Crotalus oreganus helleri* and *C. o. oreganus*) often hold onto small ectotherm prey and therefore do not have the task of relocating prey after venom has taken its course. Hayes (1991) showed that juvenile *C. v. viridis* released small endotherm prey after the strike; however, the duration of holding onto prey was much longer than that seen in medium or large *C. v. viridis*. Therefore, although extended contact with prey may increase risk of retaliation from struggling prey, the prolonged holding behavior may be advantageous for neonate feeding success, perhaps by allowing more venom to enter prey or by limiting the distance released prey can retreat before succumbing.

The prairie rattlesnake *Crotalus v. viridis* is one of three species of rattlesnakes found in Colorado, with a broad distribution throughout most of the state at elevations under 2,890 m (Hammerson, 1999). The diet of *C. v. viridis* in Colorado consists primarily of the lesser earless lizard *Holbrookia maculata*, prairie lizard *Sceloporus undulates* and plains spadefoot toad *Spea bombifrons*, as well as endotherms such as western harvest mouse *Reithrodontomys megalotis* and deer mouse *Peromyscus maniculatus*; other prey are also taken (Hammerson, 1999). As with many other species of rattlesnakes, ontogenetic shifts are seen in prey type taken, and neonates primarily consume ectotherms, while adults primarily specialize on small mammals (Mackessy, 1988; Hammerson, 1999). Prairie rattlesnakes therefore represent a species for which

many aspects of behavior, sensory processes, ecology and venom toxinology have been well studied, and it can serve as a model for behavior of rattlesnakes generally.

Tongue flicking in snakes is a stimulus-seeking behavior and is the main process for delivering volatile and non-volatile cues to the vomeronasal organs (Halpern, 1992), which mediate definitive analysis of chemical information (Cowles and Phelan, 1958; Schwenk, 1995). Tongue flicking is activated by detection of volatile chemical cues by the nasal olfactory system and by visual, thermal, or vibratory stimulation (Burghardt, 1970; Chiszar et al., 1981; Ford and Burghardt, 1993; Saviola et al., in Press); therefore, the rate of tongue flicking can be used as a convenient measure of a snake's response to any or all of these stimuli. In this paper we present data from laboratory experiments examining the responses of neonate, subadult, and adult wild-caught prairie rattlesnakes to aqueous extracts from two ectotherms (*S. undulatus* and *H. frenatus*) and two endotherms (*P. maniculatus* and *M. musculus*) to determine if an ontogenetic shift in response to chemical cues occurs within this species. In addition, we examine if *C. v. viridis* can discriminate between prey extracts of natural (*S. undulatus* and *P. maniculatus*) and non-natural (*H. frenatus* and *M. musculus*) prey items. This latter point is of interest because both of these non-natural prey are taken by *C. v. viridis* in captivity; indeed, *M. musculus* of various sizes are probably the most common food items in captivity.

Materials and Methods

Study Animals

The snakes used in this study consisted of 65 wild-caught *C. v. viridis* from Weld Co., Colorado, USA. Snakes were classified as neonates (*n*=25), subadults (*n*=20),

and adults (*n*=20) based on snout-vent length (SVL) and body mass (Table 2.1), which were measured immediately after testing. Snakes were randomly collected using snake hooks from two den sites that were frequently visited by our lab group. All snakes were housed in groups based on age (size) classification from visual inspection, as actual measurements and associated handling were not conducted until after testing. Snakes were tested approximately 48 hrs and within 96 hrs after being brought into captivity. Snakes were released at the exact location of capture and were in captivity for no more than 10 days. Snakes were maintained with water *ad libitum*, and none of the snakes were fed during their time in captivity. The laboratory was maintained at 26–28°C and the photoperiod was automatically controlled on a 12: 12 Light: Dark cycle.

Experimental Design

The test cages for neonate and subadult rattlesnakes consisted of glass terraria (51×28×48 cm); adults were tested in larger glass terraria (122×33×35 cm). Testing cages contained paper flooring, and paper was wrapped around the transparent sides. Cages were cleaned prior to and between tests with Quatricide-PV®, a commercial disinfectant and deodorant. Before testing, a snake was placed into the test cage, the top was affixed, and the subject was allowed to acclimate undisturbed for 10 min.

Trials began by gently opening the lid of the testing cage, and using forceps we placed a cotton-tipped applicator containing one of five chemical cue extracts 1 cm from the snake's snout (Cooper and Burghardt, 1990). Trials were of 60 sec duration, and we counted tongue flicks directed at the cotton-tip applicator simultaneously using a hand counter. To minimize stress to the animals and to keep them in captivity for as little time as possible, trials were separated by 10 min, during which the lid was affixed to the cage

and the snake was left undisturbed. However, this short duration between trials did not influence subsequent tongue flicking, as indicated by an absence of trials effects in statistical tests.

Chemical extracts of deer mouse *P. maniculatus*, lab mouse *M. musculus*, prairie lizard *S. undulatus*, and house gecko *H. frenatus*, were prepared by placing intact prey (entire animal) in 1ml of distilled water per gram of prey for 10 minutes (Clark, 2004). Extracts were always used within 1 hr of preparation. Distilled water was used as a control, and we added an additional control treatment of opening and closing the lid of the testing cage. This additional control consisted of an observer opening the lid of the testing cage for 60 secs, counting tongue flicks, and closing the lid of the cage. This treatment was included to take into consideration any effect of opening the test cage as well as the observer being present in front of the snake that may have led to elevated levels of tongue flicking.

Statistical Analysis

Data were analyzed by Chi-square (χ^2) and repeated-measures, and mixed analyses of variance (ANOVA) followed by Newman-Keuls Range Test (NKRT, 5%). Repeated-measures ANOVA and mixed ANOVA (treating conditions as a repeated measures factor and age as a between-subjects factor) were completed since we have multiple observations on each individual and three age-groups of individuals. The concept of analyzing data by both non-parametric and parametric statistics is based on the desire to see different methodologies converge on a common outcome, thus strengthening our conclusions (Siegel, 1956).

Results

The mean number of tongue flicks toward chemical extracts during the 60 sec trials varied markedly among treatments and age groups of snakes (Fig. 2.1). For neonates, 19 of 25 snakes directed more tongue flicks at *S. undulatus* extract than toward any other prey extract ($\chi^2_1 = 6.76$, $P < 0.01$). Repeated-measures ANOVA revealed a significant effect of prey cue type ($F_{5, 120} = 16.74$, $P < 0.01$) and NKRT showed that *S. undulatus* extract received significantly more tongue flicks than all other treatments. Also, *H. frenatus* extract received more tongue flicks than the two control treatments; no other pairwise comparisons were significant. The six means, ordered from low (open-and-close control) to high (*S. undulatus* extract), were 1.4, 2.3 (water control), 4.2 (*P. maniculatus* extract), 4.3 (*M. musculus* extract), 7.9 (*H. frenatus* extract), and 13.7, respectively. Fifteen pairwise comparisons are possible. The differences (observed ranges, ORs) between the highest mean and all others were significant, since the least significant range (LSR) was 5.08, and all ORs exceeded this value. The two control means were significantly lower than the mean for *H. frenatus* extract, because the ORs were greater than the LSR of 4.86 (all *Ps* <0.05).

For subadult *C. viridis*, response to *S. undulatus* and *P. maniculatus* extracts were significantly higher than some but not all other treatments. Response to *S. undulatus* extract was as strong as was seen in the neonates, but response to *P. maniculatus* extract was equally strong in the subadults. A significant effect of treatments (extracts) was revealed by ANOVA ($F_{5, 95} = 8.64$, $P < 0.01$) and NKRT revealed that the means for the *S. undulatus* extract and for the *P. maniculatus* extract were significantly higher than the means for the two control treatments. No other differences between treatments were

significant. The mean numbers of tongue flicks, ordered from low (open-and-close control) to high (*S. undulatus* extract), were 2.8, 3.6 (water control), 7.7 (*M. musculus* extract), 8.8 (*H. frenatus* extract), 12.7 (*P. maniculatus* extract), and 14.4, respectively. The LSRs for comparisons with *P. maniculatus* and *S. undulatus* extracts were 6.19 and 6.47, respectively, and the ORs for both control means were greater than these values; hence, these differences were significant (all *Ps* <0.05). All treatments except for *P. maniculatus* and *H. frenatus* extracts had ORs with *S. undulatus* extract that were greater than LSR = 6.47. Therefore, these three treatment means (the two controls and *M. musculus* extract) differed significantly from the mean for *S. undulatus* extract (all *Ps* <0.05). Mean response to *M. musculus* extract did not differ significantly from *P. maniculatus* extract (OR=5.0, LSR=6.19, *P* >0.05). Mean response to *H. frenatus* extract differed significantly from the open-and-close control (OR=6.0, LSR=5.83, *P*<0.05). No other pairwise comparisons were significant.

For adult *C. v. viridis*, ANOVA indicated a significant effect of treatments ($F_{5, 95}$ = 12.24, *P* <0.01). NKRT showed that the two controls did not differ from each other, but both of these means differed significantly from all others. Most conspicuously, the mean for responses to *P. maniculatus* extract was significantly higher than all other means. This was confirmed by Chi-square (χ^2_1 = 5.00, *P* <0.05) and NKRT. In addition, 17 of 20 adults had higher scores for *P. maniculatus* than for *M. musculus* extract (χ^2_1 = 9.8, *P* <0.01), indicating that adults discriminated between natural and non-natural prey cues. The six means, arranged from low (open-and-close control) to high (*P. maniculatus* extract), were 2.8, 3.3 (water control), 7.0 (*M. musculus* extract), 7.1 (*H. frenatus* extract), 8.1 (*S. undulatus* extract), and 12.9. The LSR for comparisons involving the

latter mean was 4.26, and all ORs were higher than this value, including that for *M. musculus* extract. Hence, *P. maniculatus* extract generated significantly more tongue flicks than all other conditions. The LSR for comparisons involving *S. undulatus* extract was 4.07, and both controls had ORs that were larger (all *Ps* <0.05). The mean for the open-and-close control generated ORs that were higher than the LSRs for *M. musculus* and *H. frenatus* extracts (3.49, 3.83, respectively, *Ps* < 0.05). The mean for the water control had an OR with the mean for *M. musculus* extract that exceeded the corresponding LSR (3.49; *P* <0.05). No other pairwise comparisons were significant.

Integrating all three sub-analyses into a single 3 (age) x 6 (treatments) mixed ANOVA, treating age as a between-subjects factor and treatments as a repeated-measures factor, demonstrated that the age effect was not significant ($F_{2, 62} = 1.47$, $P > 0.05$). The overall means across extracts for the age groups were similar and did not differ significantly. The significant effect of extracts ($F_{5, 310} = 54.89$, $P < 0.01$) was primarily due to the fact that the controls were lower than the other treatments. The interaction of age × treatments was significant ($F_{10, 310} = 38.21$, $P < 0.01$). When comparing the 18 means with each other using NKRT, the *S. undulatus* extract had the highest means for neonates and subadults (significantly higher than for the adults); conversely, *P. maniculatus* extract had the highest means for adults (significantly higher than for neonates). Applying NKRT to the 18 means (see the lists of means from the three previous sub-analyses) from this 3× 6 mixed ANOVA produced 153 pairwise comparisons. First, all control means (from both controls and all three age groups) had ORs that did not exceed the corresponding LSRs. The highest OR in this subset of 15 contrasts was 2.2, whereas the corresponding LSR was 4.76. So, none of these treatments

differed from each other. The highest means were for the *S. undulatus* extracts presented to subadults and to neonates, and these means had significantly higher ORs with all means except for *P. maniculatus* extracts presented to adult and subadult snakes. The highest of these latter ORs was 4.8, while the corresponding LSR was 5.10. Hence, the means for *S. undulatus* extracts presented to subadults and to neonates were significantly higher than all but these last two means. Additionally, the means for *S. undulatus* presented to subadults and neonates did not differ from each other (OR = 0.3, LSR = 5.18). The mean for *P. maniculatus* extract presented to adult snakes was higher than all other means except for *P. maniculatus* extract presented to subadults, and *S. undulatus* extracts presented to neonates and subadults (ORs = 0.2, 0.8 and 1.5, respectively; LSRs = 5.49, 5.45, and 5.57, respectively). In short, this set of comparisons agrees with those reported above in showing that the youngest snakes tongue flicked most strongly towards *S. undulatus* extract, whereas the adult snakes tongue flicked the most to *P. maniculatus*. Subadult snakes tongue flicked approximately equally to both *S. undulatus* and *P. maniculatus* extracts.

Discussion

Natural selection can be expected to influence chemosensory responses of snakes to stimuli that are most likely to lead to capture of prey (Tinbergen, 1951; Cooper, 2008). Evolutionary shifts in snake diet have been well documented and are further correlated with shifts in response to specific cues associated with such prey (see Cooper, 2008). Whether this response is a learned or heritable trait has been examined in several species, and studies have indicated that prey generalists demonstrate learning behavior such that increased response is directed toward cues of the diet being fed to the snake (Burghardt,

1993). Prey specialists, on the other hand, may exhibit chemosensory responses only to specific prey, with such behavior exhibiting little flexibility, as is characteristic of heritable responses (Arnold, 1981; see also Cooper, 2008). The majority of rattlesnakes demonstrate different feeding strategies at different life stages; for example, gut content analyses indicated that juvenile pacific rattlesnakes had a diet consisting of more than 50% ectotherms, whereas adult snakes had exclusively mammals in their diet (Mackessy, 1988).

Neonate *C. v. viridis* showed a significantly higher rate of tongue flicking for chemical extract of *S. undulatus* when compared to all other treatments, including a non-prey lizard species *H. frenatus* extract. These results indicate that neonates discriminated between natural *S. undulatus* and non-natural *H. frenatus* lizard prey chemical cues. Interestingly, Chiszar and Radcliffe (1977) found that neonate *C. v. viridis* born in captivity exhibited no significantly different responses to chemical extracts of lizard and lab mouse *M. musculus* when compared to the control. However in this study, snakes were naïve, never having been fed prior to testing. Further, these snakes had never seen nor smelled prey, and snakes were tested immediately following the shed of natal skins (about 10 days following parturition). In comparison, in the current study, snakes certainly had feeding experience before they were caught, and that the observed difference between the results of these two studies is likely due to prior predatory experience among snakes in our study.

Subadult *C. v. viridis* responded with a higher rate of tongue flicking to both *S. undulatus* and *P. maniculatus* extracts and adult *C. v. viridis* tongue flicked significantly to the extract of *P. maniculatus* when compared to all other treatments. As with the

neonates, rate of tongue flicking to chemical extract of native prey *P. maniculatus* was higher than that seen for non-native prey (*M. musculus*), indicating that adults also discriminated between natural and non-natural endothermic prey cues. Collectively, these results reveal a shift in chemosensory responses, presumably correlated with prey commonly taken between neonatal and adult life stages of *C. v. viridis* (Hammerson, 1999). Subadult snakes showed elevated levels of tongue flicking to both *S. undulatus* and *P. maniculatus*, indicating that the emergence of the adult response is not associated with an immediate decline of earlier dietary predilections. Indeed, although adult *C. v. viridis* mostly take endothermic prey, they are known to take *S. undulatus* and other ectotherms opportunistically throughout life (Hammerson, 1999).

Responses of rattlesnakes to chemical stimuli during ambush site selection demonstrate that rattlesnakes rely on prey chemical cues for ambush foraging strategy. Prairie rattlesnakes assumed ambush postures, both in the field and in the laboratory, in response to potential prey extracts (Duvall et al., 1990; Theodoratus and Chiszar, 2000), and dusky pygmy rattlesnakes *Sisturus miliarius barbouri* in the field selected ambush sites based on presence of extracts of their most taken prey, leopard frogs *Rana pipiens* (Roth et al., 1999). In addition, yearling timber rattlesnakes *C. horridus* born in captivity responded significantly to chemical extracts of natural prey items, even though snakes were fed exclusively lab mice (Clark, 2004). LaBonte (2008) further showed that ambush site selection shifted from ectotherm to endotherm cues with increased snake age in southern pacific rattlesnakes *C. o. helleri*. All of these results are consistent with our data reported here which indicate a shift in responsiveness to age-appropriate prey.

In addition to shifts in diet and chemosensory responses to prey, ontogenetic shifts in venom composition have also been documented, as neonate venoms often contain more toxic components, while adult venoms, which are less toxic, contain higher levels of pre-digestive and digestive enzyme toxins (Fiero et al., 1972; Mackessy, 1988, 2008). This shift in venom composition is correlated with prey surface-to-volume ratios. With smaller body sizes and longer limbs, ectotherms are structurally easier to digest than endotherm prey, and many of the higher mass lytic components of rattlesnake venoms (specifically metalloproteinases) are more abundant in venoms of adult snakes (Mackessy, 1988, 2008). It therefore appears that concomitant changes in several aspects of behavior and physiology occur as rattlesnakes age.

Our results support the hypothesis that chemosensory responses stages of *C. v. viridis* are highly correlated with chemical cues of prey commonly taken at different life. Similarly, Mushinsky and Lotz (1980) found that the plain-bellied water snake *Nerodia erythrogaster* shifted response preference from fish to frog extract at approximately 8 months of age. Anurans make up nearly 85% of the diet of large *N. erythrogaster*, whereas fish are primarily taken by smaller snakes, indicating that the shift in chemosensory response as snakes mature closely follows actual dietary shifts (Mushinsky and Lotz, 1980). Additionally, feeding experience has been shown to lead to shifts in chemosensory responses. Burghardt et al. (2000) showed that an isolated population of *Thamnophis sirtalis* with a natural diet primarily of earthworms, but fed exclusively fish in captivity, exhibited a stronger response to fish cues at the end of the experimental period. It should be stressed that the present study was not designed to assess the extent of flexibility within each of the life stages. These studies are needed before we can

conclude that neonates and adults exhibit different specialized strategies, each relatively resistant to modification. However, prairie rattlesnakes are clearly differentially responsive to chemical cues derived from prey typical of a given life stage, and this ontogenetic change may be typical of other rattlesnakes which show age-related changes in diet.

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Table

Table 2.1: Mean snout-vent length and body mass (± *SEM*) for the 65 rattlesnakes tested.

	Snout-vent length (mm)	Mass (g)
Neonate	280.9 (3.6)	13.4 (0.5)
Subadult	552.0 (7.0)	103.0 (4.9)
Adult	789.5 (10.9)	334.5 (15.1)

Figure

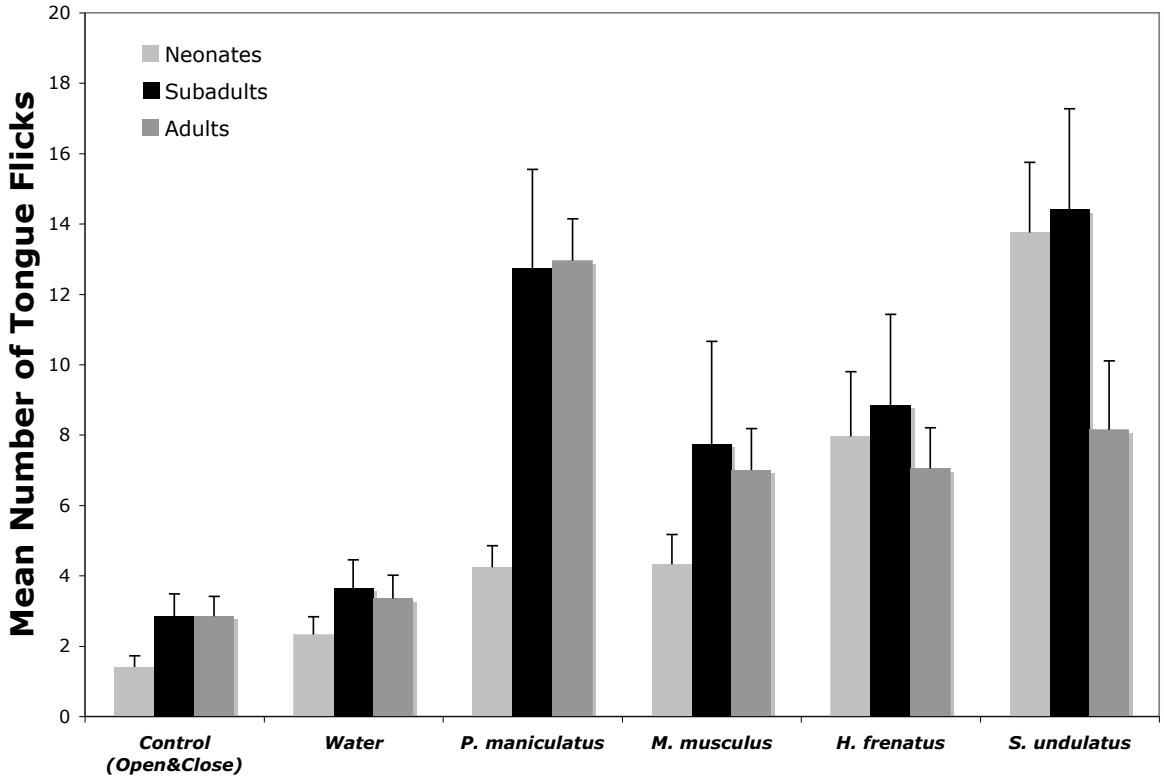

Figure 2.1: Mean Number of Tongue Flicks Over 60 Seconds. Mean number of tongue flicks per minute ± standard error of the mean (SEM) from neonate, subadult, and adult prairie rattlesnakes toward extracts of four different prey types and two control treatments.

CHAPTER III

CHEMOSENSORY RESPONSE IN STUNTED PRAIRIE RATTLESNAKES (*CROTALUS VIRIDIS VIRIDIS)*

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Abstract

Rattlesnakes use chemical stimuli in ambush site selection and for relocation of envenomated prey through strike-induced chemosensory searching. Shifts in responsiveness to prey chemicals have been documented in many snakes, and often correlate with prey commonly taken as snakes increase in age and size as well as geographical locations of the species. For instance, neonate rattlesnakes that prey primarily on ectotherms responded most strongly to chemical cues of commonly taken lizard prey, whereas adult rattlesnakes that prey primarily on small mammals responded significantly to chemical cues of commonly taken rodents. In the current study, 11 Prairie Rattlesnakes *Crotalus viridis viridis* which were classified as large neonates based on measures of snout-vent length (SVL) and body mass, yet chronologically were at or near adulthood, were tested for their responsiveness to chemical extracts of natural and non-natural prey items. Although the snakes had eaten only neonate lab mice (*Mus musculus*), they responded significantly more to chemical cues of natural prey items and particularly to chemical cues of prey normally taken by subadults (*Peromyscus* mice and *Sceloporus* lizard). These results suggest that ontogenetic shifts in responsiveness to natural prey chemical cues are innately programmed and are not based on body size or feeding experience in *C. v. viridis*. This does not imply, however, that growth and experience are without effects, especially with novel prey or rare prey that have experienced recent population expansion.

Introduction

It is well established that many squamate reptiles, especially snakes, are excellent at recognizing and discriminating between chemical cues of prey sources (Burghardt,

1970; Cooper et al., 1990, Chiszar et al., 1992; Clark, 2004). Behavioral plasticity has also been documented, as shifts in responsiveness to prey-derived cues often correlate with shifts in diet as snakes increase in age and size (Mushinsky and Lotz, 1980; Cooper, 2008; Saviola et al., 2012a). Saviola et al. (2012a) found that neonate prairie rattlesnakes *Crotalus viridis viridis* responded most strongly to chemical cues from prairie lizard prey *Sceloporus undulatus*, whereas adult *C. v. viridis* responded less strongly to these cues and more strongly to chemical cues from deer mice *Peromyscus maniculatus*. Subadults responded strongly to both lizard and rodent cues. These chemosensory response patterns also correlated with the natural prey most commonly taken by the three age groups (Hammerson, 1999; Mackessy, 1988).

Rattlesnakes are ambush predators that use chemical cues in ambush site selection and visual-thermal cues to deliver the envenomating strike (Chiszar et al., 1981; Kardong and Mackessy, 1991; Kardong, 1992; Clark, 2004). Following the strike and release of prey, rapid tongue flicking (strike-induced chemosensory searching) detects volatile and non-volatile chemical cues, allowing for relocation of the envenomated carcass (Chiszar et al., 1977, 1992). Prey-handling behaviors also vary as neonates typically hold on to lizard prey (Mackessy, 1988), whereas adults release rodent prey immediately after the strike (Mackessy, 1988; Hayes, 1991). Likewise, shifts in venom composition are often seen, as neonate rattlesnakes tend to have higher concentrations of toxic venom components, and less pre-digestive enzymes such as metalloproteinases, which are typically in much higher abundance in venoms of adult rattlesnakes (see Mackessy, 2008 for a review).

The present sample of *C. v. viridis* were collected as neonates in 2008 from the same population and location as the snakes studied by Saviola et al. (2012a), but the present animals had been underfed and, as a consequence, had not grown at a normal rate. It has been documented that prey preference correlates with skull shape and size in snakes (Vincent et al., 2004, 2009), and with maximum gape and head size being limiting factors in prey consumption, underfeeding and consumption of small prey may drastically inhibit snake growth. Based on measures of snout-vent length (SVL) and body mass, the present snakes were between the neonate and subadult categories defined by Saviola et al. (2012a), whereas chronologically the animals were at or near adulthood (Klauber, 1972). Hence, we were provided with a unique opportunity to assess whether the same shift in response to chemical cues occurred in these snakes as occurred in the snakes studied by Saviola et al. (2012a). This question is of additional interest for at least two reasons: (1) the present snakes had never encountered *P. maniculatus* or *S. undulatus*, as they had eaten only neonatal and adolescent lab mice (*Mus musculus*; 3–10g, 2–3cm in length) in captivity, and (2) because of the size of the stunted snakes, they were incapable of swallowing adult *P. maniculatus* (or adult *M. musculus*). Hence, if the stunted snakes nevertheless respond strongly to chemical cues derived from adult *P. maniculatus*, the implication would be that the transition described by Saviola et al. (2012a) was not dependent upon experience with natural prey but may be innately programmed.

Materials and Methods

Eleven *C. v. viridis* captured in Weld County, CO, October 8, 2008 (approx. one month after parturition) were maintained in captivity for approximately four years (at the time of this study). The snakes were underfed, but cage sanitation and drinking water

were not neglected. During this period, snakes were offered *M. musculus* neonates and adolescents, as the snakes were and still are incapable of ingesting adults. Saviola et al. (2012a) classified wild caught neonates, subadults and adults as shown in Table 3.1. The present snakes had mean SVL of 349.1 mm and mean mass of 27.4 g, significantly higher than the means for the neonates of Saviola et al. (t_{34} = 9.52 and 9.75, respectively, *Ps* <0.01) but also significantly lower than the means for subadults (t_{29} = 18.73 and 11.09, respectively, *Ps* <0.01). Accordingly, the present sample would be classified as large neonates or small subadults, but not as adults, although chronologically these snakes would be nearing adulthood (Klauber, 1972). All snakes were maintained in individual plastic terraria (51 × 28 × 48 cm) and provided hide boxes, paper floor coverings and water *ad libitum*. The laboratory was maintained at 26–28 °C, and the photoperiod was automatically controlled on a 12:12 light-dark cycle.

Chemical extracts of five adult potential prey organisms (*P. maniculatus, M. musculus, S. undulatus,* Side-blotched Lizard, *Uta stansburiana,* and Common House Gecko, *Hemidactylus frenatus*) were prepared by placing intact prey in one ml distilled water per gram of prey for 10 min (Clark, 2004). Extracts were always used within one hr of preparation, and distilled water was used as a control. Trials began by gently opening the lid of the terrarium. Using forceps, a cotton-tipped applicator containing one of the extracts or water was placed one cm from the snake's snout (Cooper and Burghardt, 1990). During the next 60 sec we counted the number of tongue flicks aimed at the applicator and the latency to strike if this occurred. Tongue flicking in squamates is activated by detection of chemical stimuli by the nasal olfactory system, or by visual, thermal or vibratory cues and is the main process for delivering volatile and non-volatile

cues to the vomeronasal organs, which mediates definitive analysis of chemical information (Burghardt, 1970; Halpern, 1992; Schwenk 1995; Saviola et al., 2011, 2012b). Therefore, measuring tongue flicking in snakes is a useful assay for measuring a snake's response to any or all of these cues. Trials were separated by at least 24 hr, and the six chemical cues were presented in a different random order for each snake. Each cage was cleaned prior to and between trials with Quatricide-PV®, a commercial disinfectant and deodorant. Snakes were always left undisturbed after a trial and allowed to come to rest after the cage lid was opened in preparation for the next trial.

Data were converted to tongue-flick-attack (TFA) scores in order to account for strikes (Cooper and Burghardt, 1990); four strikes occurred during all trials. So, TFA scores were essentially the same as the number of tongue flicks during the 60 sec tests. We used a log-10 transformation to normalize the data and to achieve homogeneity of variance in the six conditions.

Inferential analyses used Chi-square (χ^2) and repeated-measures analyses of variance (ANOVA) followed by non-orthogonal contrasts. Alpha was set at 0.01 for these contrasts to control type I errors. Use of both nonparametric and parametric tests was predicated on our desire to see different statistical tools converge on the same conclusions. Comparisons of characteristics of the present snakes with those of Saviola et al. (2012a) used *t*-tests.

Results

Nine of the eleven snakes had their highest TFA scores with *P. maniculatus* or *S. undulatus* cues as opposed to the other four cues ($\chi^2_1 = 4.44$, $P < 0.05$), including *M. musculus*, the only prey offered in captivity and likely the only prey ever eaten by these

snakes. All snakes had higher TFA scores for the three potential prey extracts (*M. musculus, H. frenatus, U. stansburiana*) as opposed to the water control ($\chi^2_1 = 11.00$, $P < 0.01$).

The six means shown in Figure 3.1 differed significantly by repeated-measures ANOVA ($F_{5,50} = 3.92$, $P < 0.01$). Non-orthogonal contrasts revealed that the mean of the five extracts combined differed significantly from the mean for water ($F_{1,50} = 9.06$, $P < 0.01$). The means of *P. maniculatus* and *S undulatus* extracts were significantly higher than the means of all other extracts combined, including the mean for *M. musculus* extract ($F_{1,50} = 22.30$, $P < 0.01$). The latter means did not differ significantly among themselves ($F_{1,50} = 3.56$, $P > 0.05$).

Discussion

Rattlesnakes use chemical stimuli in both ambush site selection (Duvall et al., 1990; Clark, 2004; LaBonte, 2008) and relocation of prey during strike-induced chemosensory searching (Chiszar et al., 1977). Therefore, understanding how chemosensory responsiveness varies during the development of the snakes is important for understanding the behavior and ecology of these species. Although the present snakes had eaten only *M. musculus*, the response to *P. maniculatus* had developed more-or-less on the same schedule as that of normally fed adult snakes, while the response to *S. undulatus* extract had not dropped significantly, as is seen in wild-caught snakes (see Saviola et al., 2012a). Further, the only strikes observed during trials were toward these native prey extracts (*P. maniculatus* and *S. undulatus*, two strikes towards each). Hence, these snakes behaved like normal subadults, even though they were closer to the size range of neonates and could not ingest adult mice.

Development of response to chemical cues of natural prey might be innately programmed, or experience with these or similar items might be required. In the present case, the responses to *P. maniculatus* and *S. undulatus* extracts were of normal intensity, even though the snakes probably had never seen or eaten these prey. This is suggestive of and might be taken as evidence for innate ontogenetic programming. It remains possible that feeding on *M. musculus* provided sufficient surrogate cues to promote development of response to *P. maniculatus* extracts. However, if this is true, then why did the snakes not respond strongly to *M. musculus* extracts? We cannot resolve this conundrum on the basis of present data, but we can provide two hypotheses: (1) response to *P. maniculatus* cues developed innately, perhaps even earlier than usual because of hunger and underfeeding, and (2) this sensitivity to rodent cues may have been the reason the snakes accepted *M. musculus* (rather than the other way around). Although captivity may influence chemosensory responses it has been documented that long-term captive rattlesnakes (*C. atrox, C. durissus, C. horridus, C. vegrandis, C. unicolor*) demonstrate no significant differences in strike-induced chemosensory searching when compared to wild-caught rattlesnakes (Chiszar et al., 1985). Similarly, long-term captivity did not appear to affect the ability of *C. o. oreganus* to strike, dispatch, or relocate prey normally (Alving and Kardong, 1994).

There is evidence that snakes can acquire responsiveness to new or unusual foods if the snakes are fed such foods but not their usual fare (Burghardt et al., 2000), and our data for *M. musculus* extracts agree with this finding. It is also known that naïve neonatal snakes respond to chemical extracts of natural foods (Cooper et al., 1990), and our data for *S. undulatus* extracts agree with those of Burghardt (working with garter

snakes). The data for *P. maniculatus* extracts suggest that ontogenetic appearance of response to prey normally taken later in life does not require experience with that prey. Similarly, adult *C. horridus* born and raised in captivity showed significant responses to chemical cues of preferred natural prey, even though these snakes were fed entirely *M. musculus,* never encountering natural prey items (Clark, 2004).

Being gape-limited forces snakes to consume prey that are small enough for them to swallow, and chemosensory responses to prey cues have been shown to correlate with the most commonly consumed prey and with the foraging ecology of the snake (see Cooper, 1995, 2008; Saviola et al., 2012a, b). In Colorado, neonate *C. v. viridis* prey primarily on small ectotherms such as the lesser earless lizard *Holbrookia maculata*, prairie lizard *S. undulates* and plains spadefoot toad *Spea bombifrons*; however, shifts to endotherm prey such as the western harvest mouse *Reithrodontomys megalotis* and deer mouse *P. maniculatus* occur in subadult and adult snakes (Hammerson, 1999). Although responsiveness to chemical cues changes over snake age, which typically correlates with increased size, our study demonstrates that *C. v. viridis* respond to chemical cues of prey most commonly taken in adulthood, regardless of the snakes' size, and we infer that changes in chemosensory responsiveness by *C. v. viridis* are innate.

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Table

Table 3.1: Mean Snout-vent Length and Body Mass (± *SEM*) for the 11 *C. v. viridis* Tested. **Bold** lettering indicates subject classifications and data from Saviola et al. (2012a; Chapter II).

	Snout-vent length (mm)	**Mass (g)**
Current Subjects (n=11)	349.1 (7.1)	24.7 (2.0)
Neonate (n=25)	**280.9 (3.6)**	**13.4 (0.5)**
Subadult (n=20)	**552.0 (7.0)**	**103.0 (4.9)**
Adult (n=20)	**789.5 (10.9)**	**334.5 (15.1)**

Figure

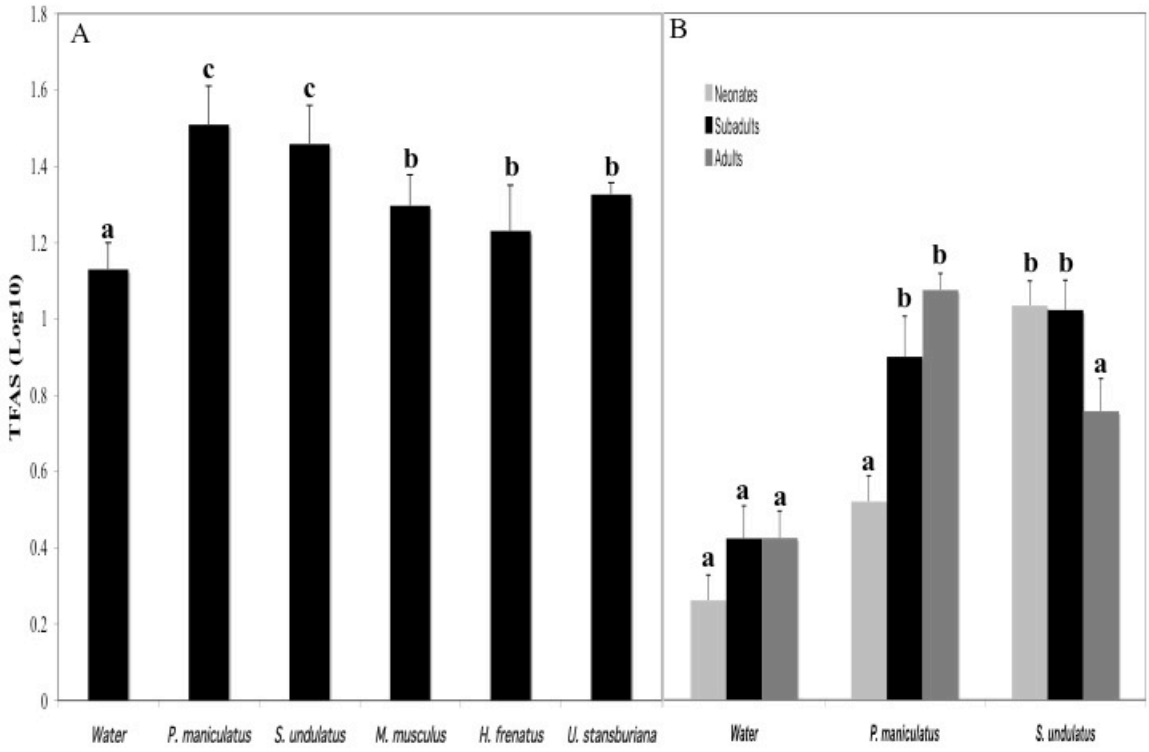

Figure 3.1: Comparison of Log10 Transformed TFAS. A. Log10 transformation of mean tongue flicks attack score (TFAS) ± standard error of the mean (SEM) for 11 stunted *C. v. viridis* toward 5 different prey types and water control. B. Log10 transformation of mean TFAS ± SEM towards chemical cues of natural prey items for wild-caught neonate, subadult, and adult *C. v. viridis* from Saviola et al. (2012a). Dissimilar letters above histogram bars indicate significant differences between responses; same letters indicate no significant differences.

CHAPTER IV

MOLECULAR BASIS FOR PREY RELOCATION IN VIPERID SNAKES

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Abstract

Vertebrate predators use a broad arsenal of behaviors and weaponry for overcoming fractious and potentially dangerous prey. A unique array of predatory strategies occur among snakes, ranging from mechanical modes of constriction and jaw-holding in non-venomous snakes, to a chemical means, venom, for quickly dispatching prey. However, even among venomous snakes, different prey handling strategies are utilized, varying from the strike-and-hold behaviors exhibited by highly toxic elapid snakes to the rapid strike-and-release envenomation seen in viperid snakes. For vipers, this mode of envenomation represents a minimal risk predatory strategy by permitting little contact with or retaliation from prey, but it adds the additional task of relocating envenomated prey which has wandered from the attack site. This task is further confounded by trails of other unstruck conspecific or heterospecific prey. Despite decades of behavioral study, researchers still do not know the molecular mechanism which allows for prey relocation. During behavioral discrimination trials (vomeronasal responsiveness) to euthanized mice injected with size-fractionated venom, *Crotalus atrox* responded significantly to only one protein peak. Assays for enzymes common in rattlesnake venoms, such as exonuclease, L-amino acid oxidase, metalloproteinase, thrombin-like and kallikrein-like serine proteases and phospholipase A_2, showed that vomeronasal responsiveness was not dependent on enzymatic activity. Using mass spectrometry and N-terminal sequencing, we identified the proteins responsible for envenomated prey discrimination as the non-enzymatic disintegrins crotatroxin 1 and 2. Our results demonstrate a novel and critical biological role for venom disintegrins far beyond their well-established role in disruption of cell-cell and cell-extracellular matrix

interactions. These findings reveal the evolutionary significance of free disintegrins in venoms as the molecular mechanism in vipers allowing for effective relocation of envenomated prey. The presence of free disintegrins in turn has led to evolution of a major behavioral adaptation (strike-and-release), characteristic of only rattlesnakes and other vipers, which exploits and refines the efficiency of a pre-existing chemical means of predation and a highly sensitive olfaction system. This system of a predator chemically tagging prey represents a novel trend in the coevolution of predator-prey relationships.

Background

Coevolution within predator-prey interactions has led to adaptations that are advantageous for either prey capture or predation avoidance. In predators, these traits may be under strong selection leading to successful capture of prey [1, 2], but they are relatively under-studied compared to the mechanisms involved in anti-predator adaptations [3]. Darwin [4] suggested that diversification of predators may be largely based on selection on predatory behaviors, and adaptations to observable phenotypic characteristics that are advantageous to prey capture are commonly examined. For example, evolution of craniofacial asymmetries has shown to increase predation success in scale-eating cichlids [5] as well as in snail-eating snakes [6]. Phenotypic plasticity undoubtedly plays a critical role in diversification of predators and prey, often leading to adaptations in behavior, life history, physiology and morphology of species [7]. Further, competition, predation and utilization of dangerous prey have been proposed as the most significant factors of selection on organisms [8]. The ability of predators to adapt to dangerous prey, such as garter snake (*Thamnophis sirtalis*) resistance to tetrodotoxin (TTX) of *Taricha* newts [2], provides strong evidence for a coevolutionary arms race

between predators and prey. However, adaptations in predatory behaviors to avoid complete retaliation from dangerous prey may be rare. Nevertheless, natural selection can be expected to lead to adaptations influencing behaviors that are most advantageous to prey capture [1], and further examination of the molecular mechanisms allowing for these large scale behavioral adaptations is critical for understanding coevolution between predator-prey interactions. Many studies examining phenotypic plasticity in species address various forms of plasticity separately, yet this variety may have significantly different ecological consequences [9]. Among venomous snakes, venom characteristics are under positive directional selection [10], and the presence of specific venom components may have played a critical role in diversification of predatory behaviors of several snake taxa.

Rattlesnakes and other vipers demonstrate one of the most advanced modes of predation among vertebrates, utilizing a strike-and-release mode of envenomation. This behavior provides the benefit of minimal contact or retaliation from potentially dangerous prey, but adds the additional task of locating the trail left behind by the envenomated prey that may wander several meters or more from the attack site. By using rapid tongue flicking (strike-induced chemosensory searching) to detect, and the vomeronasal organs to analyze volatile and non-volatile chemical cues [11], snakes must then differentiate between the trail deposited by the prey before and after envenomation has occurred, as well as the trails left inadvertently by other potential prey and non-prey sources. Several hypotheses have addressed the source of chemical cues used to discriminate between trails of struck and unstruck prey. Cues emanating from the mouse when it is punctured during the envenomating strike, as well as other potential chemical cues, such as urine or

volatiles from venom left on the prey's integument, have been examined, yet are not utilized by snakes [12-15]. These previous results indicate that venom must be injected into tissues to initiate a release of chemical odor(s), permitting discrimination of envenomated prey and their trails. A convenient bioassay of vomeronasal chemoreception was previously developed for evaluating preference towards envenomated (E) vs. non-envenomated (NE) mouse carcasses, with snakes showing high rates of tongue flicking directed toward E carcasses (strike-induced chemosensory searching, SICS [15-18]). This preference holds when envenomation occurs by a conspecific or by a closely related heterospecific [17], or when lyophilized conspecific venom is injected into previously euthanized prey [18]. Therefore, venoms represent not only a rapid-acting chemical means of dispatching potentially fractious prey [19]; they also greatly increase the perceptibility of the envenomated prey carcass [15, 18]. However, the specific component(s) of snake venom allowing for successful recovery of prey and further diversification of prey handling behaviors has not been identified.

Results

To determine which component(s) of venom allows for rattlesnakes to differentiate between envenomated (E) and non-envenomated (NE) prey, we offered western diamondback rattlesnakes (*Crotalus atrox*) E and NE mouse carcasses; E mice were injected with either crude venom or with fractionated protein or peptide peaks of crude venom (extracted from conspecifics). Non-envenomated mice were injected with a saline control. When artificial envenomation occurred with whole crude venom, the mean number of tongue flicks was significantly greater for the E mouse ($t = 3.67$, df = 6, $P < 0.01$; Table 4.1). When total number of tongue flicks were converted to percentage of

tongue flicks (to control for natural variation in absolute tongue flick rate between snakes), results confirmed that *C. atrox* directed significantly more tongue flicks at the E than at NE mice ($t = 3.76$, df = 6, $P < 0.01$) (Table 4.1). These results agree with numerous studies of vomeronasal response of rattlesnakes to E versus NE prey [15, 17, 18], including a previous study performed using the same pool of *C. atrox* venom as used in this report [18].

To test snake responses toward fractionated protein and peptide peaks, crude *C. atrox* venom was separated using low-pressure size exclusion liquid chromatography, and four major protein peaks, labeled I, IIa, IIb and III, as well as three downstream peptide peaks, were resolved (Figure 4.1A). When mouse carcasses were envenomated with either Peaks I, IIa, IIb or the peptide peaks, there was no significant difference between the mean number of tongue flicks or the percentages of tongue flicks directed towards either the E or NE carcasses (Table 4.2; see also Table 4.3). However, for Peak III, there were significantly more tongue flicks directed towards the E mouse ($t = 4.24$, df = 10, $P < 0.01$; Table 4.2), and the mean percentage of tongue flicks toward the envenomated carcass (68%) was also significantly higher than the null ($t = 5.78$, df = 10, $P < 0.01$; Table 3.2). Analysis of variance (ANOVA) indicated a significant main effect of conditions ($F = 4.63$, df = 4, 54, $P < 0.01$). The Newman-Kewls range test also revealed that the mean for Peak III was significantly higher than the means for Peaks I, IIa, IIb and the peptide peaks ($P < 0.05$), which did not differ significantly among themselves ($P > 0.05$). Further, in 10 out of 11 Peak III trials, snakes tongue flicked more towards the E mouse ($\chi^2 = 3.68$, df = 1, $P = 0.05$), whereas for Peaks I, IIa, IIb and the combined

peptide peaks, there was no preference shown over the E mouse or the NE mouse (χ^2 = 0.264, 0.045, 0.2 and 0.05, respectively; all df's = 1, all *Ps* >0.05).

We next sought to examine the components in Peak III that produced this significant vomeronasal response. Because metalloproteinase enzymes are prevalent components of most viper venoms [20] and because they would still catalyze degradation of non-living E mouse tissues, we hypothesized that these enzymes would be responsible for "tagging" of E prey. Assays for enzymes common in rattlesnake venoms (exonuclease, L-amino acid oxidase, metalloproteinase, thrombin-like and kallikrein-like serine proteases, and phospholipase A_2: [21]) indicated that all of these activities were confined to Peaks I through IIb (Figure 4.1A). SDS-PAGE (Figure 4.4) and mass spectrometry of Peak III (Figure 4.1B) revealed only peptides with masses of approximately 7.5 kD. Further analysis of Peak III through reverse-phase high pressure liquid chromatography (HPLC) yielded two peaks (Figure 4.2A) that were subjected to Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometer analysis. These results yielded masses of 7,440.35 Da (Figure 4.2B) and 7,383.29 Da (Figure 4.2C), respectively, indicating that the proteins isolated were the disintegrins crotatroxin 1 and crotatroxin 2. N-terminal sequencing of Peak III proteins confirmed the identity of these disintegrins (Figure 4.3).

Discussion

Determining the molecular mechanisms leading to large-scale adaptations of predatory behaviors, including, in this case, relocation of prey, is critical for understanding predator-prey interactions, evolutionary biology and natural history of pit vipers. Our findings show that the venom disintegrins crotatroxin 1 and 2 alone allowed

C. atrox to distinguish between envenomated and non-envenomated prey sources, presumably by altering the chemical odor of prey integument. Crotatroxins are medium-sized monomeric disintegrins with approximate masses of 7.4 kDa and contain 71 to 72 amino acids with six disulfide bonds, differing only by the presence of an additional N-terminal alanine in crotatroxin 1 ([22]; see also Figure 4.3). Disintegrins are non-enzymatic and are produced by the proteolytic posttranslational processing of the C-terminal domain of P-II snake venom metalloproteinases [23]. The presence of dimeric disintegrins in other viperid venoms has also been documented; however, only medium-sized monomeric disintegrins appear to be present in *C. atrox* venom [24]. It is currently unknown if dimeric disintegrins will produce the same type of vomeronasal response as the monomeric disintegrins did in this current study. A primary activity of disintegrins is the inhibition of platelet aggregation by selectively binding integrin receptors expressed on cell surfaces [25]. The majority of monomeric disintegrins, including crotatroxins 1 and 2, contain an active Arg-Gly-Asp (RGD) sequence [26], which has been shown to block numerous classes of integrin receptors with a high degree of selectivity. Therefore, the action of crotatroxins which results in successful relocation of envenomated prey via SICS likely involves an integrin binding mechanism and further release of volatile cues detectable by rattlesnakes.

Rattlesnake venoms are classified as either type I venoms, containing high metalloproteinase activity and lower toxicity, or type II venoms, containing low metalloproteinase activity and higher toxicity [21]. Although some strike-and-release rattlesnakes, such as *C. scutulatus scutulatus* (type A) and *C. tigris,* contain less than 0.1% venom metalloproteinases, proteomic studies have identified disintegrins in their

venoms [27, 28]. These species possess type II venoms with potent lethal toxicity, so the possibility of prey wandering a significant distance from the attack site before it has succumbed to venom is much less likely than species exhibiting type I venom, making relocation following a strike less challenging for these highly toxic rattlesnakes. Disintegrins make up approximately 2% (by mass) of the total venom proteins/peptides of crude *C. atrox* venom, though the abundance of this protein (and other venom compounds) may vary between individual snakes. The utilization of a relatively minor venom component to "tag" envenomated prey may also explain the "overkill method" [29] employed by venomous snakes. It has long been observed that many taxa of venomous snakes inject prey with amounts of venom which vastly exceed the mouse model LD_{50}, often by several orders of magnitude [14, 30]. In part, this "excessive" dosage is explained by differential sensitivity of various prey to specific toxins [31] and venoms [32], induced by coevolutionary responses of both prey and their snake predators [33]. For example, some prey species are much less affected by venoms, while others are highly sensitive (cf. frogs and lizards [34]). However, another important factor, in particular, among the strike-and-release predators, such as most viperids, is the need to discriminate between competing prey trails (E and NE rodents), selecting the one leading to the previously envenomated prey. This is likely a main reason why rattlesnakes use apparently large quantities of venom - to achieve a "minimum perceptible dose" [18].

Venoms consist of a myriad of proteins and peptides that may vary based on age, geographic locations and prey preference of the snake [35]. This complexity of venom composition, coupled with the fact that many species specialize on specific prey, likely result in selective pressures on venom characteristics, leading to the evolution of

advantageous venom phenotypes and predatory behaviors [36]. On a trophic level, the roles of disintegrins and many other proteins found in venoms still remain relatively unknown. In whole venom, disintegrins which have not been proteolytically processed could potentially assist in the targeting of PII snake venom metalloproteinases (SVMPs) to specific integrin receptors in cell membranes [37], giving rise to chemical changes recognized by the snakes. Lys49 phospholipase A_2s have also been suggested to act as a tag of envenomated prey [38]; however, we have demonstrated that neither the metalloproteinase-containing nor the PLA_2-containing fractions of *C. atrox* venom elicited prey relocating responses.

To the best of our knowledge, all pit vipers that have been tested have shown significant preference for envenomated prey [for example, 15-18], indicating that disintegrins in other venoms, not just those in *C. atrox*, assist in prey relocation for other pit viper species. But not all snake venoms contain disintegrins. How are prey relocated in these cases? *Atractaspis* species (mole "vipers") use a unilateral slashing envenomation behavior to feed on neonatal rodents within nests and burrows [39], and prey escape after envenomation is highly improbable. Elapids are typically strike-and-hold predators [40], with venoms rich in rapid-acting three-finger toxins [35], and so the presence of a "relocator protein" in these venoms is not likely advantageous. Similarly, neonate rattlesnakes that generally strike-and-hold prey [41] produce much smaller amounts of venom and have significantly lower concentrations of metalloproteinases, the protein family that releases free disintegrins, when compared to venoms of subadult and adult rattlesnakes [21, 41].

A major selective advantage for the evolution of free disintegrins among viperid venoms (apparently exclusively) is provided by their role in prey relocation.

Natural selection undoubtedly has influenced snake responses to stimuli that are most likely to lead to successful capture or, as in this case, successful relocation of prey [1, 42]. Further, this preference for envenomated prey is an adaptive mechanism that facilitates optimal foraging efforts, leading to rapid relocation of prey after it has succumbed. Snakes often will not attend to a second prey offered after the initial envenomating strike, suggesting that chemical cues arising from the struck prey may be focusing foraging efforts and redirecting the snake from additional, potentially confounding chemical cue sources [43]. Our results strongly indicate that for *C. atrox*, disintegrins have evolved into multifunctional proteins which evoke vomeronasally-salient cues, enabling the snake to relocate envenomated prey after the strike. Therefore, in addition to immobilizing, killing and predigesting prey, another biological role of venoms in rattlesnakes is for prey relocation.

Conclusions

These findings provide an important biological role for a non-lethal venom protein which has little apparent relevance to the well-characterized roles of disintegrins in disrupting cell-cell and cell-extracellular matrix interactions. Thus, in order to understand the evolution of animal venoms and venom compositional variation, it will be important to consider possible selective advantages conferred by specific venom components to the behavior and ecology of the animals which produce them, in addition to the more apparent pharmacological effects. At present, it is unknown how the crotatroxins create an olfactory “mark” that snakes are able to recognize, but we

hypothesize that integrin-mediated release of chemical cues from prey stimulate the vomeronasal system of snakes. Studies now in progress are aimed at determining the mechanism(s) by which disintegrins interact with prey tissues and facilitate relocation of envenomated prey by rattlesnakes.

Methods

Materials

BioGel P-100 resin was obtained from BioRad, Inc. (San Diego, CA, USA). Matrix for MALDI-TOF-MS, enzyme substrates, buffer salts and all other reagents were analytical grade or better and were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Experimental Animals

Behavioral trials were performed as approved by the Institutional Animal Care and Use Committee of the University of Colorado at Boulder. Eight *C. atrox*, all adult long-term captive snakes, were fed bi-weekly on live or pre-killed mice (*Mus musculus*). Snakes were never fed on the day of trials, which occurred 7 to 10 days after the last feeding session, and all trials were randomized and separated by at least 14 days. Snakes were housed individually in glass aquaria (61.0 x 41.0 x 44.5 cm) containing a paper floor, water bowls and hide boxes. We maintained the snakes on a 12:12 L:D cycle and at 26 ± 2°C. Inbred Swiss/Webster mice (*Mus musculus*) were culls from colonies maintained by the University of Colorado Department of Molecular, Cellular and Developmental Biology and were euthanized by CO_2 asphyxiation and frozen at -20° until used in this study [17]. The magnitude of SICS towards natural rodent prey such as *Peromyscus maniculatus* (deer mice) does not differ compared to lab mice (*M. musculus*)

[44], and the strain of lab mice used also does not influence results. On testing days, similar size and sex mice were thawed and warmed by electrical heaters until skin temperature was 38 ± 1°C before injection and subsequent testing.

Experiment 1

Venoms were manually extracted, centrifuged to pellet insoluble material, frozen, lyophilized and stored at -20°C until used [41]. Lyophilized venom was reconstituted on the day of testing by dissolving 10 mg of crude venom in 100 μL of deionized water. During a test day, *C. atrox* were allowed to strike and envenomate prey carcasses suspended from long forceps to initiate strike-induced chemosensory searching [15]. Since rattlesnakes release prey after the strike, this envenomated mouse was removed from the snakes' cages and discarded, and that mouse never touched the floor or walls of the cage. The test apparatus, a 4 x 10 cm metal base with two wire mesh baskets approximately 4.0 cm apart, containing both an envenomated mouse injected with 100 μL of reconstituted venom and a non-envenomated mouse [17, 18], was placed into the snake's cage. The 100 μL volume of reconstituted venom is comparable to the volume of venom injected during a predatory strike [14]. Two injections (each containing 50 μL) were made in the thoracic region, dorsal and ventral to the shoulder blade, in areas most commonly struck during predatory episodes [45]. The control (non-envenomated) mouse was injected in the same regions with 100 μL of deionized water. Trials (10-minute trial duration) started as soon as the test apparatus was placed in the cage, with observers counting tongue flicks directed within 1 cm of either the envenomated or the non-envenomated mouse. All tongue flicking was recorded double blind to the condition; therefore, the observer was unaware of which mouse carcass was injected with the

control or venom sample, as well as which condition was being tested. Tongue flicking in snakes represents a stimulus-seeking behavior that is the main process for delivering volatile and non-volatile cues to the vomeronasal organs [11]. Since tongue flicking is activated by the detection of volatile cues by the nasal olfactory system, or visual, thermal or vibratory stimuli, measuring the rate of tongue flicking is an accurate and convenient assay of nasal as well as vomeronasal chemoreception in snakes [11, 46]. Cages and test apparatus were cleaned between trials.

Experiment 2. Low-Pressure Size Exclusion Chromatography

Lyophilized venom (250 mg, from the same venom pool used in Experiment 1) was dissolved in 1.0 mL HEPES buffer solution (10 mM, pH 6.8, with 60 mM NaCl and 5 mM $CaCl_2$) and briefly centrifuged at 9,000 rpm to pellet and remove insoluble material. This solution was then fractionated by size exclusion chromatography using a 90 x 2.8 cm column of BioGel P-100 equilibrated with the same HEPES buffer. Fractionation occurred at a flow rate of 6.3 mL/hr at 4°C, and 30-minute fractions were collected. Elution of size-fractionated protein and peptide peaks was monitored at 280 nm.

Enzyme Assays of Fractionated Venom

All BioGel fractions (10 μL/assay, in duplicate) were assayed for several enzymes common to most rattlesnake venoms [21], including exonuclease (phosphodiesterase), L-amino acid oxidase, caseinolytic metalloproteinase, thrombin-like and kallikrein-like serine proteinases and phospholipase A_2, as described previously [47].

Behavior Trials Using Fractionated Venom

Fractions of Peaks I to III were pooled separately, dialyzed in a 14 kDa cutoff membrane tubing (Peak I) or in a 3.5 kDa cutoff membrane tubing (Peaks IIa, IIb and III) against 2 x 2 liters of ddH_2O, lyophilized and stored frozen at -20°C until use. Similar to Experiment 1, the experimental ("envenomated") mouse was injected with one of the four fractionated protein peaks (1.25 mg protein in 100 μL, reconstituted in ddH_2O) or the combined peptide peaks (1.5 mg in 100 μL), and a non-envenomated control was injected with 100 μL ddH_2O. When testing with fractionated venom, the number of subjects was limited by the quantity of protein in each peak. To induce SICS, each snake struck a mouse suspended by forceps just prior to placement of the apparatus; again, this mouse was immediately removed and discarded, never having touched the floor or walls of the cage. Trials began when the test apparatus containing E and NE carcasses was placed into the cage, again with 10-minute trials.

The mean number of tongue flicks directed towards the E and NE mouse carcasses for whole crude venom and each peak were compared using a two-sample t-test and Chi-square analysis (χ^2). For all trials, the numbers of tongue flicks were converted to percentages (that is, percent tongue flicks emitted to E and NE mice) by dividing the number of tongue flicks aimed at the E carcass by the total number of tongue flicks for both carcasses. These data were analyzed by single sample *t*-tests in which mean percent tongue flicks directed toward envenomated mice were compared to 50%, the expected value under the null hypothesis. Rate of tongue flicking can be highly variable among

snakes, so converting rate of tongue flicking to percentages places all snakes on the same scale. In addition, to achieve homogeneity of variance among conditions, we used a Log_{10} transformation to normalize data, which was analyzed by analyses of variance (ANOVA) followed by Newman-Keuls range test.

Mass Determination by Mass Spectrometry

Peak III from size exclusion (BioGel P-100 column) was desalted using C_4 ZipTips (Millipore Inc., Billerica, MA, USA) and analyzed using a Bruker Ultraflex MALDI-TOF mass spectrometer (Proteomics and Metabolomics Facility, Colorado State University, Fort Collins, CO, USA) operating in linear mode. Protein (approximately 0.5 µg) was spotted onto a sinapinic acid matrix (10 mg/mL 50% acetonitrile, 0.1% trifluoroacetic acid; 1.0 µL) and spectra were acquired in the mass range of 3.0 to 25 kDa.

Purification by Reverse-phase High Performance Liquid Chromatography (RP-HPLC)

Peak III was then further fractionated by reverse-phase high pressure liquid chromatography. Two hundred microliters (1.0 mg/mL) were injected onto a Grace Vydac Reverse Phase C_{18} (4.6 x 250 mm) column equilibrated with buffer A (0.1% Trifluoroacetic acid (TFA) in water). Absorbance was measured at 280 nm and proteins were eluted using a shallow gradient of 20% to 28% buffer B (80% acetonitrile in 0.1% TFA) over 50 minutes, with a flow rate of 1.0 ml/min. Peaks eluting at approximately 23% buffer B (fraction 13 - major peak; fraction 14 - minor following peak) were collected, dried in a Savant speedvac (ThermoScientific, Rockford, IL, USA), and stored

at -20°C. Masses of proteins in fractions 13 and 14 were determined using a Bruker Ultraflex MALDI-TOF mass spectrometer (Bruker Corporation, Fremont, CA, USA) as above.

N-terminal Sequencing of RP-HPLC Purified Proteins

Samples of Peak III for sequencing were reduced with dithiothreitol and alkylated with iodoacetamide as described previously [48]. The first 30 resides of sequence were obtained using an ABI Procise sequencer (Life Technologies/Applied Biosystems, Grand Island, NY, USA), and sequence obtained was subjected to Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (Bethesda, MD, USA) [49].

Abbreviations

E, Envenomated; HPLC, high-pressure liquid chromatography; MALDI-TOF, Matrix-assisted laser desorption/ionization time-of-flight; NE, Non-envenomated; RGD, Arg-Gly-Asp acid; SICS, strike-induced chemosensory searching; SVMP, Snake venom metalloproteinase; TFA, Trifluoroacetic acid; TTX, tetrodotoxin

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49. **Basic Local Alignment Search Tool (BLAST)**

[http://blast.ncbi.nlm.nih.gov/Blast.cgi]

Tables

Table 4.1: Rattlesnakes Discriminate Between Non-envenomated and Envenomated mice. directed at non-envenomated (NE) and envenomated (E) mice by *Crotalus atrox* when mice were envenomated by whole crude venom. Single-sample *t*-test was conducted on mean percentages where mean percent to E mice were compared with 50%, the value expected under the null hypothesis; df = 6. Because the two means are not independent, the same *t* value but with the opposite sign would be obtained for each mean. For raw data, see Additional file 1, Table S1. ** $P < 0.01$.

Sample	**NE**	**E**	***t***
Venom (n = 7)	32 (8.45)	83 (15.9)	3.67**
	29	71 (5.65)	3.76**

Table 4.2: Prey Discrimination is Associated with Non-enzymatic Fractions. Mean number of tongue flicks and mean percent (lower values) tongue flicks (s.e.m.) directed at non-envenomated (NE) and envenomated (E) mice by *Crotalus atrox* when mice were envenomated using BioGel Peaks I, IIa, IIb, III or combined peptide peaks. Single-sample *t*-test was conducted on mean percentages in which mean percent tongue flicks to E mice were compared with 50%, the value expected under the null hypothesis. Because the two means within each paired comparison are not independent, the same *t*-value but with the opposite sign would be obtained for each mean. For raw data, see Additional file 1, Table S1. ** $P < 0.01$.

Fraction	**NE**	**E**	***t***	**df**
Peak I	67.4 (11.9)	68.7 (12.3)	0.09	16
	51	49 (5.3)	0.22	
Peak IIa	72.9 (15.6)	59.0 (12.7)	0.70	10
	50	50 (7.0)	0.01	
Peak IIb	73.3 (18.4)	69.2 (15.3)	0.22	9
	49	51 (4.3)	0.25	
Peak III	25.3 (5.1)	53.6 (7.7)	4.24**	10
	32	68 (3.2)	5.78**	
Small peptide peaks (combined)	33.0 (7.7)	52.7 (24.6)	0.79	8
	49	51 (11.7)	0.12	

Table 4.3. Supplemental Table: Raw data: Number of tongue flicks toward envenomated (E) or non-envenomated (NE) mice.

Experiment 1 - Whole Venom

Subject	E mouse	NE mouse	Proportion to Experimental
1	46	50	.48
3	119	33	.78
4	129	71	.65
5	86	16	.84
6	17	13	.57
7	66	9	.88
8	119	35	.77

Experiment 2 – Peak I

Subject	E mouse	NE mouse	Proportion to Experimental
1	32	61	.34
2	138	1	.99
3	129	175	.42
5	107	138	.44
6	59	115	.34
7	204	89	.70
1	53	69	.43
6	41	20	.67
1	56	48	.54
3	40	20	.67
2	0	11	.00
8	96	72	.57
8	37	114	.25
1	66	108	.38
1	24	45	.35
8	53	27	.66
8	33	33	.50

Experiment 2 – Peak IIa

Subject	E mouse	NE mouse	Proportion to Experimental
1	67	56	.54
8	31	183	.14
3	30	36	.45
4	39	116	.25
5	25	30	.45
6	11	0	1.0
7	63	126	.33
3	122	63	.66
3	69	86	.45
1	148	64	.70
3	45	42	.52

Experiment 2 – Peak IIb

Subject	E mouse	NE mouse	Proportion to Experimental
1	47	51	.48
5	32	32	.50
7	38	21	.64
8	56	28	.67
1	189	71	.73
3	22	17	.56
2	64	92	.41
1	98	124	.44
8	91	204	.31
3	55	93	.37

Experiment 2 - Peak III

Subject	E mouse	NE mouse	Proportion to Experimental
1	89	20	.82
2	40	18	.69
3	51	51	.50
4	67	52	.56
5	37	11	.77
6	105	48	.69
7	14	12	.54
8	48	13	.79
3	33	20	.62
1	51	27	.65
9	55	7	.89

Experiment 2 – Peptide Peaks

Subject	E mouse	NE mouse	Proportion to Experimental
1	120	82	.59
2	46	25	.65
3	15	2	.88
6	23	24	.49
4	7	39	.15
5	9	19	.32
1	30	34	.47
6	225	19	.92
2	0	53	.00

Figures

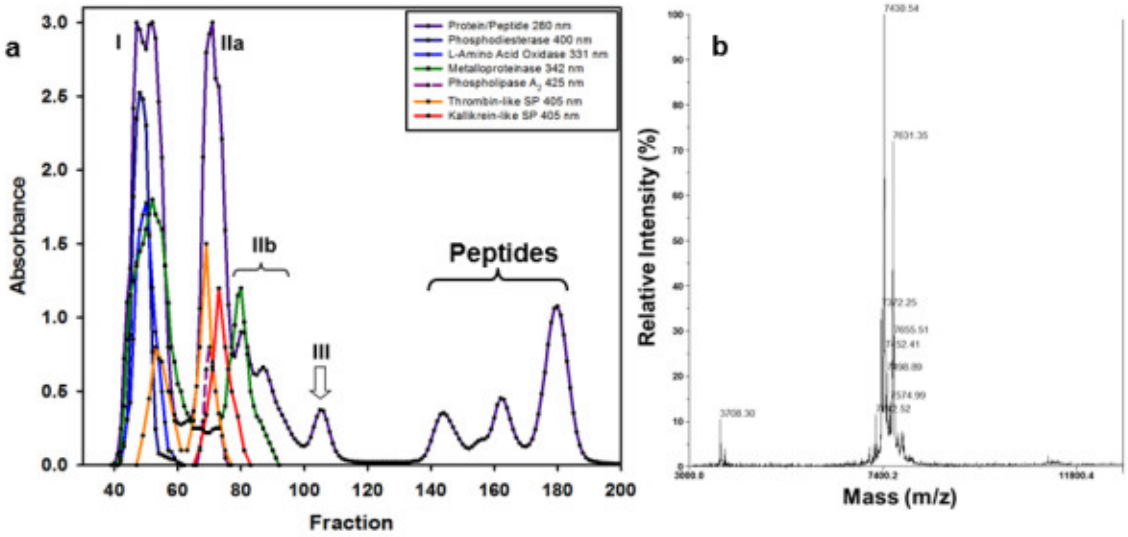

Figure 4.1: Discrimination of Envenomated Prey is not Dependent on Enzymatic Toxins. (A) Size exclusion fractionation of 250 mg crude *C. atrox* venom on a 90 x 2.8 cm BioGel P-100 column equilibrated with HEPES/NaCl/$CaCl_2$ buffer. Fractionation occurred at a flow rate of 6.3 mL per hour at 4ºC, and eluting proteins/peptides were followed by absorbance at 280 nm. Enzyme activities common to rattlesnake venoms were assayed and are limited to the first two peaks. Arrow indicates the peak containing crotatroxins 1 and 2 (Peak III). (B) MALDI-TOF-MS analysis of peptides in BioGel size exclusion Peak III. Approximately 0.5 µg protein was spotted onto sinapinic acid matrix and analyzed using a mass window of 3 to 25 kD. Several peptides with masses typical of monomeric disintegrins (7,245 to 7,655 Da) were present, but no larger proteins were observed.

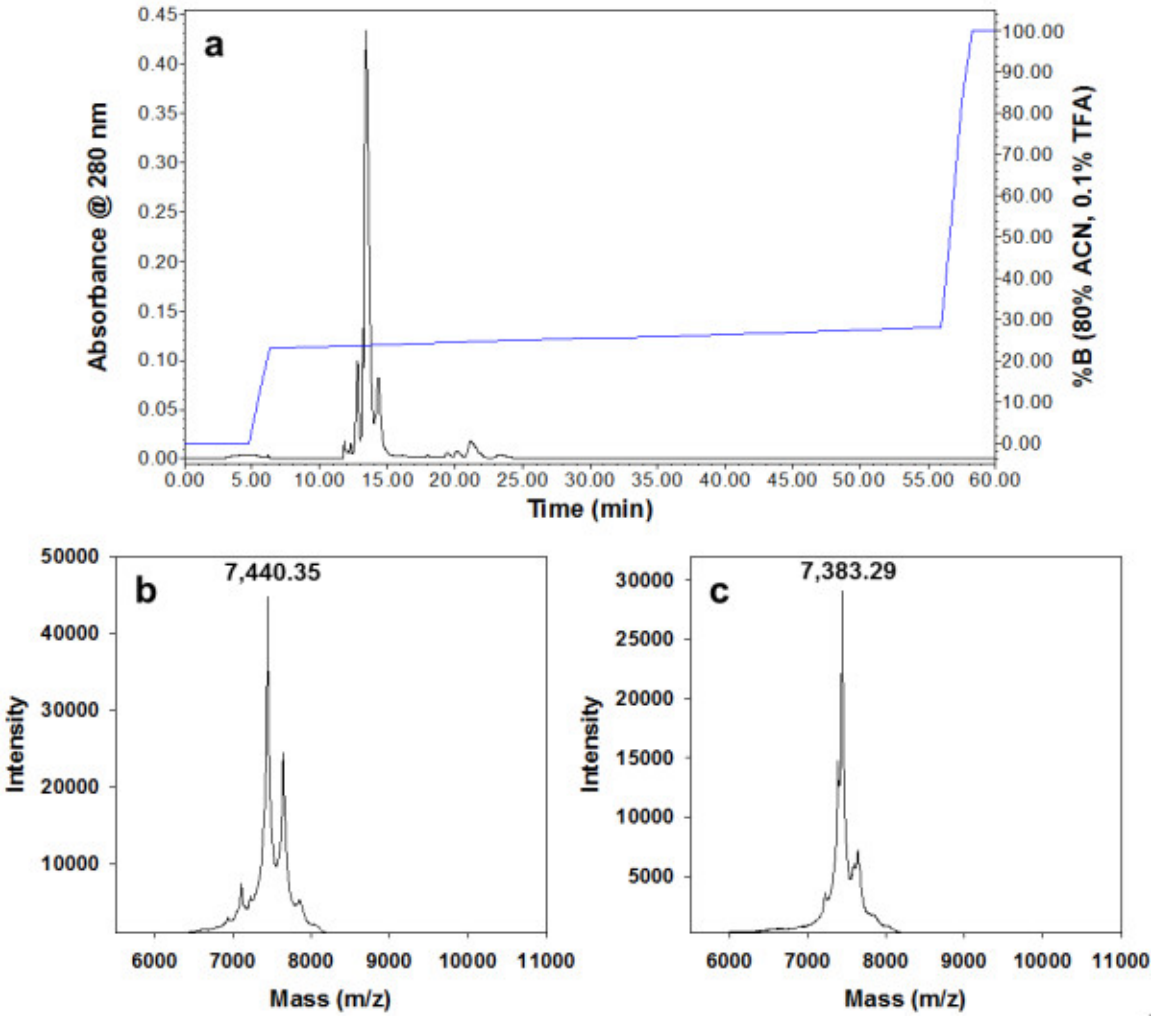

Figure 4.2: Peak III Consist of Only of 7 kDa Peptides. (A) Reversed-phase chromatography of Peak III from the gel filtration step (BioGel P-100). Two hundred microliters was injected onto a Vydac C_{18} (4.6 x 250 mm) column, and disintegrin peaks were eluted at 23% buffer B (13 to 14 minutes). (B) MALDI-TOF-MS analysis of crotatroxin 1 from the reverse-phase chromatography purification step (fraction 13). Mass of 7,440.35 was observed for crotatroxin 1. (C) MALDI-TOF-MS analysis of crotatroxin 2 from the reverse-phase chromatography purification step (fraction 14). Mass of 7,383.29 was observed for crotatroxin 2.

```
Relocator      (A)GEECDCGSP ANPCCDAATC KLRPGAQCAD G...
Crotatroxin 1    AGEECDCGSP ANPCCDAATC KLRPGAQCAD GLCCDQCRFI
Crotatroxin 2     GEECDCGSP ANPCCDAATC KLRPGAQCAD GLCCDQCRFI

Crotatroxin 1    KKGTVCRPAR GDWNDDTCTG QSADCPRNGL YG
Crotatroxin 2    KKGTVCRPAR GDWNDDTCTG QSADCPRNGL YG
```

Figure 4.3: N-terminal Sequence of Peak III Peptides. N-terminal sequencing confirms identity with crotatroxins (CT) 1 and 2. Note that CTs 1 and 2 are identical in sequence except for the additional N-terminal alanine residue in CT1. Protein sequencing of the relocator peak showed lower yield (approximately 3 pmol, compared to approximately 6.5 pmol for residues 2 to 6) and presence of an N-terminal alanine at residue 1, indicating that both CTs were present. No secondary sequence (indicative of potential contaminant proteins) was observed.

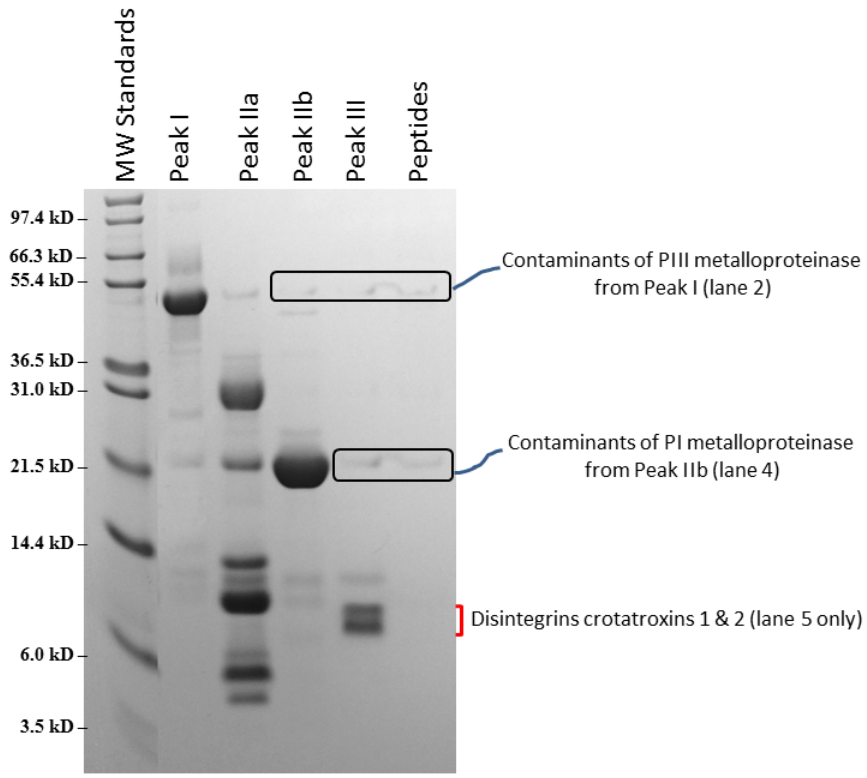

Figure 4.4: Reducing SDS-PAGE Analysis of Size Exclusion Chromatography Fractions. Ten micrograms of protein (reduced with DTT) from each size exclusion peak (BioGel P100) were loaded onto a 12% acrylamide NuPage gel. Following electrophoresis, the gel was fixed and stained with 0.1% Coomassie Brilliant Blue R250 using standard methods, destained and photographed. MW standards = Invitrogen Mark 12. Circled faint bands indicate carryover contamination of metalloproteinases (darkest bands) from lanes 2 and 4, respectively. Note that lane 5 is the only peak containing disintegrin bands (dark pair, red bracket); peptides were not visualized and are smaller than the resolution capability of the gel.

CHAPTER V

DISINTEGRINS OF *CROTALUS SIMUS TZABCAN* VENOM: ISOLATION CHARACTERIZATION AND EVALUATION OF THE CYTOTOXIC AND ANTI-ADHESION ACTIVITIES OF TZABCANIN, A NEW RGD DISINTEGRIN

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[In revision at *Biochimie*]

Abstract

Disintegrins are small non-enzymatic proteins common in the venoms of many viperid snakes. These proteins have received significant attention due to their ability to inhibit platelet aggregation and cell adhesion, making them model compounds in drug development and design investigations. The present study used a combination of molecular and proteomic techniques to screen the venom of the Middle American Rattlesnake (*Crotalus simus tzabcan*) for novel disintegrins. Six disintegrin isoforms were identified, and the most abundant, named tzabcanin, was further isolated and characterized. Tzabcanin consists of 71 amino acids, has a mass of 7105 Da (by MALDI-TOF mass spectrometry) and contains the canonical RGD binding domain. Tzabcanin was not cytotoxic to MCF-7 cells but showed weak cytotoxicity to Colo-205 cells following a 24 hr incubation period. Tzabcanin inhibited cell adhesion of both cell lines to immobilized fibronectin and vitronectin, and cell adhesion to immobilized tzabcanin was inhibited when cells were incubated with a cation chelator (EDTA), indicating that integrin-tzabcanin binding is specific. This study provides a detailed analysis of the purification and characterization of tzabcanin and provides sequence and mass data for the multiple disintegrins present in the venom of *C. s. tzabcan*.

Introduction

Snake venoms are a complex mixture of proteins and peptides exhibiting an array of biochemical and pharmacological functions [1]. These bioactive molecules have allowed for a trophic transition from a mechanical (constriction) to a chemical (venom) means of subduing prey [2] via the dysregulation of many homeostatic mechanisms simultaneously. Because venoms consist of ‘usurped’ regulatory compounds, they have

also been subjected to detailed screenings in the search for novel compounds which may be utilized as biomedical tools and reagents [3–5]. Further, research into venomous systems provides unique insights of the biological roles of venom compounds [6–8] and increases the ability to devise effective clinical treatment of human envenomations [9,10].

Venoms likely evolved from the modification and differential expression of endogenous proteins with normal physiological functions early in the evolution of advanced snakes [11–13]. The vast majority of venomous snakes belong to three families: the highly toxic Elapidae and Viperidae, and the diverse, but generally less toxic, polyphyletic clade "Colubridae" [1,14,15]. Proteomic analyses of species within the family Viperidae demonstrate that venoms may contain up to 100 different compounds (including various isoforms), yet the majority can classified into a small number of protein families which dominate overall venom protein composition [1,16]. While snake venom metalloproteinases (SVMPs), serine proteinases, phospholipases A_2 and myotoxins account for the majority of proteins in most rattlesnake venoms, technological advances in proteomic [17] and transcriptomic [18–20] methodologies have allowed for the identification of less abundant and lesser known venom compounds, such as ohanin-like toxins, phospholipase B, and glutaminyl cyclase, in several viperid venoms [e.g. 21–23].

Disintegrins are common constituents of viperid venoms and are small (4-16 kDa) non-enzymatic, cysteine-rich proteins that result from a post-translational cleavage of the P-II class of SVMPs [24]. From a predation perspective, disintegrins may aid in the circulation of other venom compounds throughout prey by binding integrins $\alpha_{IIb}\beta_3$ and

inhibiting platelet aggregation [25,26]. We have recently shown that disintegrins also have an important functional role during envenomation, facilitating prey relocation by altering chemical cues emanating from envenomated prey and allowing for prey recovery via strike-induced chemosensory searching [8]. Structurally, disintegrins are classified based on their polypeptide length and number of disulfide bonds [27]. Short disintegrins consist of 41 to 51 amino acid residues and 4 disulfide bonds, whereas medium disintegrins are approximately 70 amino acids and have 6 disulfide bonds. The vast majority of disintegrins that have been characterized and studied belong to this medium size class. The third group, long disintegrins, is composed of 84 amino acids and 7 disulfide bonds. The fourth group, which consists of the homo- and heterodimeric disintegrins, has subunits of approximately 67 amino acids, including 10 cysteines which are involved in 4 intra-chain disulfide bonds and 2 interchain cysteine linkages [27,28].

Despite the fact that disintegrins are relatively conserved, significant differences are seen with respect to their binding affinity towards integrin receptors. These disintegrin-integrin interactions are primarily mediated by the disulfide-defined integrin-binding loop containing an RGD, KGD, MVD, MLD, MGD, WGD, or VGD sequence [27,29,30]. Although the amino acid residues adjacent to this binding motif also influence binding affinity [31], it has been suggested that the conserved aspartate residue in the tripeptide binding site is responsible for binding to the specific β integrin subunit, whereas the first two residues determine the affinity to the specific α integrin subunit [32]. The majority of characterized disintegrins contain an Arg-Gly-Asp (RGD) sequence which has been shown to block integrin $\alpha_{IIb}\beta_3$ on platelet membranes, in addition to integrins $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$ and $\alpha_v\beta_3$ (among others) which are expressed on many cell

membranes [33]. These disintegrin-integrin interactions have been shown to disrupt adhesion between cells and various ECM and plasma proteins such as fibronectin vitronectin, fibrinogen, laminin, and certain collagen [34 –37]

The use of venom compounds as potential therapeutics has long been an area of interest among venom researchers. Disintegrins in particular have been explored for biomedical applications due to their potent integrin blocking activity. In fact, two anti-platelet drugs currently on the market, tirofiban (aggrastat), and integrilin (eptifibatide), were both designed based on the structures of the venom disintegrins echistatin [38] and barbourin [31], respectively. Further, disintegrins have received significant attention for their anti-metastatic and anti-angiogenic properties [34–36,39–43] demonstrating their potential applications as an anti-cancer therapeutic. Contortrostatin, a homodimeric RGD disintegrin purified from the venom of *Agkistrodon contortrix contortrix* [34,35,39], and several monomeric RGD-containing disintegrins, such as colombistatin [36], have been shown to significantly inhibit experimental metastasis and cellular adhesion to specific ECM proteins. The pharmacological potential of these compounds provides a strong motivation to examine snake venoms for novel disintegrins that may have application in biomedical research and drug discovery efforts.

The current study was aimed at screening the venom of the Middle American Rattlesnake (*Crotalus simus tzabcan*) for novel disintegrins by a combination of cDNA analysis of transcripts and multistep liquid chromatography and mass spectrometry-based analysis of venom proteins. We isolated and characterized the most abundant disintegrin present in *C. s. tzabcan* venom and examined its cytotoxicity and ability to inhibit cell

adhesion of human colon adenocarcinoma (Colo-205) and breast adenocarcinoma (MCF-7) cell lines.

Materials and Methods

Snakes, Venoms and Biochemicals

Two adult *Crotalus simus tzabcan* were housed individually at the University of Northern Colorado Animal Resource Facility (UNC-IACUC protocol #0901C-SM-ML). Venom was extracted as previously described [44], pooled, and samples were centrifuged (1000 rpm for 5 min), lyophilized and stored at -20 °C until use. Matrigel (356234) was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Fibronectin (F0895), vitronectin (V8379), and all additional buffers and reagents (analytical grade or better) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Protein gels, mass standards and electrophoretic reagents were obtained from Invitrogen-Life Technologies (Grand Island, NY, USA).

Size Exclusion Liquid Chromatography (SE-LC)

Two-hundred and fifty milligrams of lyophilized venom were dissolved in 3.0 mL HEPES buffer solution (10 mM, pH 6.8, with 60 mM NaCl and 5 mM $CaCl_2$) and centrifuged at 3000 rpm for 5 min to pellet and remove insoluble material. This solution was then fractionated by low pressure size exclusion chromatography using a 90 x 2.6 cm column of BioGel P-100 medium (BioRad Inc., Hercules, CA, USA) equilibrated with the same HEPES buffer. Fractionation occurred at a flow rate of 6.0 mL/hr at 4 °C, and 30 min fractions were collected. Elution of size-fractionated protein and peptide peaks was monitored at 280 nm using a Beckman DU640 spectrophotometer.

Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

Using two additional RP-HPLC methods, peak 6 from SE chromatography was further purified using a Phenomenex Jupiter C_{18} (250 x 4.6 mm, 5 µm) column on a Waters HPLC system using Empower software. The C_{18} column was equilibrated with 95% of 0.1% TFA in water (solvent A) and 5% of 80% acetonitrile in 0.1% TFA in water (solution B) and elution was achieved as follows: 95% solvent A and 5% solvent B for 10 minutes; linear gradient to 85% A and 15% B over 1 minute; linear gradient to 60% A and 40% B over 65 minutes; linear gradient to 100% B over 2 minutes; isocratic at 100% B for 5 minutes; linear gradient to 95% A and 5% B over 2 minutes. The peak containing tzabcanin (RP-HPLC peak 2) was further purified using the same C_{18} column and a shallower gradient. Elution was achieved as follows: 95% A and 5% B, with a linear gradient to 79% A and 21% B over 10 minutes; linear gradient to 75% A and 25% B over 40 minutes; linear gradient to 100% B over 2 minutes; isocratic at 100% B for 5 minutes; linear gradient to 100% A over 2 minutes; isocratic at 100% A for 5 min. Fractions were collected using a Waters Fraction Collector II at a flow rate of 1.0 ml/min, and protein peaks were monitored at 220 and 280 nm using a Waters 2487 Dual Absorbance Detector. Fractions were collected, lyophilized, and stored at -20 °C until further use.

One-dimensional SDS-PAGE Electrophoresis

Crude *C. s. tzabcan* venom and SE-LC and RP-HPLC fractions were assessed for the number and relative molecular masses of protein components by SDS-PAGE under reducing (DTT) conditions using NuPAGE 12% Bis-Tris gels and MES running buffer

(electrophoresed for approx. 45 min) as described previously [8]. Twenty-four µg of crude venom or 3 µL SE-LC aliquots were added to each lane, and Mark 12 standards were used for mass estimation. For RP-HPLC fractions, 3 µg of lyophilized protein were resuspended in 1X reducing LDS buffer, placed into lanes on a NuPage 12% Bis-Tris gel and electrophoresed as above. Gels were stained with Coomassie Brilliant Blue overnight, destained and scanned with an HP Scanjet 4570C.

Mass Analysis (MALDI-TOF-MS)
And N-terminal Sequencing

Mass determinations of lyophilized protein samples obtained by RP-HPLC were analyzed using a Bruker Ultraflex MALDI-TOF/TOF mass spectrometer (Proteomics and Metabolomics Facility, Colorado State University, Fort Collins, CO) operating in linear mode. Protein samples (~0.5 µg) were spotted onto a sinapinic acid matrix (10 mg/mL 50% acetonitrile, 0.1% trifluoroacetic acid; 1.0 µL) and spectra were acquired in the mass range of 3.0-25 kDa. For N-terminal sequencing, approximately 50 µg of purified tzabcanin was reduced with dithiothreitol and alkylated with iodoacetamide as previously described [45]. Clean up and isolation of the reduced and alkylated product was accomplished by RP-HPLC using a Vydak C_{18} column as above, with a flow rate of 1.0 mL/min and a gradient (1%/min) of 5-50% ACN. Protein fractions were collected and dried via a Speed Vac and N-terminal sequencing (Edman degradation) was performed on an ABI Procise 494 protein sequencer (Protein Structure Core Facility, University of Nebraska Medical Center, Omaha, NE) to obtain the first 30 amino acid residues. The sequence obtained was subjected to Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

**RNA Isolation, cDNA Synthesis,
And 3' RACE**

RNA isolation, cDNA synthesis, and 3′ RACE of the *C. s. tzabcan* disintegrins was accomplished following methods of Modahl et al., 2015 (in review). Briefly, RNA was purified from approximately 6 mg of lyophilized venom resuspended in 1 mL of TRIzol (Life Technologies, CA, USA) following the manufacturer's recommended RNA protocol, with the addition of a 4 °C overnight incubation in 300 µL ethanol containing 40 µL 3 M sodium acetate to increase RNA yields. cDNA synthesis from total RNA was accomplished using the 3′ RACE System (Life Technologies, CA, USA) following the manufacturer's protocols. The oligo(dT) adaptor primer (provided with the kit) initiates reverse transcriptase cDNA synthesis at the poly(A) region of mRNA and effectively selects for polyadenylated mRNAs from the total RNA preparation. Two sets of 3′ RACE sense primers were designed to obtain *C. s. tzabcan* disintegrin sequences. The first sense primer (5′-GGAGAAGARTGTGACTGTGGC-3′) was designed from the tzabcanin N-terminal sequence, and the second sense primer (5′-GAGGTGGGAGAAGAWTGYGACTG-3′) was modified from a previous primer [46] by the addition of degenerate base pairs, determined from multiple sequence alignments of a diversity of disintegrins from the NCBI database, including multiple *C. adamanteus*, *C. viridis viridis, Agkistrodon piscivorus, Bothrops neuwiedi, Echis pyramidum, Echis coloratus* and *E. carinatus* metalloproteinase PII sequences. Each sense primer was used in a reaction with the 3′ RACE system AUAP antisense primer 5′-GGCCACGCGTCGACTAGTAC-3′. Twenty-three µL of PCR SuperMix High Fidelity polymerase (Life Technologies, CA, USA) was used with 1 µL of cDNA template and 0.5 µL of each primer (sense and antisense). PCR was done with seven touchdown cycles

of 94 °C for 25 seconds, 52 °C for 30 seconds, and 68 °C for two minutes. Thirty additional cycles followed at 94 °C for 25 seconds, 48 °C for 30 seconds, and 68 °C for two minutes with a final 68 °C extension for five minutes. The amplified products were electrophoresed on a 1% agarose gel, and bands of the estimated disintegrin size were removed and purified using the Wizard SV gel and PCR clean-up system (Promega, USA).

Cloning and Sequencing of Disintegrins

Amplified cDNA was ligated into the pGEM-T Easy Vector System (Promega, Inc.) and transformed into *Escherichia coli* DH5 α competent cells and grown on nutrient-rich agar plates overnight at 37°C. Recombinant plasmids were selected from agar plates, ten *E. coli* colonies were picked from each PCR and placed into 2 mL LB broth treated with 1 μL/mL ampicillin, and shaken overnight at 37°C. Plasmids of each *E. coli* colony were than purified using the Quick Clean 5M Miniprep Kit (GenScript, Inc) and sent for sequencing at the DNASU facility (Arizona State University, AZ, USA) using Big Dye V3.1 chemistry with samples processed on an Applied Biosystems 3730XL Sequence Analysis Instrument.

Cell Lines and Culture Conditions

Cancer cells, growth media, fetal bovine serum (FBS) and cell viability assay kits were purchased from American Type Cell Culture (ATCC; Manassas, VA, USA). Human colorectal adenocarcinoma cells (Colo-205; ATCC CCL-222) were maintained with ATCC-formulated RPMI-1640 medium supplemented with 10% FBS, and human breast adenocarcinoma cells (MCF-7; ATCC HTB-22) were maintained with Eagle's Minimum Essential Medium (EMEM) growth medium supplemented with 10% FBS and

10 µg/mL human recombinant insulin. Both cell lines were maintained in 75 cm^2 flasks as a monolayer culture in a humidified 5% CO_2 air incubator at 37 °C. Subcultivation of cells was performed according to ATCC instruction, using trypsin-EDTA (0.05% trypsin and 0.02% EDTA).

Cytotoxicity Assays

To examine the possible cytotoxicity of tzabcanin, as well as crude *C s. tzabcan* venom, the colorimetric MTT [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-teyrazolium bromide] assay was performed [47; see also 48]. One hundred µL aliquots of Colo-205 and MCF-7 cells, at a density of 5.0 x 10^5 cells/ml with complete media, were plated in 96-well cell culture plates and incubated with various concentrations of tzabcanin (0.22–14 µM), crude *C. s. tzabcan* venom (20 µg), or 10 µL of 0.01 M phosphate buffered saline (PBS, pH 7.2, as a control) at 37 °C for 24 hr. After 24 hr, 10 µL of MTT was added to the treated cells and plates were returned to 37 °C for 2 hr. Following incubation, 100 µL of Detergent Reagent (ATCC) were added to cells, which were then incubated at room temperature overnight in the dark. The plate was gently shaken and the absorbance read at 570 nm using a SpectraMax 190 plate reader. Assays at each tzabcanin concentration were performed in triplicate and each assay was repeated at least twice. Percent cell viability was calculated by the following formula: [(absorbance of treatment cells) - (absorbance of medium blank)/(absorbance of control cells) - (absorbance of medium blank)] x 100, and all values are reported as mean ± standard error of the mean.

Cell Adhesion Inhibition Assays

Inhibition of Colo-205 and MCF-7 cell binding to the extracellular matrix proteins fibronectin and vitronectin, and the basement membrane matrix Matrigel was measured as previously described [49]. Triplicate wells of Immulon-II 96 well microtiter plates were coated with either 100 µl of fibronectin (0.5 µg per well), vitronectin (0.3 µg per well), or Matrigel (0.5 µg per well) dissolved in 0.01M PBS, pH 7.2, and allowed to incubate overnight at 4 °C. Fluid was then removed and unbound proteins were removed by washing wells three times with 1% bovine serum albumin (BSA) in PBS, and unbound sites were blocked with 2.5% BSA in PBS (1 hr incubation at 37 °C). Both Colo-205 and MCF-7 cells were harvested as mentioned above and resuspended in serum-free medium containing 1% BSA at concentrations of 5 x 10^5 cells/mL. Cells were treated with various concentrations of tzabcanin (0.22-14 µM) and allowed to incubate at 37 °C for 1 hr immediately prior to seeding. The blocking solution was aspirated, wells were washed 2X with 100 µL 1% BSA in PBS, and 100 µL cells were seeded in the coated microtiter plate wells. Following a 1 hr incubation at 37°C, unbound cells were removed by gentle washing (3X) with 1% BSA in PBS, and then 100 µL of serum-free medium containing MTT (5:1, vol/vol) and 1% BSA was added to the wells. Following incubation at 37°C for 2 hr, 100 µL of Detergent Reagent was added to the wells and cells were incubated overnight in the dark at room temperature. The plate was gently shaken and the absorbance read as above. The percent inhibition was calculated by [(absorbance of control-absorbance of treatment)/absorbance of control] x 100. Assays at each tzabcanin concentration for all cell lines were performed in triplicate and each assay was repeated at least three times to confirm results.

Inhibitions of Cell Binding to Immobilized Tzabcanin

Because integrin binding interactions are dependent on divalent cations [50,51], cells were incubated with varying concentrations of a cation chelator (disodium EDTA) to examine if tzabcanin binding occurs specifically via integrin receptors. Purified tzabcanin (20 µg/mL) was resuspended in PBS and 100 µL aliquots were add to Immulon-II 96 well microtiter plates and incubated overnight at 4°C. Following overnight incubation, wells were washed and blocked as described above. Further, both Colo-205 and MCF-7 cells were treated as mentioned above except before addition to the disintegrin treated wells, cells were incubated for 60 min at 37°C with serum-free medium containing 1% BSA and various concentrations of EDTA. Cells were then added to the disintegrin treated wells and the adhesion assay was performed as described above. All assays were completed in triplicate and replicated at least 3 times.

Molecular Modeling

Three dimensional structure of tzabcanin was modeled from the primary structure using the I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) as described previously [7]. A viperid venom disintegrin with known solution structure was identified by I-TASSER as an appropriate template model (salmosin: Protein Database accession number 1L3X A). Figures were created using Discovery Studio Visualizer v3.1.1.11157 (Accelerys Software Inc., San Diego, CA).

Statistical Analyses

Data were analyzed by Analysis of Variance (ANOVA) followed by Tukey's post-hoc test using R version 2.15.2. All p values <0.05 were considered as statistically significant.

Results

Isolation and Masses of *C. s. Tzabcan* disintegrins and N-terminal Sequencing of Tzabcanin

Following SE chromatography, 9 major peaks were collected (Fig. 5.1) which were assessed by reduced SDS-PAGE (Fig. 5.1 inset) to identify proteins in the ~7kDa range, as this is the general mass of monomeric disintegrins [28,36,37,40]. Peak 6 yielded one protein band in this mass range (Fig. 5.1 inset), and was further fractionated by RP-HPLC chromatography, yielding 5 additional protein fractions that eluted between 22 and 28% solution B (Fig. 5.2a). Following reducing SDS-PAGE, all five fractions showed masses of approximately 7 kDa (Fig. 5.2b). The spectra obtained by MALDI-TOF indicate the presence of six disintegrin isoforms in *C. s. tzabcan* venom (Fig. 5.2C). The most prominent of these disintegrins (RP-HPLC Peak 2) was further purified by an additional RP-HPLC step (Fig. 5.3a), yielding one protein peak eluting at approximately 23% solvent B. This protein, with molecular mass of 7105.0 Da (Fig. 5.3b), was named tzabcanin. It was subjected to Edman degradation N-terminal sequencing, yielding the sequence GEECDCGSPANPCCDAATCKLRPGAQCADGLCCD and was assessed for cytotoxicity and inhibition of Colo-205 and MCF-7 cell adhesion to fibronectin, vitronectin and Matrigel.

cDNA Sequencing Analysis

Six cDNA sequences were obtained, ranging from 210 to 222 bp in length and coding for six protein isoforms varying from 69 to 73 amino acids (Fig. 5.4). NCBI protein BLAST analyses indicated that all amino acid sequences obtained belong to the disintegrin family. Five of the six sequences contain twelve cysteine residues,

characteristic of medium sized disintegrins [28]; tzbdis-4 contains only 11 cysteine residues. All six sequences also express the canonical RGD binding motif. Fig. 5.5 shows the alignment of the amino acid sequences from the six disintegrins and the N-terminal sequence in comparison with sequences of disintegrins purified from other viperid venoms. Tzabcanin, the most abundant of the six disintegrin isoforms, contains 71 amino acids, begins with a Gly, ends with an Ala, and shares 97% sequence identity to the disintegrin basilicin [P31981].

Cytotoxicity

Crude *C. s. tzabcan* venom (20 µg/100 µL) exhibited potent cytotoxicity towards both Colo-205 and MCF-7 cell lines, with approximately 35 and 26% cell viability, respectively, remaining after 24 hr incubation (both $p< 0.001$; Fig. 5.6). However, tzabcanin caused only a slight decrease in Colo-205 cell viability at concentrations of 1.75 µM and 3.5 µM (both $p<0.05$) and at 14 µM ($p<0.01$); 7 µM tzabcanin also resulted in a decrease in cell survival, but this treatment level failed to reach significance ($p>0.05$). Tzabcanin failed to exhibit significant cytotoxicity towards MCF-7 cells ($p>0.05$).

Inhibition of Cell Adhesion *In vitro*

Fibronectin and vitronectin support adhesion to Colo-205 and MCF-7 cells. Results indicate that tzabcanin inhibits adhesion of both cell lines to fibronectin and vitronectin in a dose-dependent manner (Figs. 5.7a & b). The greatest inhibition was observed for MCF-7 cells binding to fibronectin (81% binding inhibition at 14 µM; IC_{50} = 6.9 µM), and although adhesion of Colo-205 cells to fibronectin was hindered, the IC_{50} was twice as high (14 µM). Similarly, tzabcanin inhibited adhesion of both cell lines to

vitronectin (Fig. 5.7B); the highest binding inhibition was observed with Colo-205 cells (62% binding inhibition at 3.5 μM; IC_{50} = 6.6 μM), but inhibition of MCF-7 cell adhesion failed to reach 50% (44% binding inhibition at 14 μM). Matrigel supported adhesion in both cell lines, but tzabcanin failed to inhibit adhesion (data not shown).

Tzabcanin Binds to Colo-205 and MCF-7 Cells via Integrins(s)

Binding of both Colo-205 cells and MCF-7 cells to immobilized tzabcanin was inhibited when cells were incubated with varying concentrations of EDTA (Fig. 5.8). Approximately 95% inhibition of MCF-7 cell binding to immobilized tzabcanin was achieved at 5 mM EDTA (IC_{50} = 2.01 mM), and 55% binding inhibition of Colo-205 cells to tzabcanin was achieved at 5 mM EDTA. These results suggest that tzabcanin binds to both of these cell lines via integrin receptors.

Molecular Modeling of Tzabcanin

Like other medium disintegrins, tzabcanin adopts a semi-globular configuration, and the RGD integrin-binding domain is presented on the surface of the molecule (Fig. 5.9). The accessibility of this integrin-binding loop is believed to be critical to the binding efficiencies of disintegrins; in addition, a similar integrin-binding motif is found in the disintegrin-like domain of many P-III SVMPs [52] and is also likely functionally very important [53].

Discussion

Due to their potential as lead compounds for binding and blocking integrin receptors, disintegrins have become one of the most studied venom protein families to date. In the current study, a combination of molecular and proteomic techniques were utilized to screen the venom of *C. s. tzabcan* for potentially novel disintegrins. In addition

to disintegrins, SDS-PAGE of crude *C. s. tzabcan* venom and SE fractions showed masses indicative of venom compounds such as LAAOs, SVMPs, serine proteases and PLA_2s, which were confined to SE-HPLC peaks 1-5*;* these venom proteins were also observed in a recent proteomic analysis of *C. s. tzabcan* venom [54]. These enzymes have also been documented in the venom proteome of the closely related *C. simus* [55] and *C. simus simus* [56], and they are common to the venoms of numerous other *Crotalus* species [1]. SE peak 6 yielded one protein mass of approximately 8 kDa by SDS-PAGE and was subjected to an additional RP-HPLC step, yielding 5 additional protein peaks, each exhibiting molecular masses in the range of monomeric disintegrins as determined by both SDS-PAGE and MALDI-TOF mass spectrometry. When combined with cDNA analyses, results suggest the presence of numerous disintegrin isoforms with masses ranging from 7105 to 7637 Da, and 69 to 73 amino acids in length. The molecular masses, cysteine pattern, and polypeptide length places these disintegrins in the medium-size classification as described by Calvete et al. [27,28]. Further, although MALDI-TOF mass analysis indicates the presence of six isoforms which correlate with the six cDNA sequences, caution must be taken with attempting to assign the RP-HPLC peaks to the specific translated amino acid sequences reported here without further independent amino acid sequence data. Only RP-HPLC peak two, the most abundant of the disintegrins present, was further characterized by N-terminal sequencing and correctly assigned to the complete amino acid sequence obtained from cDNA sequences.

Disintegrin functionality is primarily due to interactions between the tripeptide binding loop, often containing an RGD sequence, and the specific integrin receptor. Molecular modeling demonstrates that this motif in tzabcanin is accessible to the solvent

and is therefore available for ligand binding, as expected among disintegrins which are proteolytically processed from P-II SVMPs. In P-III SVMPs, which express a disintegrin-like domain, it has been suggested that the distinct disulfide bond arrangement near the integrin-binding motif may lead to a very different functionality [52,57]. Further, although the P-III SVMP BjussuMP-I exhibits an RGD sequence in the disintegrin-like domain [58], the majority of P-III SVMPs express an XCD sequence [57], likely contributing to different integrin-binding affinities. For example, the disintegrin-like domain of alternagin-C contains an ECD binding motif and exhibited potent activity against integrin $\alpha_2\beta_1$, yet failed to show activity towards integrins $\alpha_{IIb}\beta_3$, $\alpha_1\beta_1$, $\alpha_5\beta_1$, $\alpha_4\beta_1$, $\alpha_9\beta_1$, or $\alpha_V\beta_3$ [59]. Likewise, it has been suggested that in P-II SVMPs that fail to release the disintegrin domain, the presence of the metalloproteinase domain may sterically alter the accessibility of the RGD motif, lowering integrin-binding activity [57]. Binding specificity and affinity is also influenced by the amino acid residues flanking this tripeptide sequence, as well as characteristics of the C-terminal region of the protein [28,60]. Of the six disintegrins reported here, four contain an RGDW binding motif, and two, including tzabcanin, contain an RGDN binding region. One of these disintegrins, tzbdis-1, contains a Glu29 in the N-terminal region of the protein, whereas the other five disintegrins contain an Asp at this position. The presence of this Glu, in addition to a Lys at position 43 in tzbdis-1, are the only dissimilarities between this disintegrin and tzabcanin, both of which contain an RGDN binding domain and identical C-terminal sequences. However, these two amino acid differences in the N-terminal region of the protein likely do not influence activity of the disintegrins. Tzbdis-4, on the other hand, contains an additional N-terminal Glu at position 1, Val at position 2, an Arg at position

26, and completely lacks Cys27 which, when present, forms a disulfide bond with Cys58 located in the C-terminal region of the disintegrin. As integrin inhibitory activity is dependent on the appropriate pairing of cysteine residues [61], the deletion of this disulfide bond likely abolishes inhibitory activity of this specific disintegrin [62].

Differences in the C-termini regions are apparent when comparing RGDW to RGDN disintegrins. All RGDW disintegrins (tzbdis-3-6) reported here contain an Asn at position 53, Thr at position 58, and the sequence GLYG at the C-terminal end of the protein. Further, tzbdis-6 contains an Arg at position 62, whereas all other *C. s. tzabcan* disintegrins contain a Gln at this position. In contrast, the two RGDN disintegrins, tzbdis-1 and tzabcanin, contain a Pro at position 53, Arg at position 58, and end with the sequence HFHA. Combined with the substitution of a Trp with Asn at a position carboxyl to the RGD sequence, these additional differences in the C-terminal regions may significantly influence disintegrin activities. Disintegrins expressing RGDW domains often show high affinity to $\alpha_{IIb}\beta_3$ integrins, whereas RGDN show higher selectivity towards both $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins [31,32,60].

Tzabcanin has a mass of 7105 Da and shares high amino acid identity with basilicin from *C. basiliscus* venom. Basilicin has an additional alanine at the N-terminus, a Glu at position 29 and Lys at position 43, whereas tzabcanin contains an Asp and Thr at these positions, respectively. Both disintegrins also have identical RGDN and C-terminal sequences. Yet even with identical binding regions, structural discrepancies in the C-terminal region can alter biological activity. Differences in ADP-induced platelet aggregation have been documented between colombistatin and cotiarin, both RGDN disintegrins that differ only by the presence of a Tyr72 in colombistatin, whereas cotiarin

exhibits His72 [36]. Of the RGDW disintegrins present here, it is hypothesized that tzbdis-3 and 5, due to their identical RGDW and C-terminal regions, may exhibit very similar, if not identical, biological activity, yet may be somewhat different from tzbdis-6. Likewise, due to the identical RGDN and C-termini regions, both tzbdis-1 and tzabcanin likely exhibit very similar biological activity.

Although crude *C. s. tzabcan* venom was highly toxic to both Colo-205 or MCF-7 cell lines, purified tzabcanin showed very low levels of cytotoxicity to Colo-205 cells and failed to exhibit cytotoxicity at concentrations as high as 14 μM following 24 hr treatment of MCF-7 cells. Disintegrin-induced apoptosis in HUVEC cells has been documented with rhodostomin [63] as well as with accutin [64]; however, the ability of disintegrins to induce apoptosis varies between cancer cell lines. Lucena et al. [42] found that the recombinant RGDN disintegrin, r-viridistatin, failed to induce apoptosis in SK-12 melanoma cells, and the disintegrin rhodostomin did not induce apoptosis of MDA-MB-231 cells [65]. Similarly, the homodimeric disintegrin contortrostatin was found to lack cytotoxicity toward MDA-MB-435 cells *in vitro* [35]. However, recently Lucena et al. [43] showed that recombinant disintegrins r-virdistatin 2 and r-mojastin 1 induced apoptosis in approximately 20% of human pancreatic adenocarcinoma (BXPC-3) cells. Therefore, the slight decrease in Colo-205 cell viability in the presence of high concentrations of tzabcanin could be due to induction of apoptosis, or a loss of membrane integrity, ultimately leading to antiproliferative effects. On the other hand, the potent toxicity of crude venom towards both cell lines is likely due to the presence of LAAOs, SVMPs, and PLA_2s, which are abundant in *C. s. tzabcan* venom and have been shown to exhibit a combination of cytotoxic and apoptotic activities [66–68]. Similarly, Bradshaw

et al. [48] also showed that the venom of *C. s. tzabcan* venom was significantly cytotoxic to both MCF-7 and human melanoma (A-375) cell lines.

Integrins are critical to cell attachment, migration and invasion, and their significance in cancer progression is being examined extensively [69,70]. Integrin engagement to ECM proteins induces cell proliferation and may prevent apoptosis in some cancers, demonstrating the significance of cell adhesion in tumor progression and survival. α_v integrins, in addition to $\alpha_5\beta_1$, $\alpha_8\beta_1$, and $\alpha_{IIb}\beta_3$, mediate cell adhesion to various ECM proteins, often by recognition of the tri-peptide RGD binding motif [71]. These integrin-ligand interactions are dependent on divalent cations, especially $Mn^{2+}$and Mg^{2+}, to support ligand binding [50]. Ca^{2+}, on the other hand, fails to support binding, yet it greatly regulates ligand adhesion supported by $Mn^{2+}$and Mg^{2+} [50]. The inhibition of both Colo-205 and MCF-7 cell binding to immobilized tzabcanin in the presence of a cation chelator (EDTA) indicates that tzabcanin-integrin binding was occurring, likely through the presence of the RGD domain. The low binding inhibition seen in Colo-205 cells (~55% binding inhibition at 5 mM EDTA) compared to MCF-7 cells suggests that tzabcanin binding is occurring in this cell line through a higher number of integrins, requiring a higher concentration of EDTA to produce inhibition. Although tzabcanin binds to both Colo-205 and MCF-7 cells, tzabcanin-induced binding inhibition of these cell lines to fibronectin and vitronectin is not as potent as has been reported for some other disintegrins. Differential potency may be cell line dependent; for example, colombistatin had a potent inhibitory effect (IC_{50} = 33 nM) on SK-Mel-28 cell adhesion to fibronectin, yet much higher concentrations (IC_{50} = 4.4 μM) of this disintegrin were needed to inhibit T24 cells from binding to fibronectin [36]. A recombinant form of

viridistatin-2 also showed varying binding inhibition of numerous cell lines to fibronectin, with IC_{50} values ranging from 11 to 4450 nM [42]. Further, although inhibition of cell adhesion was not as potent as other disintegrins, tzabcanin may exhibit additional anti-metastatic properties by binding different integrin receptors. For example, crotatroxin 2 failed to inhibit 66.3p cell adhesion to fibronectin, yet it significantly inhibited cell migration *in vitro* and lung tumor colonization *in vivo* [40].

Colo-205 and MCF-7 cells express integrins $\alpha_5\beta_1$, $\alpha_v\beta_5$ and $\alpha_v\beta_6$, as well as several other α/β subunits [72–75]; however, the expression of these integrins and specific subunits may drastically vary. For instance, Colo-205 cells show low levels of α_5, but moderate levels of α_v, α_1, and α_3, and high levels of α_2, α_6 and β_1 subunits [72], each displaying specificity to one or several discrete ligands. Fibronectin is recognized by an array of integrins including $\alpha_5\beta_1$ and $\alpha_v\beta_6$, which were first characterized for their ability to bind to this ECM protein [76–78]. Integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_3\beta_1$ $\alpha_4\beta_1$ and $\alpha_8\beta_1$ are additional receptors of fibronectin [79]. Fibronectin-integrin interactions contribute to numerous stages of tumor development, including tumor migration, invasion and metastasis [80]. $\alpha_v\beta_5$ has classically been known as a vitronectin receptor [81,82] however, integrin $\alpha_v\beta_6$ has also been shown to adhere to vitronectin [83]. Therefore, Colo-205 and MCF-7 cells express an array of integrins that show recognition to both fibronectin and vitronectin. Because many disintegrins have the capability to recognize an array of integrins, the relatively weak potency of tzabcanin suggest that this disintegrin may inhibit cell adhesion by binding to a more select group of integrins, leaving other receptors available for integrin-ECM interactions. Further, tzabcanin also contains an RGDN binding domain which has been shown to exhibit higher affinity to integrin $\alpha_v\beta_3$ [31,60], an integrin not

expressed on either Colo-205 or MCF-7 cells. Therefore, studies utilizing cell lines expressing $\alpha_v\beta_3$ are predicted to demonstrate greater potency of tzabcanin.

Conclusions

This study reports the isolation and characterization of the most abundant disintegrin, tzabcanin, from the venom of *C. s. tzabcan*. In addition, we report molecular masses and cDNA sequences of five additional medium-sized disintegrin isoforms from the same venom. The documented differences in integrin-binding affinity between RGDW and RGDN disintegrins could represent distinct biological roles for these integrin homologs and differential anti-cancer effects may also exist. Tzabcanin, a new RGD disintegrin, was not cytotoxic to MCF-7 cells but produced a slight decrease in cell viability in Colo-205 cells at high concentrations. This could be due to induction of apoptosis by binding to an integrin(s) specifically expressed on Colo-205 cells that may be absent on the MCF-7 cell line. By binding to integrins, tzabcanin also inhibited cell adhesion of both cell lines to fibronectin and vitronectin. Although this inhibition was not as potent as reported for other disintegrins, the binding domain (RGDN) of tzabcanin suggests that it may have higher affinity towards $\alpha_v\beta_3$ integrins which are not present in either Colo-205 or MCF-7 cells.

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Figures

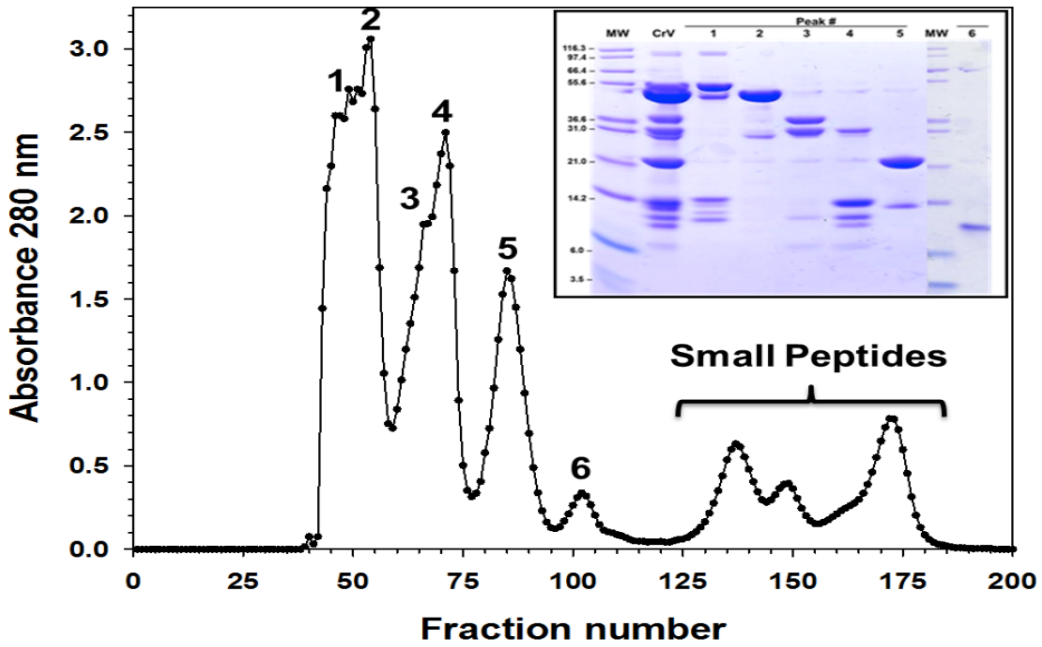

Figure 5.1: Size Exclusion Chromatography of Crude *C. s. tzabcan* Venom. A total of 250 mg of crude venom was fractionated on a BioGel P-100 column. Fractions collected and crude venom (CrV) were analyzed by SDS-PAGE (inset) under reducing conditions; fraction 6 contained disintegrin-sized peptides.

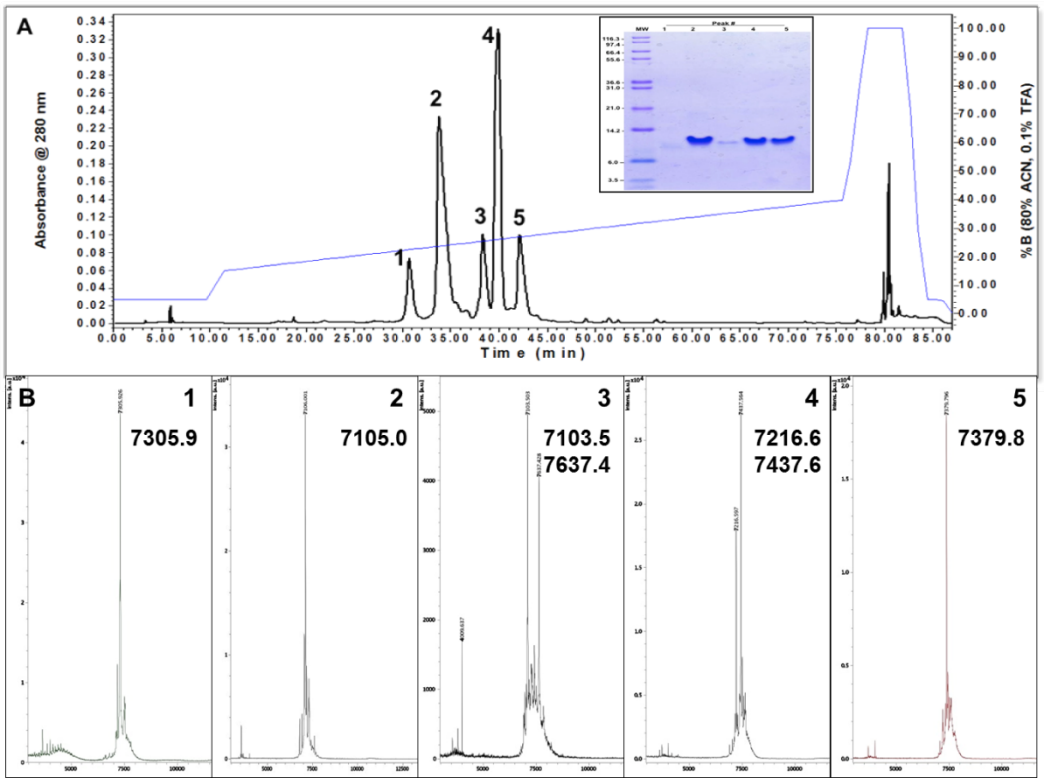

Figure 5.2: RP-HPLC Purification and Mass Determination by MALDI-TOF-MS of *C. s. tzabcan* Venom Disintegrins. (a) RP-HPLC of SE peak 6. 2 mL samples were injected into a Phenomenex Jupiter C_{18} column and separated on a Waters 515 HPLC system. Five protein peaks eluted between 22 and 26% solvent B. Fractions were collected and analyzed by SDS-PAGE (inset) under DTT-reducing conditions. (b) Mass spectra of RP-HPLC peaks 1-5; tzabcanin was present in peak 2 (m = 7105 Da), and peaks 3 and 4 yielded two masses as indicated.

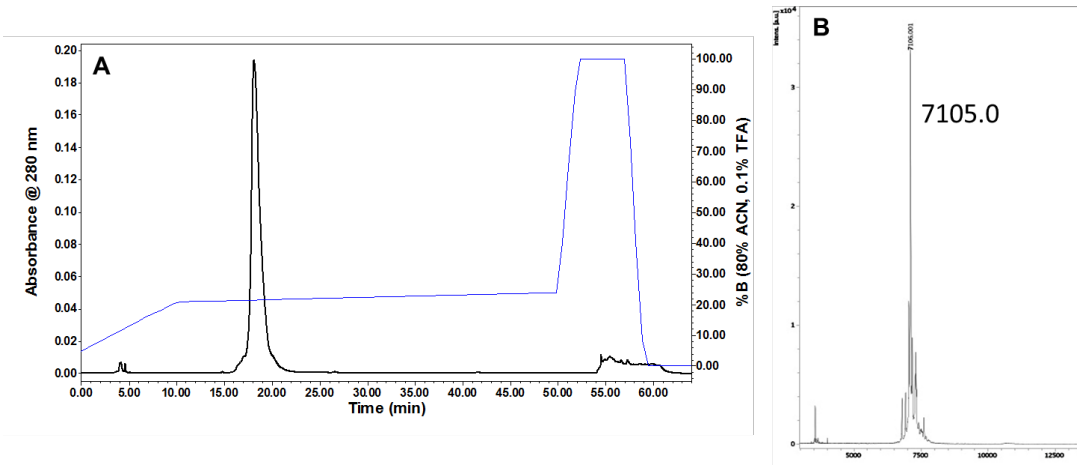

Figure 5.3: RP-HPLC Polishing and Mass Determination of Tzabcanin. (a) RP-HPLC peak 2 was further purified using a very shallow gradient. One peak eluted at 23% ACN and was assessed by MALDI-TOF mass spectrometry. (b) Mass determination of tzabcanin by MALDI-TOF mass spectrometry analysis in positive linear mode indicated a native mass of 7105 Da.

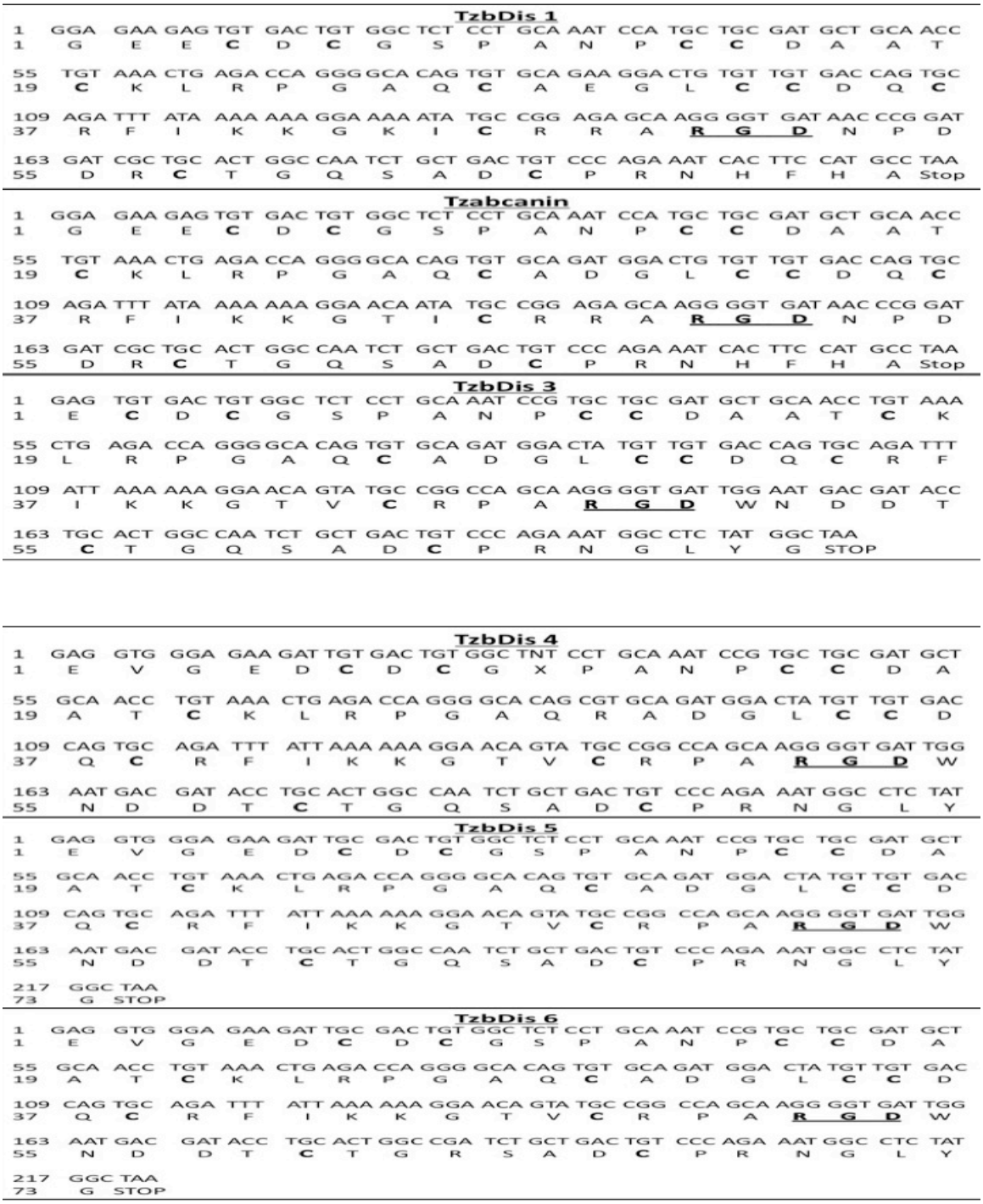

Figure 5.4: cDNA Sequence and Predicted Amino Acid Sequence of *C. s. tzabcan* Disintegrins. The cDNA sequence is located on the upper line and the corresponding amino acid sequence is below. The cysteine residues are in bold print and the RGD binding motif is in bold print and underlined.

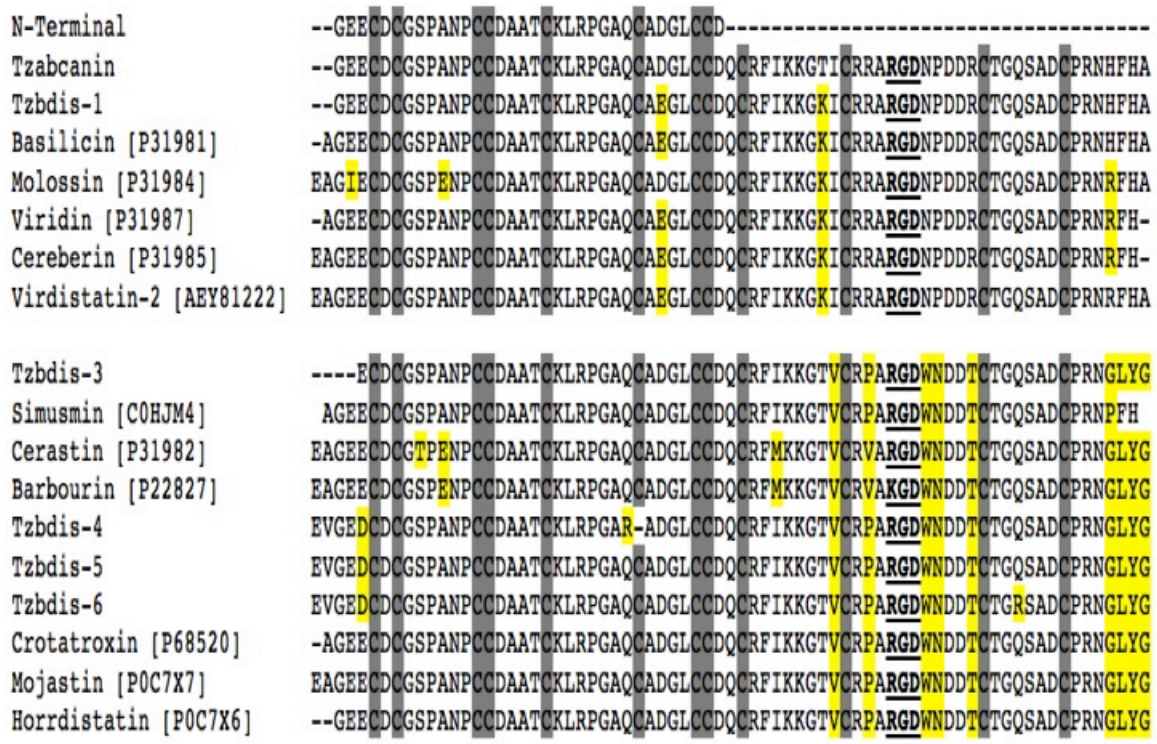

Figure 5.5: Amino Acid Sequence Comparison of *C. s. tzabcan* Disintegrins with Selected Disintegrins. RGDN disintegrins are in the top group and RGDW disintegrins are in the bottom diagram. One-letter code for amino acids is used. Cysteine residues are shaded in gray, and the RGD binding motif is in bold print and underlined. Residue differences from tzabcanin are shaded in yellow.

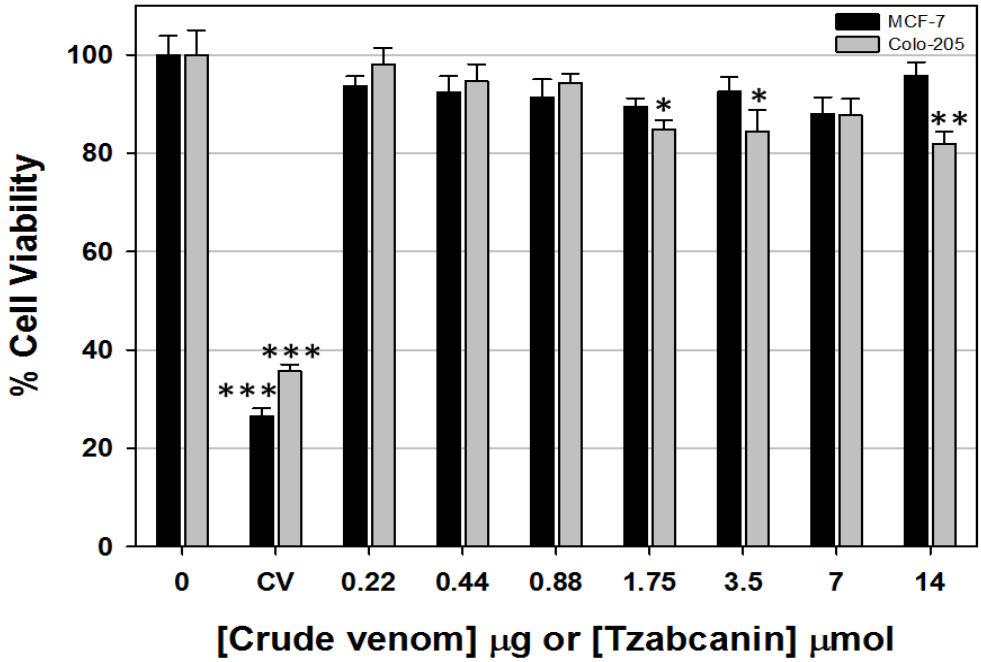

Figure 5.6: Percent Cell Viability of Colo-205 and MCF-7 Cells. Cells were incubated with either crude *C. s. tzabcan* venom (CV) or purified tzabcanin. * $p < 0.05$, **$p < 0.01$, *** $p < 0.001$, compared to controls.

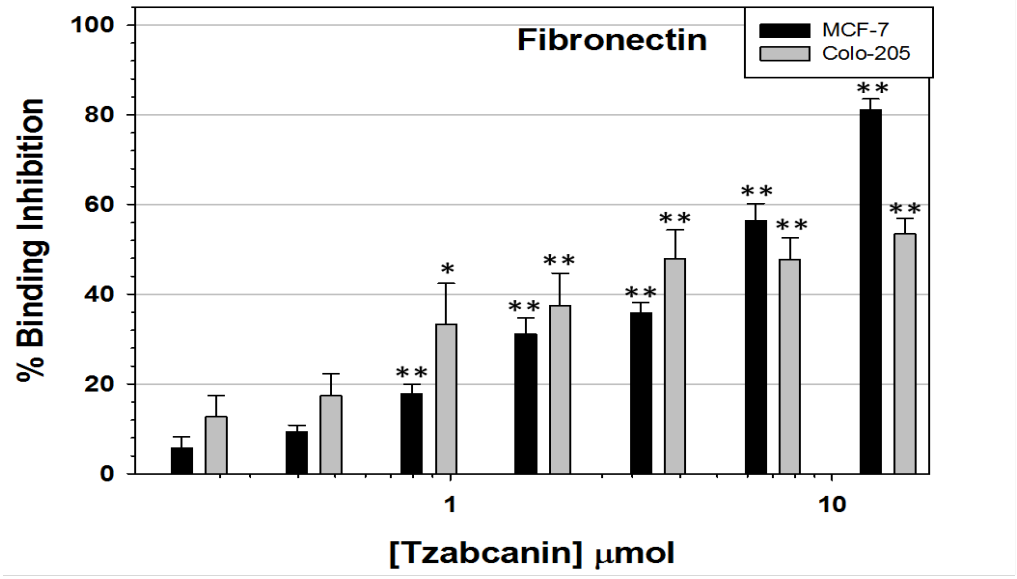

Figure 5.7a: The Effect of Tzabcanin on Colo-205 and MCF-7 Adhesion to Fibronectin; semilog plot. Various concentrations of tzabcanin (0.22 μM – 14 μM) were incubated with Colo-205 and MCF-7 cells (5.0 x 10^5 cells/mL) prior to addition to 96-well culture plates containing immobilized fibronectin. * $p < 0.01$, ** $p < 0.001$

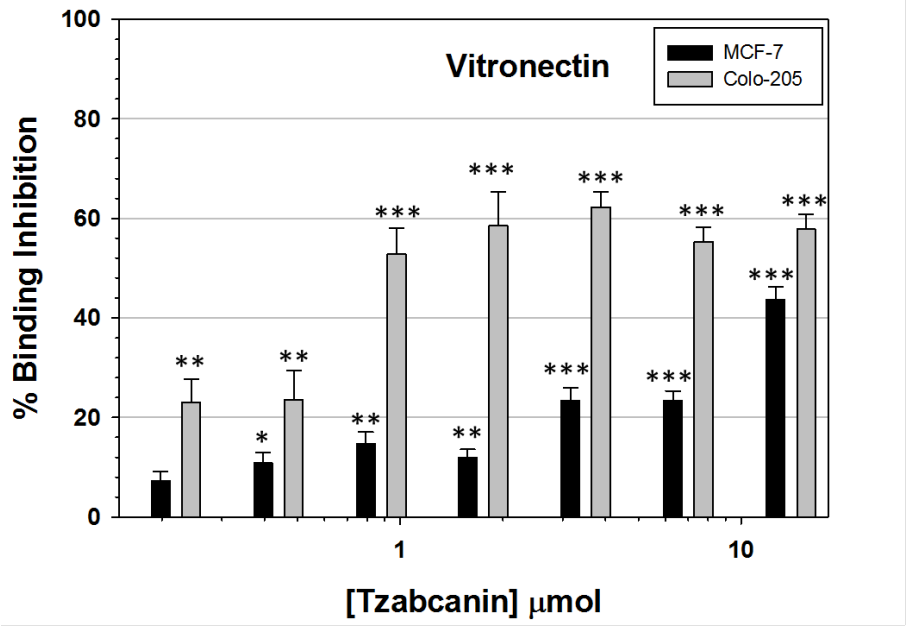

Figure 5.7b: The effects of tzabcanin on Colo-205 and MCF-7 adhesion to Vitronectin; semilog plot. Various concentrations of tzabcanin (0.22 μM – 14 μM) were incubated with Colo-205 and MCF-7 cells (5.0 x 10^5 cells/mL) prior to addition to 96-well culture plates containing immobilized vitronectin. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, relative to controls.

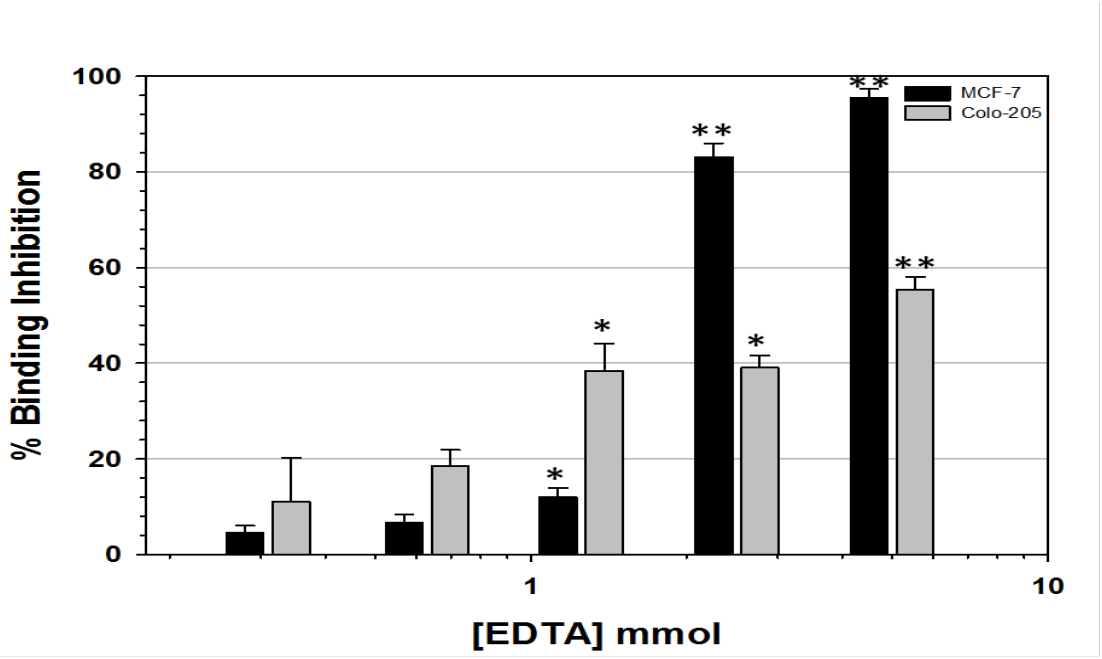

Figure 5.8: Percent Binding Inhibition of Cells to Immobilized Tzabcanin. Inhibition of Colo-205 and MCF-7 cells to immobilized tzabcanin was measured following cell incubation with the cation chelator EDTA; semilog plot. Colo-205 and MCF-7 cells (5.0 x 10^5 cells/mL) were incubated with various concentrations of EDTA (0.3 – 5 mM) prior to addition to wells containing immobilized tzabcanin. * $p < 0.01$, ** $p < 0.001$, relative to controls

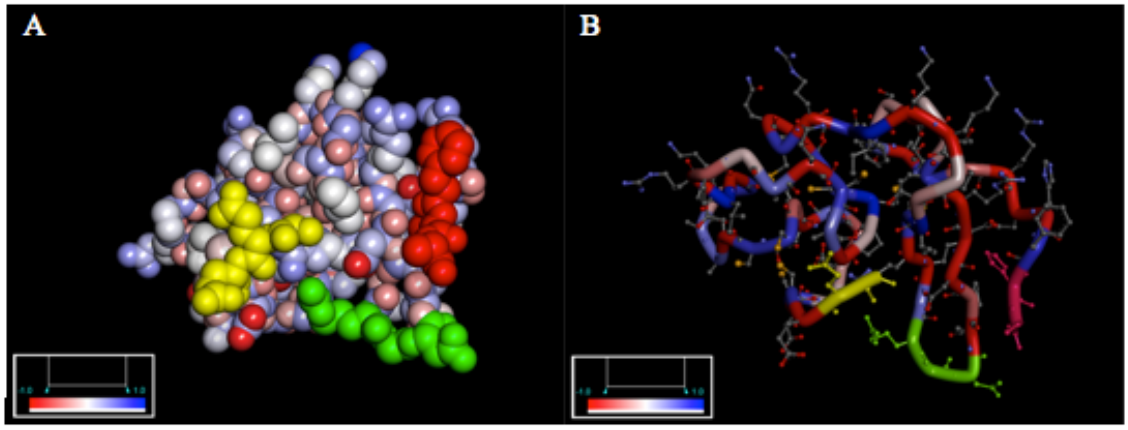

Figure 5.9: Molecular Modeling of Tzabcanin. Space filling model (a) and stick model (b) of tzabcanin showing the same face of the molecule. In both figures, the RGD domain is shown in green, the N terminus is in yellow and the C terminus is shown in red. Surface charge features of other residues are shown in red to blue shading. Models are based on I-TASSER-derived modeling of tzabcanin and were constructed using Accelerys Discovery Studio 3.1 software.

CHAPTER VI

TZABCANIN, A SNAKE VENOM DISINTEGRIN FROM *CROTALUS SIMUS TZABCAN*, INHIBITS $\alpha_V\beta_3$ – MEDICATED CELL ADHESION AND MIGRATION IN MELANOMA (A-375) AND LUNG (A-549) CANCER CELLS

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Abstract

The role of integrins in cancer progression and invasion is critical to the development of the disease, making these transmembrane proteins attractive targets in drug development and design. Disintegrins are small (4-16 kDa), Arg-Gly-Asp-containing proteins, common in the venoms of many viperid snakes. These non-enzymatic proteins inhibit integrin-mediated cell-cell and cell-extracellular matrix interactions, making them potential candidates as therapeutics in cancer and numerous other human disorders. The present study examines the anti-adhesion and migration effects of a recently characterized disintegrin, tzabcanin, towards melanoma (A-375) and lung (A-549) cancer cell lines. Tzabcanin inhibits adhesion of both cells lines to vitronectin and exhibited weak cytotoxicity towards A-375 cells; however, it had no effect on cell viability in A-549 cells. Further, tzabcanin inhibited adhesion of both cell lines to the extracellular matrix protein vitronectin, and it significantly inhibited cell migration in cell scratch wound assays. Flow cytometric analysis indicates that both cell lines express integrin $\alpha_v\beta_3$, a critical integrin in tumor motility and invasion which is identified here to be a binding site of tzabcanin. Tzabcanin blocks $\alpha_v\beta_3$, inhibiting cell adhesion to vitronectin and migration in A-375 and A-549 cell lines, suggesting that it may have utility for developing anticancer therapies.

Introduction

Integrins comprise an important family of cell surface receptors that mediate cell-cell and cell-extracellular matrix (ECM) interactions (Hynes, 1987; Albelda and Buck, 1990). To date, 24 distinct integrin heterodimers have been described, based on the appropriate noncovalent pairing of one of 18 α subunits with one of 8 distinct β-subunits

(Hynes, 2002). The specific pairing of these subunits regulates which substrates a cell will adhere to and migrate on, which will influence the activity of the cell (Aplin et al., 1999). Typically the α-subunit dictates ligand specificity, whereas the β-subunit associates with the downstream signaling pathway (Hynes, 2002; Barczyk et al., 2010). Further, integrins have the ability to recognize a single, or several, ECM ligands or cell membrane proteins, each contributing to the regulation of an array of cellular functions (Plow et al., 2000; van der Flier and Sonnenberg, 2001; Humphries et al., 2006). For example, integrins $\alpha_5\beta_1$, $\alpha_4\beta_1$ and $\alpha_v\beta_3$ recognize fibronectin, and $\alpha_v\beta_3$ in addition to $\alpha_v\beta_5$ show high affinity to vitronectin and fibrinogen. Integrins $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_6\beta_1$ bind laminin, and both $\alpha_1\beta_1$ and $\alpha_2\beta_1$ recognize collagen (Humphries et al., 2006). The integrin $\alpha_{IIb}\beta_3$, which is expressed in platelets, binds to fibrinogen or von Willebrand factor, where it assists in platelet aggregation (Bennet et al., 1982; Humphries et al., 2006). Approximately one-third of the 24 integrins recognize these adhesive molecules through the tripeptide Arg-Gly-Asp (RGD) binding sequence, while others bind the triple helical GFOGER sequence present in collagen (Barczyk et al., 2010), or YIGSR in laminin (Gehlsen et al., 1988). As integrins are critical to numerous aspects of cell function, mutations targeting integrin receptors or integrin-related pathways are known to contribute to numerous human disorders (Wehrle-Haller and Imhof, 2003).

It is well documented that several integrins play critical roles in cancer progression (Desgrosellier and Cheresh, 2010; Rathinam and Alahari, 2010; Sun et al., 2014), and they have a significant role in tumor angiogenic activity, proliferation, survival and metastasis (Clark and Brugge, 1995; Serini et al., 2006). Further, expression of these cell membrane proteins may vary significantly between normal and cancerous

tissue, increasing their potential as selective targets in cancer therapy (Goodman and Picard, 2002; Kumar 2003; Sun et al., 2014). Integrins $\alpha_6\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_6$ are almost undetectable in normal epithelial tissue (Desgrosellier and Cheresh, 2010), yet may be highly over-expressed in cancerous cells (Gehlsen et al., 1992; Nam et al., 2010). Integrin $\alpha_v\beta_3$ has been shown to increase 50-100 fold in melanoma (A-375) cells displaying an increased metastatic phenotype, indicating that increased integrin expression is correlated with advanced cancer states (Gehlsen et al., 1992). Tumor dependence on angiogenesis is also well documented (Folkman, 1971, 2006, 2007) and this formation of new blood vessels is required for delivering nutrients as well as providing a means of waste removal for tumors. Although numerous integrins are involved in angiogenesis (Avraamides et al., 2008), evidence indicates that $\alpha_v\beta_3$ is critical for tumor angiogenic activity (Brooks et al., 1994; Varner et al., 1995), likely permitting angiogenic endothelial cells to recognize proteins present in the tumor microenvironment (Desgrosellier and Cheresh, 2010). The significance of integrins in cancer biology cannot be overemphasized, so the isolation and characterization of compounds that have integrin-blocking activity and potential anti-angiogenic functions may result in novel anti-neoplastic therapies and reveal new approaches to controlling cancer cell proliferation and metastasis (Folkman, 2006).

The use of toxins as potential therapeutics has been an increasing emphasis of biomedical research in the last decade, and several novel compounds developed from the poisons and venoms of animals are currently in clinical trials and use (Fox and Serrano, 2007; Takacs and Nathan, 2014). Snake venoms in particular have been a promising source of several protein drugs and additional protein drug leads (Vonk et al., 2011; Saviola et al., 2014). Snake venoms consist of a complex mixture of proteins and

peptides that exhibit an array of biochemical and pharmacological functions (Mackessy, 2010). As many of these proteins are often mimics of compounds with normal physiological activities, but contain dramatically different pharmacologies, venom components have been subjected to detailed examination for their potential in biomedical or therapeutic use (Fox and Serrano, 2007; Lin et al., 2010; Mukherjee and Mackessy, 2013). One class of venom proteins, the disintegrins, are small, cysteine-rich, non-enzymatic proteins that result from the post-translational proteolytic processing of the enzymatic P-II class of snake venom metalloproteinases (Kini and Evans, 1992; Calvete et al. 2005). Many disintegrins contain an RGD-binding domain in the carboxyl terminal portion of the molecule and were originally characterized due to their ability to inhibit platelet aggregation by binding integrin $\alpha_{IIb}\beta_3$ (Haung et al., 1987). RGD disintegrins have also been shown to bind integrins $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, and $\alpha_v\beta_3$, and variants of this tripeptide sequence demonstrate differing levels of selectivity to numerous integrin receptors (Calvete et al., 2009).

Due to their potent integrin binding activity, disintegrins are continuously being examined for their ability to reduce experimental metastasis. Contortrostatin, a homodimeric RGD disintegrin from the venom of the Southern Copperhead (*Agkistrodon contortrix contortrix*), has been shown to inhibit cell adhesion, migration, invasion and angiogenesis in numerous cancer cell lines (Trikha et al., 1994; Zhou et al., 2000, 2001; Lin et al., 2010). In addition, monomeric disintegrins such as crotatroxin 2 and colombistatin have also been shown to exhibit various anti-cancer effects (Galán et al., 2008; Sanchez et al., 2009). We previously reported the isolation and characterization of a novel 7.1kDa, RGD-containing disintegrin from the venom of the Middle American

Rattlesnake (*Crotalus simus tzabcan*). Tzabcanin was not cytotoxic, but it inhibited colon (Colo-205) and breast (MCF-7) cancer cell adhesion to the ECM proteins fibronectin and vitronectin (Saviola et al., in review). Analyses of cell adhesion assays suggest that tzabcanin may bind $\alpha_v\beta_5$ and $\alpha_v\beta_6$, both of which are expressed in Colo-205 and MCF-7 cell lines (Agrez et al., 1996; Taherian et al., 2011), and both recognize VN and FN, respectively. However, it has been reported that neither of these cell lines express the integrin $\alpha_v\beta_3$. Since integrin $\alpha_v\beta_3$ has been shown to contribute significantly in the progression of cancer (Gehlsen et al., 1992; Wong et al., 1998; Pecheur et al., 2002; Sloan et al., 2006), the current study was designed to examine if tzabcanin inhibits $\alpha_v\beta_3$-mediated cell adhesion and migration in two highly metastatic melanoma (A-375) and lung carcinoma (A-549) cell lines which do express integrin $\alpha_v\beta_3$.

Materials and Methods

Snakes, Venoms and Biochemicals

Venoms from two adult Middle American Rattlesnakes (*Crotalus simus tzabcan*) housed individually at the University of Northern Colorado Animal Resource Facility were extracted as previously described by Mackessy (1988). Venoms were centrifuged (10,000 rpm for 5 min), lyophilized, and stored at -20°C until use. Matrigel (356234) was purchased from BD Biosciences (Franklin Lakes, NJ, USA). $\alpha_v\beta_3$ antibody (sc-7312 FITC) conjugated with a FITC was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Fibronectin (F0895), vitronectin (V8379), and all buffers and additional reagents (analytical grade) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

Purification of Tzabcanin

Isolation and purification of tzabcanin was conducted as previously described (Chapter V; Saviola et al., in review) by a combination of multistep size exclusion and two steps of C_{18} reverse-phase liquid chromatography. Mass determination, purity and identification of tzabcanin was ascertained by both SDS-PAGE and MALDI-TOF mass spectrometry as described (Chapter V; Saviola et al., in review).

Cell Lines and Culture Conditions

Human malignant melanoma (A-375; ATCC CRL-1619) and human lung adenocarcinoma (A-549; ATCC CCL-185) cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). A-375 cells were maintained in 75 cm^2 flasks in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained as a monolayer culture ($\leq$80% confluent) in a humidified 5% CO_2/air incubator at 37°C. A-549 cells were also maintained in 75 cm^2 flasks with ATCC-formulated F-12K growth medium supplemented with 10% FBS as a monolayer culture under the same conditions mentioned above. Subcultivation of cells was performed according to ATCC instruction, using trypsin-EDTA (0.05% trypsin and 0.02% EDTA). Cells were counted manually with a hemocytometer (4x, averaged) and diluted to appropriate densities.

Cytotoxicity of Tzabcanin Towards A-375 and A-549 Cells

Cytotoxicity of tzabcanin towards A-375 and A-549 cells was measured by the colorimetric MTT [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-teyrazolium bromide] assay (Mossmann 1983; see also Bradshaw et al. in press). Both cell lines were trypsinized and resuspended in complete media at a concentration of 5.0 x 10^5 cells/ml.

One hundred microliter aliquots of A-375, and A-549 cell suspensions were plated in 96-well cell culture plates with various concentrations of tzabcanin (0.22 – 14 μM) or 20 μg of crude *C. s. tzabcan* venom and incubated at 37 °C for 24 hr. After 24 hours, 10 μL of MTT reagent (ATCC) was added to the cells which were then returned to 37 °C for 2 hr. Following incubation, 100 μL of Detergent Reagent (ATCC) were added to cells, which were then incubated overnight in the dark at room temperature. The plate was gently shaken and the absorbance read at 570 nm using a SpectraMax 190 spectrophotometer. Assays at each concentration of tzabcanin were performed in triplicate and each assay was repeated at least twice.

A-375 and A-549 Cell Adhesion Assays

Triplicate wells of Immulon-II 96 well microtiter plates were coated with 100 μl of either tzabcanin (2 μg per well), fibronectin (0.5 μg per well), vitronectin (0.3 μg per well) or Matrigel (0.5 μg per well) which was dissolved in 0.01M PBS, pH 7.2; protein was allowed to incubate overnight at 4°C. Excess proteins were washed away twice with 1% bovine serum albumin (BSA) in PBS and unbound sites were blocked with 100 μl 2.5% BSA in PBS and incubated at 37 °C for 1 hr. Cells were treated with various concentrations of tzabcanin (7.8 nM – 2 μM) and allowed to incubate at 37 °C for 1 hr immediately prior to seeding in treated plates. The blocking solution was aspirated, and excess proteins were washed away twice with 1% BSA in PBS. One hundred microliters of tzabcanin-treated cells (5 x 10^5/mL) were seeded in the coated microtiter plate wells and returned to 37 °C for 1 hr. Unbound cells were washed away 3 times with 1% BSA in PBS by filling and aspirating, and 100 μL of serum-free medium with 1% BSA containing MTT (5:1 vol/vol) was added to wells and incubated at 37 °C for 2 hrs. One

hundred microliters of Detergent Reagent was then added to the wells and cells were incubated overnight in the dark at room temperature. The plate was gently shaken and the absorbance read at 570 nm using a SpectraMax 190 spectrophotometer. The percent inhibition of cell binding was calculated by [(absorbance of control-absorbance of treatment)/absorbance of control] x 100. Assays at each tzabcanin concentration for all cell lines were performed in triplicate and each assay was repeated at least three times.

Cell Migration/Scratch Assay

To measure the effects of tzabcanin on cell migration, a modified wound healing/scratch assay was completed as previously described by Liang et al. (2007). Twelve well Immulon-II plates were seeded with 1mL of A-549 or A-375 cells (5 x 10^5/mL) and allowed to grow to confluence at 37°C. The complete media was then discarded and cells were starved in serum-free media for 48 hr at 37 °C, followed by 2 hr incubation with 10 µg/mL mitomycin C in serum-free medium at 37°C. Mitomycin C inhibits DNA synthesis and therefore was used to evaluate the contribution of cell migration in the absence of cell proliferation to scratch wound healing. A scratch in the cell monolayer was created with a 200 µL pipet tip, followed by extensive washing with serum-free medium to remove cell debris. Cells were then incubated with either 10 µL of tzabcanin (1 µg/µL) resuspended in PBS, or PBS alone as a control. Photographs were taken at the same location of the culture well using an Olympus D21 camera attached to an Olympus CKX41 inverted microscope at 4X magnification. Because tzabcanin exhibited low cytotoxicity (see Results) towards A-375 cells, the migration evaluation for this cell line was conducted at 24 hrs, with photographs taken at 0 and 24 hrs. However, tzabcanin was not cytotoxic to A-549 cells, and therefore the migration assay for this cell

line was conducted for an extended period, with photographs taken at 0, 24, 48, and 72 hr intervals. Migration was measured by taking multiple measures of the width of the scratch for each cell line, and calculated using the equation [(S-F)/S] x 100, where S is the distance (mm) of the cell edge at 0hrs, and F is the distance (mm) of the cell edge at 24, 48, and 72 hrs (when possible). Assays were performed in triplicate and each assay was repeated at least three times.

Competitive Binding Assay

A-375 and A-549 cells were resuspended in 1% BSA in PBS at a density of 1 x 10^6 cells/mL. One hundred µL cell aliquots were incubated with 0 (control) or 2 µg of tzabcanin resuspended in PBS for 30 min at 37°C, followed by the addition of mouse monoclonal anti-$\alpha_v\beta_3$ antibody conjugated with a FITC (10 µg/mL). After 30 min incubation at room temperature in the dark, cells were gently pelleted and washed 3 times with 1% BSA in PBS to remove unbound antibody, and the fluorescence intensity of the cells was analyzed using flow cytometry (FACscan, Becton Dickinson, Bedford, MA). Tests were performed in triplicate and all experiments were repeated twice.

Statistical Analyses

Cytotoxicity and cell adhesion data were analyzed by Analysis of Variance (ANOVA) followed by Tukey's post-hoc test using R version 2.15.2. Cell migration assays were analyzed using a Student's T-test, comparing the percent migration of the treatment to the percent migration for the control for the respective time interval. All p values <0.05 were considered as statistically significant.

Results

Tzabcanin Binds to Both A-375 and A-549 Cells via Integrin(s)

Integrin-ligand interactions are cation-dependent (Mould et al. 1995), and to confirm that cell recognition to tzabcanin in both A-375 and A-549 cells was via integrins, tzabcanin was immobilized in 96-well microtiter plates, and cell adhesion was measured following cell incubation with the cation chelator EDTA. A-375 and A-549 binding to tzabcanin was inhibited by EDTA in a dose-dependent manner (Figure 6.1), with IC_{50} values of 2.4 mM and 3.23 mM, respectively. These results strongly indicate that tzabcanin interacts with both cell lines through integrin receptors, likely by cell recognition of the RGD binding region of tzabcanin.

Cytotoxicity Towards A-375 and A-549 Cells In vitro

Crude *C. s. tzabcan* venom (20 µg/100 µL) showed significant cytotoxicity toward A-375 cells, with approximately 13% cell viability remaining following 24 hr of incubation (Figure 6.2; $p < 0.001$). In addition, tzabcanin appeared to exhibit a slight dose-dependent decrease (~2-6% lower) in A-375 cell viability, and results were statistically significant ($p < 0.05$, 0.44 µm to 14 µM) at all concentrations except 0.22 µM. In contrast, both crude *C. s. tzabcan* venom and tzabcanin failed to show any significant decrease in A-549 cell viability (all p's > 0.05).

Tzabcanin Inhibits Cell Adhesion of A-375 and A-549 to Vitronectin

The ECM proteins fibronectin and vitronectin, and membrane matrix Matrigel, all support adhesion to A-375 and A-549 cells. By treating both cell lines with various

concentrations of tzabcanin, its ability to inhibit cell adhesion to these matrices was evaluated. Results show that tzabcanin inhibits adhesion of both A-375 and A-549 cells to vitronectin in a dose-dependent manner (Figure 6.3); however, this inhibition is much more potent towards A-375 cells. The IC_{50} for A-375 cells was 747 nM, whereas A-549 failed to reach 50% binding inhibition even at 2 μM. Tzabcanin failed to inhibit adhesion of either cell line to fibronectin or Matrigel (data not shown).

Tzabcanin Inhibits Cell Migration

Cell migration was measured following an *in vitro* scratch/wound healing assay. Tzabcanin (10 μg/ml) inhibited A-375 cell migration by approximately 45% when compared to the untreated control over 24 hr (Figure 6.4A; $p < 0.01$). Likewise, tzabcanin inhibited cell migration of A-549 cells by 76, 47, and 37% over the 24, 48, and 72 hr time intervals, respectively (Figure 6.4B; all $p < 0.01$). At 72 hr, all of the untreated A-549 controls had 100% closed.

$\alpha_v\beta_3$ is Identified as a Binding Site For Tzabcanin on A-375 and A-549 cells

Since tzabcanin inhibits binding of A-375 and A-549 cells to vitronectin, we examined if tzabcanin binds to $\alpha_v\beta_3$, a vitronectin receptor (Horton, 1997). Flow cytometry analysis demonstrates that both A-375 and A-549 cells express integrin $\alpha_v\beta_3$ (Figure 6.5), consistent with previously published reports (Gehlsen et al., 1992; Chetty et al. 2010). When A-375 and A-549 cells were pre-treated with tzabcanin, the disintegrin significantly inhibited anti-$\alpha_v\beta_3$ antibody binding (Figure 5), indicating that tzabcanin binds to $\alpha_v\beta_3$ on both cell lines.

Discussion

In 2014, it was estimated that over 585,000 deaths were expected from cancer in the United States alone (Siegel et al., 2014). Although there appears to be declining or stable trends among most cancers, melanoma incidences appear to be increasing, and cancer of the lung and bronchus remains as the most common cause of cancer related deaths in both men and women (Jemal et al., 2010; Siegel et al., 2104). The molecular mechanisms involved in metastasis are complex, enabling cancerous cells to disseminate from the primary tumor, invade local tissue, enter into circulation, and ultimately adhere, proliferate, and stimulate angiogenesis at a distant site (Liotta et al., 1991). This ability to metastasize is a significant cause of treatment failure and death in cancer patients (Price et al., 1997). Therefore, there is a tremendous need to identify compounds that may effectively arrest the numerous factors involved in metastasis.

Integrins mediate cell adhesion, migration, invasion, proliferation and angiogenesis, and their roles in metastasis and tumor survival are now apparent (Desgrosellier and Cheresh, 2010; Rathinam and Alahari, 2010). Further, integrin expression levels may vary significantly between normal and cancerous tissues (Desgrosellier and Cheresh, 2010), and additionally they are correlated with advanced stages of disease progression. Further, it has been strongly implicated that in cancer cells possessing them, tumor invasiveness is proportional to the expression levels of integrin $\alpha_v\beta_3$ (Gehlsen et al., 1992; Liapis et al., 1997). However, numerous other integrin subfamilies, such as α_3, α_5, α_6, α_v, β_1 and β_4, also enhance tumorigenesis (Rathinam and Alahari, 2010). Our prior results demonstrated that tzabcanin, a 7.1 kDa, RGDN monomeric disintegrin, inhibited adhesion of MCF-7 and Colo-205 cells to fibronectin

and vitronectin through competitive binding to integrins (Chapter V; Saviola et al., in review), although this inhibition was not as potent as has been previously reported for other disintegrins (Zhou et al., 2001; Sánchez et al., 2009). Since the RGDN binding region of disintegrins exhibits higher affinity towards $\alpha_v\beta_3$ (Scarborough et al., 1993), an integrin not expressed on either Colo-205 or MCF-7 cells, it was postulated that tzabcanin may demonstrate significantly higher affinity and anti-metastatic properties towards cell lines expressing this receptor.

Immobilized tzabcanin supports adhesion of A-375 and A-549 cells, demonstrating that these cells bind to this disintegrin. Because a cation chelator (EDTA) significantly inhibited adhesion of both cell lines to tzabcanin, it is likely that tzabcanin-cell binding is primarily mediated via integrin receptors. Integrin-mediated cell adhesion to the ECM proteins fibronectin and vitronectin is largely due to the presence of the RGD region found in these proteins (Ruoslahti, 1992, 1996), with roughly one-third of all identified integrins recognizing this binding sequence (Barczyk et al., 2010). Tzabcanin, a RGD disintegrin, inhibited binding of both A-375 and A-549 cell lines to vitronectin, but this inhibitory effect was significantly more potent for A-375 cells (IC_{50} = 747 nM). Cell adhesion to vitronectin is mediated by integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_8$ (Barczyk et al., 2010). Using an antibody against integrin $\alpha_v\beta_3$, our flow cytometric analysis indicates that A-375 and A-549 cell lines are $\alpha_v\beta_3$ positive, results which are supported by previous reports (Gehlsen et al., 1992; Chetty et al., 2010). However, A-375 cells appear to express a higher percentage (29%, S.D. = 3.14%) of this receptor when compared to A-549 cells (16%, S.D. = 3.7%), possibly explaining the weak inhibitory effect of tzabcanin on A-549 cell binding to vitronectin. Flow cytometric analysis also indicated that

tzabcanin binds to this receptor, inhibiting the binding of the FITC labeled anti-$\alpha_v\beta_3$ antibody and therefore also inhibiting cell adhesion to immobilized vitronectin during adhesion assays. Despite this, it is hypothesized that because tzabcanin (at 2 μM) did not inhibit 100% adhesion of either A-375 or A-549 cells to vitronectin, other integrin receptors may participate in anchoring cells to this ECM protein. Therefore, complete inhibition of attachment of A-375 and A-549 cells to vitronectin would require blocking multiple integrins, and the possibility of other RGD-dependent integrins exhibiting affinity towards vitronectin in A-375 and A-549 cell lines cannot be excluded.

Tzabcanin did not disrupt adhesion of either cell line to fibronectin or Matrigel at concentrations as high as 14 μM. Fibronectin is a ligand for numerous integrins including $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$, and $\alpha_4\beta_7$, which could explain why tzabcanin failed to inhibit cell adhesion to this ECM protein (Barczyk et al., 2010). In addition, the major components of Matrigel are laminin and type IV collagen (Hughes et al., 2010), which are recognized by the β_1 subclass of integrins (Barczyk et al., 2010). Therefore, tzabcanin appears to show specificity towards $\alpha_v\beta_3$, likely accounting for the inhibitory effects of adhesion of both cell lines to vitronectin but not to fibronectin or laminin.

Crude *C. s. tzabcan* venom was significantly cytotoxic to A-375 cells, results that confirm those published by Bradshaw et al. (*in press*). However, crude venom failed to exhibit any cytotoxic effects towards A-549 cells. This outcome was surprising due to the potent toxicity of *C. s. tzabcan* venom toward several immortal cell lines and toward mice (LD_{50} = 0.74 μg/g; Castro et al., 2013). Phenotypic differences between cell lines could account for the drastic differences in toxic effects of crude venom. Although purified tzabcanin failed to show a decrease in A-549 viability, there was a dose-

dependent small decrease in A-375 cell viability. It is currently unknown if this decrease is the result of apoptosis or necrosis. However, it has recently been shown that the recombinant disintegrins r-mojastin 1 and r-viridistatin 2 induced apoptosis in approximately 20% of human pancreatic adenocarcinoma (BXPC-3) cells at concentrations of 5 μM following 24 hr incubation (Lucena et al., 2015).

Migration is a critical step in metastasis, and cancer cells express various adhesion molecules facilitating movement from the primary tumor site to remote tissues or organs. Expression and activation of $\alpha_v\beta_3$ has been shown to enhance migration significantly in numerous cancer cell lines (Rolli et al., 2003; Dang et al., 2006; Sloan et al., 2006; Fong et al., 2009). Tzabcanin (10 μg/mL) effectively inhibited cell migration in both A-375 and A-549 cell lines, likely by blocking $\alpha_v\beta_3$-mediated adhesion. Upon ligation, $\alpha_v\beta_3$ induces the myosin light chain kinase through ras/MAP kinase pathways, causing an increase in phosphorylation of the myosin light chain kinase and leading to the phosphorylation of myosin light chains, thereby influencing cell locomotion (Klemke et al., 1997). It is hypothesized that by binding to $\alpha_v\beta_3$, tzabcanin inhibits this cell signaling pathway and further reduces cell motility in A-375 and A-549 cells. Cell adhesion to fibronectin and Matrigel was not inhibited, but inhibition of cell adhesion is not always correlated with inhibition of cell migration (Bartsch et al., 2003; Galán et al., 2008). For example, the disintegrin crotatroxin 2 from *Crotalus atrox* significantly inhibited migration of the murine mammary breast carcinoma cells (by 66%) yet failed to inhibit adhesion of this cell line to fibronectin or collagen IV and VI (Galán et al., 2008).

In conclusion, results from this study demonstrate that by binding integrin $\alpha_v\beta_3$, tzabcanin supports adhesion to both A-375 and A-549 cell lines. The RGDN binding

region present in tzabcanin may show specific affinity to integrin $\alpha_v\beta_3$, and not toward other receptors, which would explain why tzabcanin failed to inhibit adhesion of either cell line to fibronectin or Matrigel. However, by blocking $\alpha_v\beta_3$, tzabcanin inhibits A-375 and A-549 cell-mediated adhesion to vitronectin, as well as migration in both cell lines over 24 (A-375) and 72 (A-549) hr periods. Further studies will assess the effects of tzabcanin *in vivo* and against numerous other cancer cell lines.

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Figures

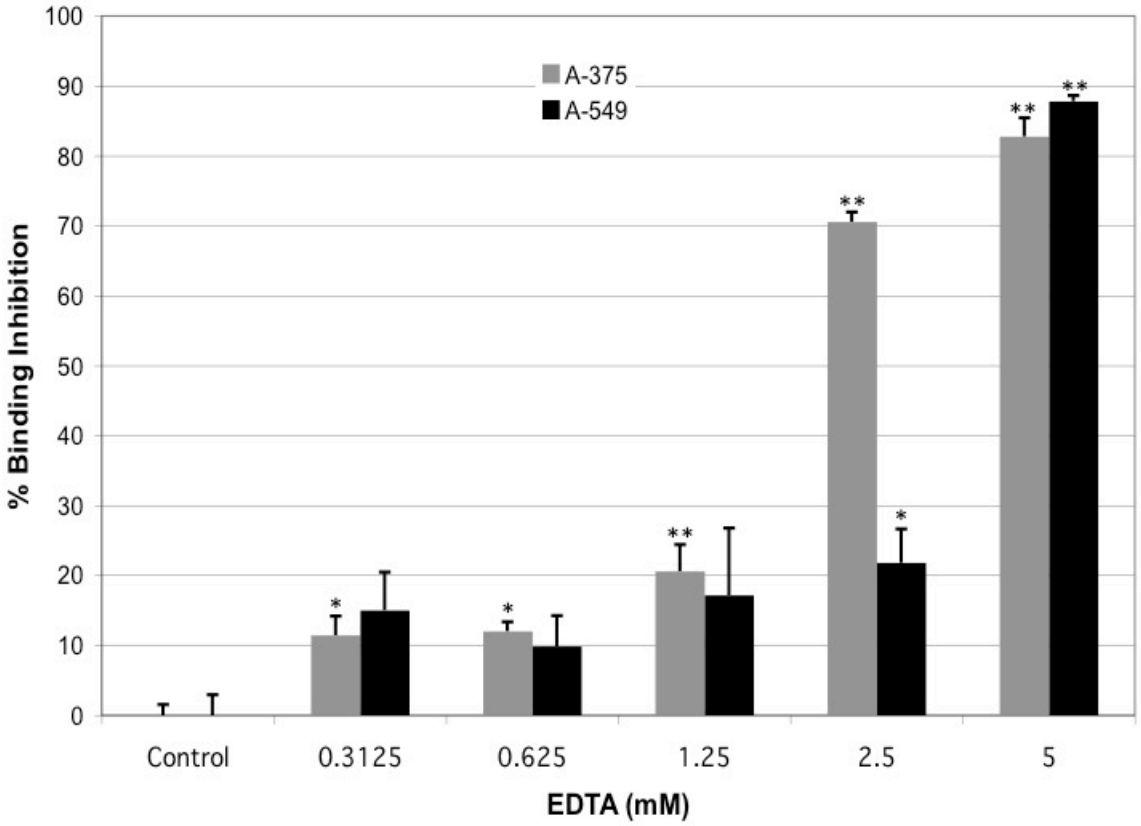

Figure 6.1: Percent inhibition of binding of A-375 and A-549 Cells to Immobilized Tzabcanin. A-375 and A-549 cells (5.0 x 10^5 cells/mL) were incubated with various concentrations of EDTA (0.3 – 5 mM) prior to addition to wells containing immobilized tzabcanin. All treatments were significantly different from controls (*, $p < 0.01$; **, $p < 0.001$).

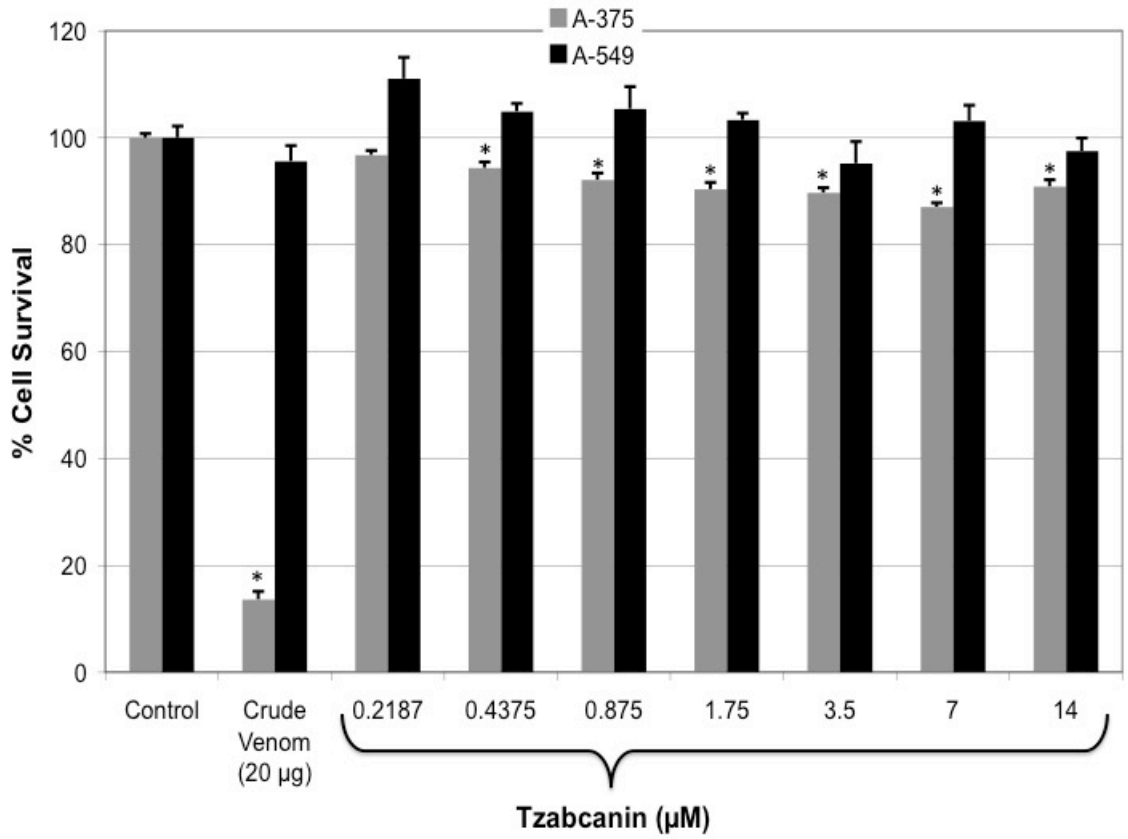

Figure 6.2: Percent Cell Viability of A-375 and A-549 cells following exposure to crude *C. s. tzabcan* venom or purified tzabcanin. *, $p < 0.05$, compared to controls.

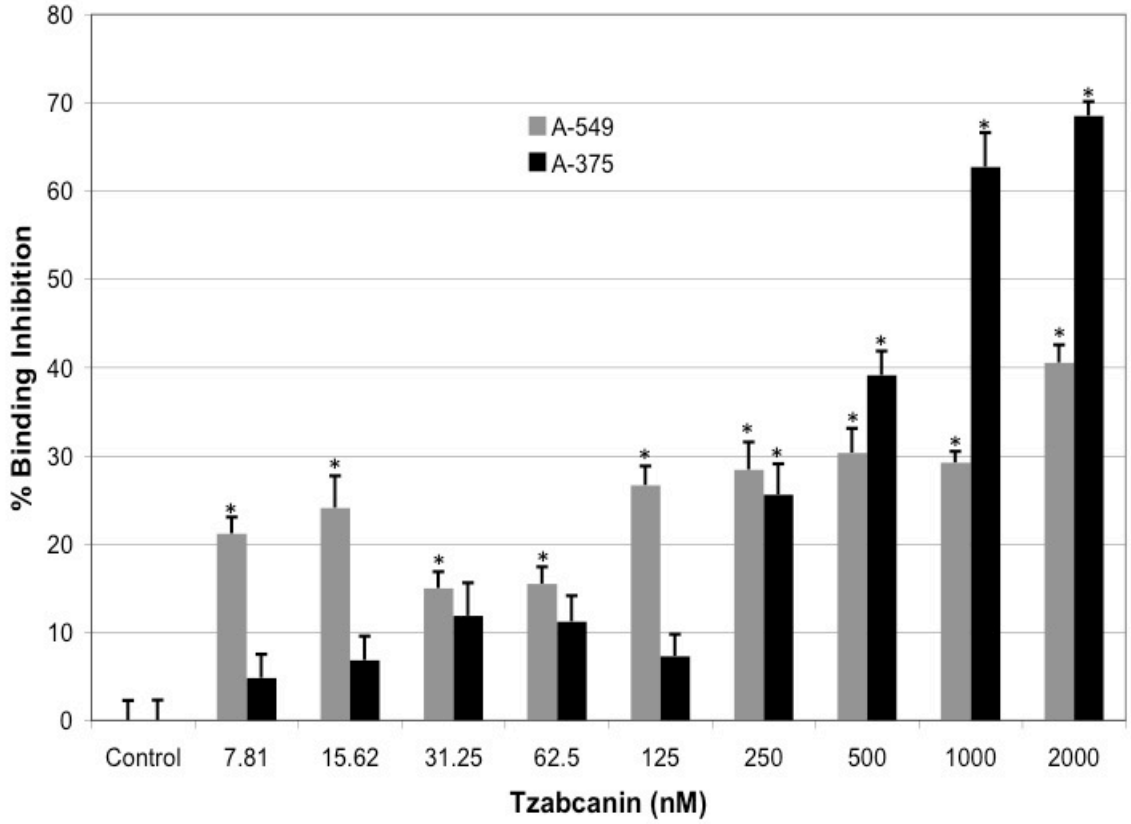

Figure 6.3: The Effects of Tzabcanin on A-375 and A-549 Adhesion to Immobilized Vitronectin. Various concentrations of tzabcanin (7.8 nM – 2 μM) were incubated with A-375 and A-549 cells (5.0 x 10^5 cells/mL) prior to addition to 96-well culture plates containing immobilized vitronectin. * $p < 0.001$.

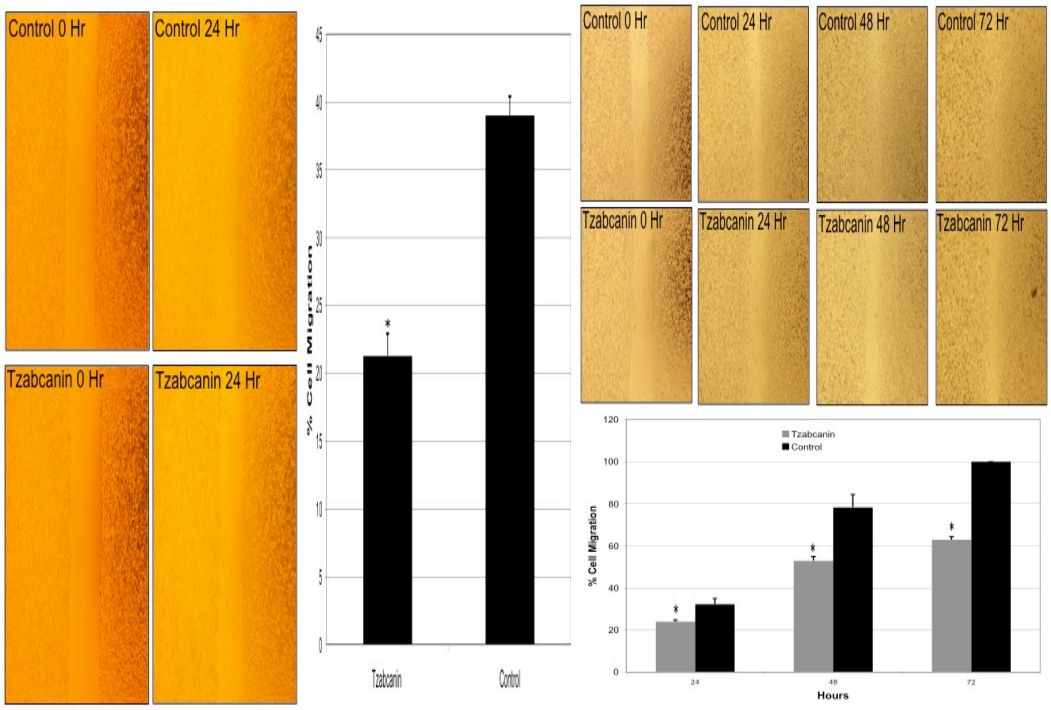

Figure 6.4: Inhibition of A-375 and A-549 Cell Migration. Cells were maintained as a monolayer in serum free medium for 48 hr before a 2 hr incubation with mitomycin-C. A line was scratched through the cell monolayer (0 hr), and cultures were allowed to migrate at 37°C in the presence of tzabcanin or a PBS control. Multiple measurements of the width of the scratch were made for for each treatment. A) cell migration for A-375 cells after 24 hr. B) cell migration of A-549 cells at 24, 48, and 72 hr.

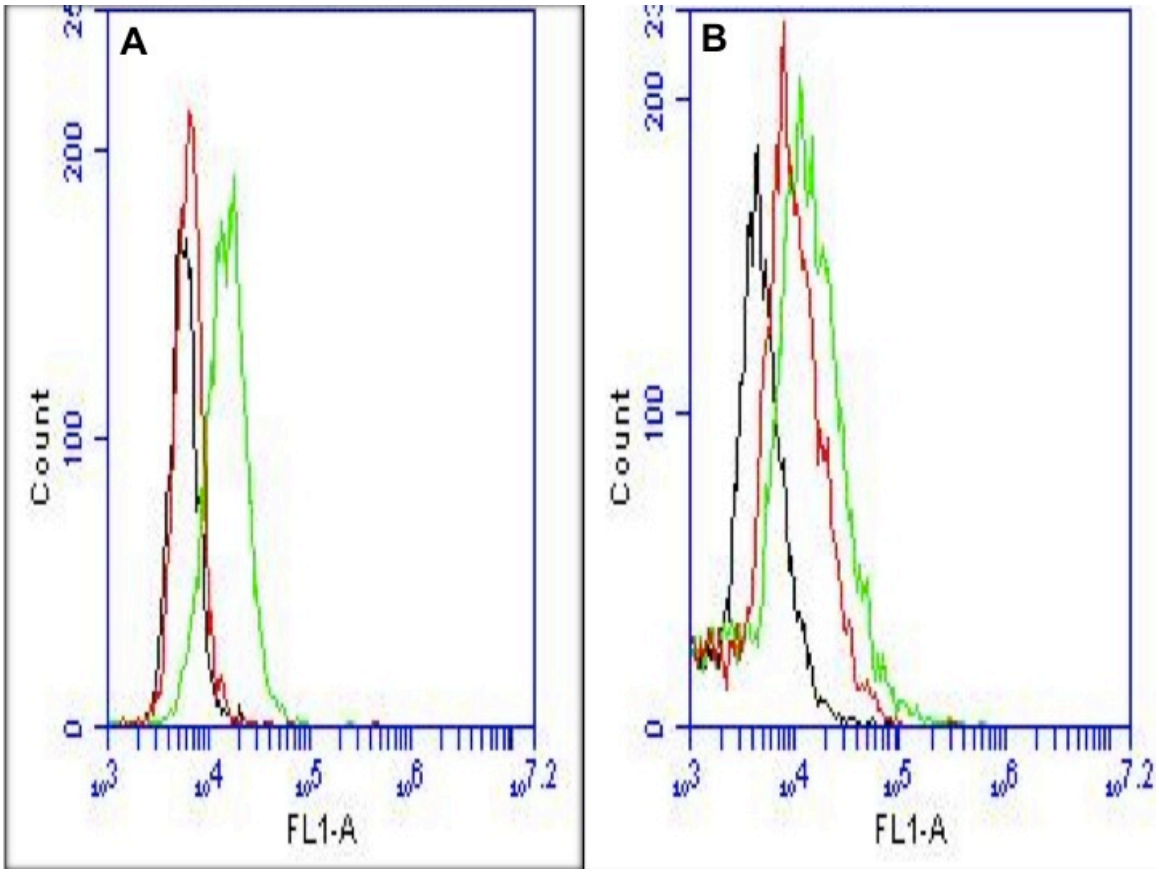

Figure 6.5: Tzabcanin Inhibits Binding of Anti-$\alpha_v\beta_3$ to A-375 and A-549 Cells. The natural fluorescence of A) A-375 melanoma cells and B) A-549 lung cancer cells is shown (black line) and the fluorescence following incubation with $\alpha_v\beta_3$ antibody (green line) indicates antibody-integrin binding. Tzabcanin added to cells prior to addition of the $\alpha_v\beta_3$ antibody effectively inhibits antibody binding (red line), as demonstrated by a shift of cell fluorescence back toward controls (black lines).

CHAPTER VII

COMPARATIVE VENOMICS OF THE PRAIRIE RATTLESNAKE (*CROTALUS VIRIDIS VIRIDIS*) FROM COLORADO

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Abstract

Here we describe and compare the venomic and antivenomic characteristics of both neonate and adult Prairie Rattlesnake (*Crotalus viridis viridis)* venoms. Although both neonate and adult venoms contain unique components, similarities among protein family content were seen. Both neonate and adult venoms consisted of of myotoxin, bradykinin-potentiating peptide (BPP), phospholipase A_2 (PLA_2), Zn^{2+}-dependent metalloproteinase (SVMP), serine proteinase, L-amino acid oxidase (LAAO), cysteine-rich secretory protein (CRISP) and disintegrin families. Quantitative differences, however, were observed, with venoms of adults containing significantly higher concentrations of the non-enzymatic toxic compounds and venoms of neonates containing higher concentrations of pre-digestive enzymatic proteins such as SVMPs. To assess the relevance of this venom variation in the context of snakebite and snakebite treatment, we tested the efficacy of the common antivenom CroFab® for recognition of both adult and neonate venoms *in vitro*. This comparison revealed that many of the major protein families (SVMPs, CRISP, PLA_2, serine proteases, and LAAO) in both neonate and adult venoms were immunodepleted by the antivenom, whereas myotoxins, one of the major toxic components of *C. v. viridis* venom, in addition to many of the small peptides, were not efficiently depleted by CroFab®. These results therefore provide a comprehensive catalog of the venom compounds present in *C. v. viridis* venom and new molecular insight into the potential efficacy of CroFab® against human envenomations by one of the most widely distributed rattlesnake species in North America.

Introduction

Produced and stored in a pair of highly specialized cephalic gland, snake venoms represent a complex mixture of bioactive proteins and peptides that exhibit diverse biochemical and pharmacological functions [1]. Venoms likely evolved via the co-opting and secondary modification of endogenous proteins with normal physiological functions early in the evolution of advanced snakes [e.g., 2 but see 3, 4], enabling the transition from a mechanical (constriction) to a chemical (venom) means of subduing prey [5]. The complexity of venoms, coupled with the fact that many snake species specialize on specific prey, has led to selective pressures resulting in the evolution of advantageous venom phenotypes that may vary based on phylogenetic affinities [1, 6, 7], geographic localities [8, 9], snake age [10, 11, 12] and diet [13, 14]. It is this variation and complexity that has continuously led researchers to examine snake venoms and the evolution of venom systems. Research into the origin and evolution of snake venoms offers remarkable insights into the biological roles of venom compounds [15, 16] and potential avenues for novel drug discovery [17, 18, 19], as well as addressing the ever-growing concern for effectively treating human snakebite [20, 21]. Proteomic analyses of venoms, termed “venomics”, is significantly expanding our knowledge and understanding of these oral secretions [e.g., 22, 23], which are not only critical to the foraging success of the snakes, but may also be of potential value or threat to humans.

Within the superfamily Caenophidia, the family Viperidae consists of approximately 260 species within four subfamilies: Azemiopinae, Causinae, Crotalinae and Viperinae. Of these subfamilies, the Crotalinae (pit vipers) is the most speciose, and currently comprises over 200 species distributed among 28 genera. In the Americas, the

only viperids are the monophyletic pit vipers, which appear to have dispersed into the New World during the late Oligocene to early Miocene approximately 22-24 mya [24]. Among New World pit vipers, the genus *Crotalus* currently comprises 30-36 species of venomous snakes distributed throughout much of South, Central and North America (http://www.reptile-database.org). The Prairie Rattlesnake (*Crotalus viridis viridis*) is a medium-sized terrestrial pitviper commonly exceeding 100 cm snout-vent length (SVL) [25]. The range of this species spans much of the Great Plains of the central United States, northwestern Mexico and southwestern Canada, making it one of the most widely distributed rattlesnake species in North America (Fig.1). Due to this wide geographic distribution, and the sometimes large home ranges, *C. v. viridis* may occur in close proximity to housing developments and are often found migrating into human-inhabited areas [26], increasing the possibility of encounters with humans. Terrestrial habitats occupied by *C. v. viridis* range from semi-desert and plains grasslands to pinion-juniper, mountain shrublands and montane woodlands, up to 2740 m in elevation [25, 27]. In grasslands habitat, *C. v. viridis* is a frequent inhabitant of prairie dog towns where burrows are commonly used for prey ambush sites, predator avoidance, and hibernation [26]. Like many other rattlesnake species, the diet of *C. v. viridis* shifts with snake age, generally focusing on small ectothermic prey and newborn rodents as neonates, and switching to larger endothermic prey (small mammals and occasionally birds) as adults [10, 27].

Viperid venoms contain an abundance of proteins which interfere with homeostasis and with the blood coagulation cascade, ultimately leading to the immobilization, killing and predigestion of prey. Individual venom may contain well over

100 proteins and peptides (including various protein isoforms); these compounds can, however, generally be classified into 10-15 protein families, such as the enzymatic L-amino acid oxidases (LAAOs), metalloproteinases (SVMPs), phospholipases A_2 (PLA_2) and serine proteases, as well as the non-enzymatic peptide myotoxins, C-type lectins, cysteine-rich secretory proteins (CRISPs) and disintegrins, among others [1]. Venom composition, especially in viperid species, can be classified based on enzymatic activity and toxicity, which are generally inversely correlated [7, 28]. For species classified as having type I venom, neonate and juvenile snakes have venoms exhibiting increased toxicity with lower SVMP and serine protease activity, whereas adults have lower toxicity (>1.0 μg/g mouse body weight) but higher SVMP activity [29]. Type II venoms, on the other hand, have been suggested to be paedomorphic [7, 28, 30, 31] since neonates, juveniles and adults all exhibit low SVMP activity but are higher in toxicity (< 1.0 μg/g mouse body weight), retaining similar venom characteristics throughout the life history of the snake.

Previous studies of the venom of *C. v. viridis* have shown moderate to high activity levels of LAAO, kallikrein, plasmin, and thrombin-like serine proteases, SVMP, PLA_2 and phosphodiesterase enzymes [28, 32]. Gel electrophoresis and mass spectrometry indicate that myotoxins, CRISPs and disintegrins are also abundant compounds in the venom of *C. v. viridis*. Venom yields from adult *C. v. viridis* may vary from 40 mg to well over 100 mg of dry venom in Colorado populations [28, 33], while neonate snakes may yield only 2-4 mg venom. Further, Mackessy [28] reported mouse intravenous LD_{50} values at 1.55 μg/g of mouse body weight, making it one of the more toxic rattlesnake species in the Western rattlesnake complex.

It is estimated that there are over 9000 venomous snake bites in the United States annually [34], with roughly 99% of these bites from snakes of the family Viperidae [35]. These human envenomations may be characterized by edema, erythema, clotting disorders, hypofibrinogenemia and local tissue necrosis [36, 37]. Bites may pose a serious or potentially deadly emergency, and early therapeutic administration of antivenom is necessary if severe envenomation is suspected. In the United States, the antivenom CroFab® (Crotaline Polyvalent Immune Fab (ovine)) is commonly administered during envenomation cases. CroFab® is produced from sheep immunized with one of the following North American snake venoms: *Agkistrodon piscivorus* (Water Moccasin), *Crotalus adamanteus* (Eastern Diamondback rattlesnake), *Crotalus atrox* (Western Diamondback rattlesnake) and *Crotalus scutulatus* (Mojave rattlesnake) [38]; serum collected from hyperimmune animals is affinity purified using columns containing the same immobilized venom, and hyperimmune sera are then mixed to produce a polyvalent antivenom. Surprisingly, in spite of its wide distribution in North America, *C. v. viridis* is not one of the species utilized for CroFab® production. Adequate treatment of snakebite is dependent on the ability of the antivenoms to reverse the pathological symptoms induced by venom by immunologically binding to venom components, facilitating their removal and degradation. Therefore, knowledge on venom composition and inter- and intra-specific venom variability is critical for assessment of antivenom efficacy and treatment of snakebite. The present work was designed to provide a comparative analysis of the venom proteomes of neonate and adult *C. v. viridis*, to determine venom composition and to investigate the immunoreactivity profile of the commercially available antivenom CroFab® against these venoms.

Materials and Methods

Venoms and Antivenoms

The venoms of fourteen neonate, twelve subadult and twelve adult *C. v. viridis* (equal numbers of female and male snakes) were manually extracted from wild-caught specimens (Weld Co., Colorado, USA). Age classes of snakes were based on snout-vent lengths from a large dataset of mark-recaptures from the same population (Mackessy, unpub. data); snakes ≤300 mm were considered neonates, snakes 500-540 mm were considered subadults and snakes ≥800 mm were considered adults. Following extraction, snakes were in captivity for no more than 3 days and were released to the exact location of capture. Venoms were immediately centrifuged at 10,000 x g for 5 min to pellet insoluble material, frozen, lyophilized and stored at -20°C until used. CroFab® was donated by Dr. Robert Palmer of the Rocky Mountain Poison and Drug Center, and anti-myotoxin a antibodies were a gift of Dr. Charlotte Ownby (Oklahoma State University).

RP-HPLC Fractionation

Venom proteins were separated by reverse-phase high-performance liquid chromatography (RP-HPLC) using a Teknokroma Europa C_{18} (250 x 4 mm, 5 µm particle size, 300 Å pore size) column and an ETTAN™ LC HPLC System (GE Healthcare). Two mg of venom from adult (2 samples, one male (specimen 281), one female (specimen 288) or 1.5 mg neonate (2 samples, one male (specimen 280), one female (specimen 249) were dissolved in 300 µL of 0.05% trifluoroacetic acid (TFA) and 5% acetonitrile, and insoluble material was removed by centrifugation in an Eppendorf centrifuge at 13,000 *g* for 10 min at room temperature. The flow-rate was set to 1 mL/min and the column was developed with a linear gradient of 0.1% TFA in water (solution A)

and acetonitrile with 0.1% TFA (solution B). Elution was achieved as follows: isocratic at 5% solution B for 5 min, followed by 5–25% B for 10 min, 25–45% B for 60 min, and 45–70% for 10 min. Protein detection was carried out at 215 nm and peaks were collected manually and dried using a Speed-Vac (Savant) for subsequent characterization. These four venom samples were considered the primary samples.

Characterization of RP-HPLC Fractions

Fractions obtained from RP-HPLC (primary samples) were further separated by SDS-PAGE under reduced and non-reduced conditions, using 15% gradient polyacrylamide gels. Chromatographic fractions containing peptides ($m/z \leq 1700$) were loaded in a nanospray capillary column and subjected to peptide sequencing using a QTrap™ 2000 mass spectrometer (Applied Biosystems) equipped with a nanospray source (Protana, Denmark). Doubly- or triply-charged ions were selected for collision-induced dissociation (CID) MS/MS analysis. Production spectra were interpreted manually or using the on-line form of the MASCOT program at http://www.matrixscience.com against a private database containing viperid protein sequences deposited in the SwissProt/TrEMBL database plus the protein sequences translated from the species-specific venom gland transcriptome. MS/MS mass tolerance was set to ± 0.6 Da. Carbamidomethyl cysteine and oxidation of methionine were fixed and variable modifications, respectively. Spectra producing positive hits were manually inspected. Good quality spectra that did not match any known protein sequence were interpreted manually to derive *de novo* amino acid sequences. Amino acid sequence similarity searches were performed against the available databanks using the BLAST

program [39] implemented in the WU-BLAST2 search engine at http://www.bork.embl-heidelberg.de.

Protein bands of interest were excised from a Coomassie Brilliant Blue-stained SDS-PAGE gel and subjected to in-gel reduction (10 mM dithiothreitol) and alkylation (50 mM iodacetamide), followed by overnight sequencing-grade trypsin digestion (66 ng/μl in 25 mM ammonium bicarbonate, 10% acetonitrile; 0.25 μg/sample) in an automated processor (using a Genomics Solution ProGest Protein Digestion Workstation) following the manufacturer's instructions. Tryptic digests were dried in a vacuum centrifuge (SPD SpeedVac®, ThermoSavant), redissolved in 15 μL of 5% acetonitrile containing 0.1% formic acid, and submitted to LC-MS/MS [40, 41]. To this end, tryptic peptides were separated by nano-Acquity UltraPerformance LC® (UPLC®) using a BEH130 C_{18} (100 μm x 100 mm, 1.7μm particle size) column in-line with a Waters SYNAPT G2 High Definition Mass Spectrometry System. The flow rate was set to 0.6 μl/min and column was developed with a linear gradient of 0.1% formic acid in water (solution A) and 0.1% formic acid in acetonitrile (solution B) at 1% B for 1 min, followed by 1-12% B for 1 min, 12-40% B for 15 min, 40-85% B for 2 min. Doubly and triply charged ions were selected for CID MS/MS. Fragmentation spectra were interpreted i) manually (*de novo* sequencing), ii) using the on-line form of the MASCOT program at http://www.matrixscience.com against the NCBI non-redundant database, and iii) using Waters Corporation's ProteinLynx Global SERVER 2013 version 2.5.2. (with Expression version 2.0) against the species-specific venom gland cDNA-derived toxin sequences. MS/MS mass tolerance was set to ± 0.6 Da. Carbamidomethyl cysteine and oxidation of methionine were selected as fixed and variable modifications, respectively.

The relative abundances (expressed as percentage of the total venom proteins) of the different protein families were calculated as the ratio of the sum of the areas of the reverse-phase chromatographic peaks containing proteins from the same family to the total area of venom protein peaks in the reverse-phase chromatogram [40, 41]. When more than one protein band was present in a reverse-phase fraction, their proportions were estimated by densitometry of Coomassie-stained SDS-polyacrylamide gels using ImageJ version 1.47 (http://rsbweb.nih.gov/ij). Conversely, the relative abundances of different proteins contained in the same SDS-PAGE band were estimated based on the relative ion intensities of the three more abundant peptide ions associated with each protein by MS/MS analysis. Finally, protein family abundances were estimated as the percentages of the total venom proteome.

To evaluate population-level variation in venom composition, and to confirm that trends observed in the primary samples were representative of the population, 34 additional samples (secondary samples) were subjected to RP-HPLC fractionation as above, using a Waters 2485 HPLC system, Empower software and a Phenomenex Jupiter C_{18} (4.0 x 250 mm, 5µm) column. Characterization of these samples was based on the detailed characterizations of the primary samples, and peak identifications were determined by comparison of elution times and visual inspections of chromatograms with the primary samples. These samples consisted of 10 adult, 12 subadult and 12 neonate venom samples for each sex, collected from the same population as the four primary samples. Data from these 34 secondary samples were used to determine protein family abundances as a percent of total venom proteins, with a particular emphasis on two of the most abundant protein families (SVMPs, peptide myotoxins). Combined samples for each

age class were also subjected to RP-HPLC fractionation as above to obtain a population average. One hundred fifty μg from each of 12 individuals (per age class) were combined, fractionated on RP-HPLC and compared to primary samples.

Antivenomics

A second-generation antivenomics approach [42] was utilized to examine the paraspecific immunoreactivity of commercially available CroFab® against both neonate and adult *C. v. viridis* venom (primary samples). For preparation of the antivenom affinity column, 500 μL of NHS-activated Sepharose 4 Fast Flow (GE Healthcare) matrix was packed in a Pierce centrifuge column and washed extensively with 10 matrix volumes of cold 1 mM HCl followed by two matrix volumes of coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) to adjust the pH of the column to 7.0-8.0. Sixty milligrams of CroFab® was then dissolved in 250 μL coupling buffer and incubated with matrix for 4 h at room temperature. The amount of antivenom coupled to the matrix was estimated by measuring the amount of non-bound antivenom by quantitative SDS-PAGE band densitometry (MetaMorph software, MDS Analytical Technologies) of CroFab®, which consists almost entirely of fragment antigen binding antibodies (Fab); the amount remaining in the coupling buffer was subtracted from the starting amount (60 mg), providing an estimate of approximately 16.4 mg (27%) of CroFab® antivenom bound to column matrix. The non-reacted groups were then blocked with 500 μL of 0.1 M Tris-HCl, pH 8.5 at room temperature for 4 h. The column was alternately washed with three 500 μL volumes of 0.1 M acetate containing 0.5 M NaCl, pH 4.0-5.0, and three 500 μL volumes of 0.1 M Tris-HCl, pH 8.5; this was repeated 6 times. The column was then equilibrated with 5 volumes of working buffer solution (20 mM phosphate buffer, 135

mM NaCl, pH 7.4; PBS). For the immunoaffinity assay, 300 µg of neonate (male) or adult (male) *C. v. viridis* venom were dissolved in ½ matrix volumes of PBS and incubated with the affinity matrix for 1 h at room temperature using an orbital shaker. As specificity controls, 500 µL of Sepharose 4 Fast Flow matrix, without or with 16 mg of immobilized control IgGs purified from non-immunized horse serum, were incubated with venom and the columns developed in parallel to the immunoaffinity experiment. Following elution of the non-retained fractions with 500 µL of PBS, the column was washed with 2.5 volumes of PBS, and the immunocaptured proteins were eluted with 5 volumes of elution buffer (0.1 M glycine-HCl, pH 2.0) and neutralized with 500 µL 1 M Tris-HCl, pH 9.0. The non-retained and the immunocaptured venom fractions were fractionated by reverse-phase HPLC using a Discovery® BIO Wide Pore C_{18} (15 cm x 2.1 mm, 3 µm particle size, 300 Å pore size) column and an Agilent LC 1100 High Pressure Gradient System equipped with a DAD detector. The flow rate was set to 0.4 mL/min and the column was developed with a linear gradient of 0.1% TFA in water (solution A) and 0.1% TFA in acetonitrile (solution B): isocratic at 5% solution B for 1 min, followed by 5-25% solution B for 5 min, 25-45% solution B for 35 min, and 45-70% solution B for 5 min. Protein detection was carried out at 215 nm with a reference wavelength of 400 nm.

Western Blot Analysis

Venoms (16 µg/lane) were from the four specimens of *C. v. viridis* characterized here, plus venom from one *C. o. helleri* and one *C. s. scutulatus* (both from Los Angeles County, CA, USA), and purified myotoxin a (from this source population of *C.v. viridis* in Colorado; 3 µg/lane); each sample was subjected to Western blot analysis following reducing SDS-PAGE on 12% acrylamide NuPAGE® Bis-Tris precast gels. Proteins were

blotted to nitrocellulose (150 mA for 1.5 hr), and the membrane was rinsed in Millipore-filtered water (18.2 MΩ·cm MilliQ™ H_2O) and then blocked in PBS-buffered 3% BSA (Sigma Fraction V) for 1hr at room temperature (RT). The membrane was cut so that one-half of the myotoxin a lane was retained on each part of the membrane. Membranes were rinsed three times in PBS and then incubated with 15 mL primary antibody (CroFab® - 1.0 mg/mL 3% BSA in PBS; or specific anti-myotoxin a antibodies raised in rabbits, 5 µL in 15 mL 3% BSA in PBS) overnight at RT with constant gentle shaking. The membranes were rinsed three times with Tris buffered saline (TBS, 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.4) and then secondary antibody (5 µL donkey anti-sheep IgG conjugated with alkaline phosphatase for CroFab®; 5 µL goat anti-rabbit IgG conjugated with alkaline phosphatase for anti-myotoxin a) in 15 mL TBS was incubated with the appropriate membrane for 60 min at RT with gentle shaking. Membranes were then washed four times with TBS and alkaline phosphatase substrate (SIGMAFAST™ BCIP®/NBT) in 10 mL of Millipore-filtered water (18.2 MΩ·cm MilliQ™ H_2O) was added. The color reaction was stopped with 20 mM disodium EDTA in PBS after ~5 min. Membranes were washed in MilliQ™ H_2O, dried and photographed. The same venoms (16 µg/lane) and myotoxin a (1, 3 and 5 µg/lane) were also run on a second 12% acrylamide NuPage gel under reducing conditions. This gel was stained with 0.1% Coomassie Brilliant Blue, destained and photographed. The 34 secondary samples were also subjected to electrophoresis using 12% acrylamide NuPage gel under reducing conditions.

SVMP Activity Assay

SVMP activity of crude neonate (n=12), subadult (n=12), and adult (n=12) *C. v. viridis* venoms was measured colorimetrically using azocasein as a substrate. Briefly, 2.5 µL of crude *C. v. viridis* venom (4 µg/µL), or 2.5 µL MilliQ H_20 as a control, was added to 247.5 µL of azocasein (2 mg/ml) resuspended in assay buffer (50 mM HEPES, 100 mM NaCl, pH 8.0). The reaction mixture was then incubated at 37 °C for 30 min. The assay was terminated by the addition of 125 µL of 0.5 M trichloroacetic acid, vortexed at room temperature, and centrifuged at 2000 x g for 5 min. Following centrifugation, 100 µL of supernatant was mixed with 100 µL of 0.5 M NaOH and the absorbance was determined at 450 nm using a SpectraMax 190 plate reader. Assays for each sample were performed in triplicate, and activity was reported as ΔA_{450nm}/min/mg protein.

Statistical Analysis

The percent abundance of myotoxin a and SVMP from all RP-HPLC runs was analyzed by Analysis of Variance (ANOVA) followed by Tukey's post-hoc test using R version 2.15.2. Similarly, SVMP activity was also analyzed by ANOVA and Tukey's post-hoc comparison. Comparisons between age classes and between sexes were also analyzed by ANOVA and Tukey's post-hoc comparison and two-tailed t-test. All p values <0.05 were considered as statistically significant.

Results and Discussion

The Venom Proteome of *C. v. viridis*

In the current study, venoms of both male and female neonate and adult *C. v. viridis*, obtained from snakes from approximately the center of the species' distribution (Fig.7.1), were characterized by venomics analysis. These four (primary) venom samples

(Fig.7.2), as well as the 34 additional (secondary) samples (Fig. 7.3), exhibited similar chromatographic profiles and toxin family composition (Table 7.1), but there is apparent variation in concentrations of specific toxins and protein families (Table 7.1). Venoms from all *C. v. viridis* examined shared compounds from 10 protein classes (Table 7.1; Fig. 7.3), which are typically abundant in rattlesnake venoms [7]. In addition, some molecules were detected in only a subset of venoms, including an ohanin-like toxin [~ *L. muta* Q27J48], PI-SVMP [~ *C. atrox* Q90392], phospholipase B [~ *C. adamanteus* F8S101], an acidic PLA_2 [P0DJM5], and the tripeptide inhibitors of SVMPs, ZNW and ZQW (Table 7.1) [43-46]. Both endogenous inhibitors were primarily detected in neonate venoms (peaks 39* and 40* in panels C and D of Fig.2). Only ZQW was observed in adult female venom (peak 4, Fig. 7.2B), whereas tripeptide inhibitors were not seen in adult male venom (Table 7.1). Consistent with previous reports [44], the concentration of endogenous inhibitors correlates with the abundance of SVMPs in the venoms, as overall SVMPs (PI, PII, and PIII classes) were detected in higher percentages in both neonate venoms when compared to adult venoms (Table 7.1). This observation supports the view that the relatively low affinity endogenous tripeptides (Ki = 0.20-0.95 mM) [43] keep SVMPs functionally silent in the venom gland, and disengagement of this control occurs spontaneously at the time of the snakebite.

The major toxins present in both adult and the neonate male venoms were peptide myotoxins (Table 7.1). There were no statistically significant differences in myotoxin a or SVMP content, or SVMP activity of crude venom, with regards to sex of the snake (all p's > 0.05). However, there was a significant age-related change in myotoxin a content of the venoms, and neonate venoms contain significantly less myotoxin a than adult venoms

(Fig. 4; $p = 0.05$). Further, there was no significant difference between neonate and subadult ($p = 0.74$) or subadult and adult ($p = 0.23$) myotoxin a concentration. Both myotoxin a [P01476] and myotoxin 2 [P63175] were detected in adult male *C. v. viridis* venom, whereas only myotoxin a was found in adult female and neonate venoms. Small basic myotoxins represent a Nearctic and Neotropical crotaline innovation of a protein fold acting on the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum [47] and voltage-sensitive Na^{+} channels [12, 48-51]. These myonecrotic toxins primarily serve two biological roles: to limit the flight of prey by causing tetanic paralysis of the hind limbs, and to promote death by paralysis of the diaphragm [52, 53].

SVMPs are present in the venoms of all families of venomous snakes, and analysis of this activity in all samples of *C. v. viridis* venom showed a significant age-related decrease (Figs. 7.4A and 7.4B). For overall SVMP abundance, ANOVA showed significant differences when comparing neonate to subadult ($p = 0.02$) and neonate to adult venoms ($p = 0.002$), yet comparison of subadult to adult venoms was not statistically significant ($p = 0.69$). SVMP activity assays further support these results with both subadult and adults venoms showing significantly less activity when compared to neonate *C. v. viridis* venoms (both p's < 0.001). There was no difference in SVMP activity between subadult and adult venoms ($p = 0.61$). Tryptic peptides recovered after in-gel digestion yielded ions matching the highly hemorrhagic PIII atrolysin-A [Q92043], first characterized from the venom of *C. atrox* [54], in the venoms of all four *C. v. viridis* examined here. Adult and neonate male venoms also yielded peptides matching an additional PIII-SVMP [Q9DGB9] from *C. atrox*, and one other PIII-SVMP in the 36 kDa range [C9E1S0] was detected in the venom of the neonate male. Peptides of PI-SVMPs,

which are less hemorrhagic than the higher molecular weight PIII-SVMPs [55], were only detected in the adult male and neonate male venoms (Table 7.1). However, analyses of peak 9 from all four individuals yielded a 3 kDa protein band (see Fig. 7.2 panel A, protein band 9) that was subjected to tryptic peptide mass fingerprinting, producing the ion YIELVVVADHR that matches a *C. atrox* PI-SVMP [Q90392]. The early HPLC elution of this peptide compared to the other SVMPs, in addition to the low molecular mass of the protein band, suggests possible degradation of these PI-SVMP enzymes, which exhibit an intact mass of 20-24 kDa.

Disintegrins are platelet aggregation inhibitors commonly found in viperid venoms as the result of the post-translational proteolytic processing of PII-SVMPs [56]. In *Crotalus*, these non-enzymatic toxins have been shown to range from 0.1% of the venom proteome of *C. tigris* [57] to over 6% of the total venom proteome in *C. atrox* [58]. Stage-dependent down-regulation of the precursor metalloproteinases in *C. viridis* may account for the lower abundance of disintegrins in adult compared with neonate venoms.

C-type lectin-like molecules (CTLs), also known as snaclecs (snake venom C-type lectins), are also present in *C. v. viridis* venoms (Table 7.1). Snaclecs have been reported to bind in a Ca^{2+}-independent manner and via protein-protein interactions with coagulation factors IX/X, X and II, impairing their physiological roles in hemostasis. Snaclecs also reduce platelet function by inhibiting surface receptors such as the von Willebrand receptor, GPIb, and the collagen receptor, integrin $\alpha_2\beta_1$, or by activating platelets via clustering of the collagen receptor GPVI so that they are removed from the circulation, producing thrombocytopenia [59]. Whether this class of toxins participates in

age- and gender-dependent prey-securing strategies, and how they participate, deserves further investigation.

Phospholipase A_2 (PLA_2) enzymes are one of the most heavily-studied venom toxin families to date [60] and contribute to local tissue damage due to myonecrosis, edema, and inflammation. However, a single venom may contain numerous PLA_2 isoforms, and each may exhibit varying biological effects. In this respect, protein masses, in addition to tryptic peptides, indicate the presence of multiple PLA_2 isoenzymes in all four venoms examined. Thus, tryptic peptides matching that of the D49-PLA_2 [Q9I8F8] were found in adult male venom (Fig.2A, peak 13); D49-PLA_2 [Q800C3] was found in venoms belonging to both adult and neonate male snakes (Figs.7.2A and C, peaks labeled 11). Peptides representing another D49-PLA_2 [Q800C4] were seen in the adult male and female venom samples (Figs.7.2A and 7.2B, peaks 19 and 19a/b, respectively), and ions for D49-PLA_2 [Q71QE8] and acidic PLA_2 [P0DJM5] were present in the adult female venom (Fig.7.2B, peak 32*).

Cysteine-rich secretory proteins (CRISPs), which comprise 1.8 to 7.3% of the venom proteome of adult and neonate *C. v. viridis* (Table 1), represent another widely distributed protein family in snake venoms [61, 62]. Reported activities of some CRISPs include inhibition of smooth muscle contraction and cyclic nucleotide-gated ion channels; however, their role in envenomation and prey capture has not been established.

L-amino acid oxidases are flavoenzymes that catalyze oxidative deamination of L-amino acids to form corresponding α-keto acids, hydrogen peroxide and ammonia. Due to their wide distribution in snake venom, LAAOs are thought to contribute to the toxicity of the venom due to the production of hydrogen peroxide during the oxidation

reaction. In addition, LAAOs have been reported to induce platelet aggregation in platelet-rich plasma [63, 64], although the overall functional contribution to the envenoming process remains elusive.

Several somewhat unusual venom constituents, including glutaminyl cyclase (GC) and phospholipase B, were found within the venoms of *C. v. viridis* and deserve further discussion. GCs may contribute indirectly to overall venom toxicity by catalyzing the N-terminal formation of pyroglutamate characteristic of several snake venom toxin families [65, 66] and thereby stabilizing them to endogenous scavenging mechanisms. These cyclases have also been documented in the proteomes of *C. atrox* [58, 67] and *C. d. terrificus* [68], as well as in the transcriptomes of *C. adamanteus* [69], *B. jararaca* [70] and the colubrids *Boiga dendrophila* and *B. irregularis* [71]. Snake venom gland GC is also likely involved in the biosynthesis of pyroglutamyl peptides such as bradykinin-potentiating peptides (BPPs) [72] that contribute to symptoms of hypotension experienced by snakebite victims [73], and of endogenous inhibitors of metalloproteinases, ZQW and ZNW, discussed above [44, 45]. Although GCs are found in low concentrations in snake venoms, the enzyme may play a significant role in post-translational modifications of functionally important and abundant venom proteins. Thus, mature PIII-SVMPs and other venom proteins, eg. svVEGF (http://www.ncbi.nlm.nih.gov/protein/?term=svVEGF) and colubrid three-finger toxins [19], usually contain an N-terminal pyroglutaminyl residue, indicating that the action of glutaminyl cyclase is downstream of the proteolytic processing of the pre-pro-precursors.

Reverse-phase peak 28 of venom samples from the adult female and both neonate *C. v. viridis* (Figs. 7.2B-D) yielded numerous ions matching a phospholipase B (PLB)

from *C. adamanteus* (F8S101, J3S4V6; supplemental Table 1). The occurrence of PLB in snake venoms was initially reported by Doery and Pearson [74] and was characterized as being responsible for the high direct hemolytic activity of several Australian elapid venoms [75-77]. PLB molecules have been identified in the venom proteome of the *C. adamanteus* [78], *B. atrox*, *B. jararacussu*, *B. jararaca*, *B. neuwiedi*, *B. alternatus*, and *B. cotiara* [79], and *Porthidium lansbergii* [80]. The functional relevance of this class of proteins in envenomation, represents another intriguing topic that requires future detailed study.

C. v. viridis Exhibits a Novel Pattern of Ontogenetic Venom Proteome Changes

The ontogenetic compositional shift in *C. v. viridis* venom is characterized by a stage-dependent decrease of the relative content of SVMPs, disintegrins, catalytically active D49-PLA_2s, and L-amino acid oxidase, and the concomitant increase in the relative abundance of small basic myotoxins, serine proteinases and an ohanin-like toxin (Table 7.1; Figs. 7.3-7.5). We focused on SVMPs and myotoxin a levels as these ontogenetic venom shifts may represent an age-dependent "strategy" for effectively securing prey, because the snake prey regime switches with age from newborn rodents and small ectothermic prey to larger endothermic prey.

PIII-SVMPs are often highly hemorrhagic, promoting prey immobilization and tissue necrosis by degradation of the basement membrane surrounding capillary vessels [81]. SVMPs occur in venoms of all families of advanced snakes, suggesting the recruitment and modification of an ADAM (A disintegrin and metalloproteinase)-like gene early in the evolutionary history of venomous snakes [82, 83]. Although these

enzymes are generally highly expressed in venoms within the Viperidae [84, 85], the venom of the Black-speckled Palm Pitviper*, Bothriechis nigroviridis*, a neotropical arboreal pit viper from Costa Rica, does not possess detectable Zn^{2+}-dependent metalloproteinases and is unique among *Bothriechis* species by possessing a high content of neurotoxic PLA_2 and vasoactive peptides [86]. These data suggest that distinct evolutionary solutions have evolved within the arboreal genus *Bothriechis* for the same trophic purpose, and it underscores the versatility of viperid venoms as adaptive traits. The evolutionary justification for the ontogenetic decrease of PIII-SVMP hemorrhagins in *C. v. viridis* is elusive, although it is tempting to hypothesize that their biological role has been successfully replaced by the paralytic action of small basic myotoxins, the locomotion-disrupting and hyperalgesia-inducing ohanin-like protein [87], and the hemostasis-disrupting serine proteinases [88]. These latter enzymes comprise the second most abundant venom protein family in both adult male (26.82%) and female (26.86%) *C. v. viridis* (Table 1).

Variation in the biochemical composition of venoms from different geographic locations and with age has long been appreciated by herpetologists and toxinologists [10, 89-91]. Stage-specific venom proteins differentially expressed during ontogenetic development have been reported in just a few species, and in each taxa investigated a somewhat different pattern of ontogenetic changes has been described. The ontogenetic shifts reported here for *C. v. viridis* represent a novel pattern of age-related venom compositional transitions among viperid species. For example, in *Bothrops asper*, major ontogenetic changes involve a shift from a PIII-SVMP-rich to a PI-SVMP-rich venom and the secretion in adults of a distinct set of PLA_2 molecules than in the neonates [8];

ontogenetic changes in the toxin composition of *L. stenophrys* venom results in the net shift from a vasoactive (bradykinin-potentiating and C-type natriuretic) peptide (BPP/C-NP)-rich and serine proteinase-rich venom in newborns and 2-year-old juveniles to a (PI>PIII) SVMP-rich venom in adults [92]; age-dependent venom changes in *C. simus* involve a shift from a neurotoxic to a hemorrhagic venom phenotype [29]; conversely, *Sistrurus m. barbouri* showed little evidence for an ontogenetic shift in venom composition [93].

Although the environmental and molecular mechanisms that generate this age-dependent venom diversity remain unclear [94], age-dependent changes in the concentration of venom gland microRNAs have recently been shown to influence the translation of venom proteins from genes transcribed in the venom gland [29]. While the generalization of this finding requires additional study in other species, posttranscriptional modulation of the venom transcriptome could conceivably contribute broadly to differential venom composition without large-scale alterations of the underlying gene expression machinery.

Assessment of the Immunoreactivity of CroFab®

In the United States, human envenomation due to snakebite is relatively rare, and CroFab® is the antivenom administered universally to treat bites. CroFab® is produced utilizing venoms from four different North American viper species, *A. piscivorus*, *C. adamanteus*, *C. atrox* and *C. scutulatus*. Venomic profiles of all four species used in producing CroFab® have been published (*A. piscivorus*: [95]; *Crotalus adamanteus*: [78]; *C. atrox*: [58]; *C. scutulatus*: [49]), and these species collectively show varying relative

concentrations of typical viperid venom protein families. For example, in *C. atrox*, the venom proteome consisted of nearly 50% PI and PIII-SVMPs, with approximately 20% serine proteases and 7% PLA_2s [58]; this species lacked small basic myotoxins, which represent approximately 22% of the venom proteome of *C. adamanteus* [78]. In addition to small basic peptide myotoxins, PLA_2s and SVMPs represent a significant proportion (~59%) of the overall venom composition of *C. adamanteus.* Further, venomics analysis of *A. piscivorus* showed that over 75% of venom proteins consisted of PLA_2 (33.6%), SVMP (33.1%), and serine protease (13.2%) [95]. However, *C. scutulatus* shows significant venom compositional diversity, with several distinct venom phenotypes varying in overall composition and toxicity [49]; venoms containing high amounts of the presynaptic neurotoxin Mojave toxin are typically used in the production of CroFab® (pers. comm., SPM: R. Straight).

Our antivenomic assessment of *C. v. viridis* venoms against CroFab® (Fig.7.6) showed that significant amounts of the peptides and proteins in early eluting HPLC fractions (1-8 of adult and neonate venoms, and peaks 39* and 40* of neonate samples) were not immunocaptured by CroFab® affinity chromatography (Figures 7.6C and 7.6F); several additional downstream protein peaks were also not immunodepleted from neonate venom (Fig. 7.6F). Our venomic analyses indicate that these non-depleted HPLC fractions consist of bradykinin inhibitory peptides, myotoxins a and 2, and SVMP inhibitors. It has recently been shown that the BPP family of venom proteins from *Lachesis* species were also not immunocaptured by antivenoms developed at Instituto Vital Brazil (IVB) and Instituto Clodomiro Picado (ICP). In spite of this, caudal vein injection of BPP proteins in mice failed to demonstrate toxicity or elicit abnormal

behavior [96], suggesting that BPPs, even if not recognized by antivenoms, may not contribute to the often severe pathologies seen in viperid envenomations.

The immunoaffinity antivenomics assessment of CroFab® indicated that it exhibits partial immunoreactivity towards small basic myotoxin a (Fig.7.6, panels C and F). However, Western blot analysis shows that CroFab® does recognize myotoxin a in the crude venoms of several species as well as the purified toxin from *C. v. viridis* venom, as does a specific anti-myotoxin a antibody (Fig. 7.7). Myotoxin a produces rapid tetanic contraction of skeletal muscles in prey [97], leading to rapid immobilization of prey, and the poor immunodepletion by the CroFab® affinity column suggests that this should be problematic during human envenomations. However, the amount of CroFab® utilized was relatively small compared to human dosages, and so if anti-myotoxin a antibodies represent only a small percentage of CroFab® antibodies, this deficit may be compensated by high clinical dosages. Further, case log data from the American Association of Poison Centers for rattlesnake bites in Colorado (*C. v. viridis* is the most probable source of bites) over four years (2010-2013) indicated no fatalities (0/175 cases); unfortunately, long-term data for snakebites is generally lacking from all health databases, so chronic effects cannot be evaluated. These data suggest that in spite of minimal immunodepletion, CroFab® did provide sufficient protection for patients. Although quantitative estimates of anti-myotoxin a antibodies are not yet available for CroFab®, our data show that CroFab® does contain significant amounts of antibody which recognize myotoxin a, whereas the antivenom previously used in the United States (Wyeth polyvalent Crotalidae) was shown to contain very low titers to myotoxin a [98]. The low recovery of SVMPs in the immunocaptured and the non-bound fractions of both

adult and neonate venoms contrasts with the clear immunoreactivity towards these components exhibited by CroFab® in Western blot analysis. This indicates that the high binding affinity of the antivenom for SVMPs likely prevents their elution from the column.

The antivenomic analysis also indicated that CroFab® effectively recognizes and depletes other potent and abundant venom components, including PLA_2s, serine proteases, LAAOs and SVMPs, indicating that the similarity in venom protein family representation in *C. v. viridis* venom and venoms of the four species utilized in CroFab® production is reflected in the immunoreactivity of this antivenom. While comparing the levels of immune recognition gathered from antivenomics with the *in vivo* neutralization capacity of an antivenom is not straightforward, since both experiments involve radically different protocols, in our experience, even a moderate immunocapturing capability of ~20%–25% correlates with a satisfactory outcome in the *in vivo* neutralization tests [99]. Consistent with these observations, CroFab® shows high efficacy in treatment of human and domestic animal envenomations by *C. v. viridis*, including snakes from Colorado [100, 101], so even partial binding/recognition of myotoxin a by Fabs appears sufficient to ameliorate symptoms effectively.

Concluding Remarks

In this study we conducted venomic and antivenomic analyses of *C. v. viridis* (Prairie Rattlesnake), one of the most widely distributed rattlesnake species in North America. The previously reported LD_{50} of 1.55 μg/g (inbred mice) for *C. v. viridis*, coupled with the SVMP concentrations detected here, confirms *C. v. viridis* as possessing type I venom as described previously [7]. Ontogenetic variation in prey preference has

been reported in *C. viridis* [10, 27] and changes in diet are correlated with ontogenetic changes in venom composition in Pacific Rattlesnakes [10]. These age-related changes in venom composition may facilitate prey handling and possibly digestion [10, 11]. Although a common ontogenetic trend documented in rattlesnake venoms is a shift from a type II venom composition (high toxicity, low SVMP activity) in neonates to a type I venom in adults (lower toxicity, high SVMP activity), our results clearly indicate the opposite relationship for *C. v. viridis*, with overall SVMP concentrations being lower in venoms from adult snakes, and myotoxin (a and 2) concentrations being higher in adult samples. Further, classic venom paedomorphism [11, 12, 30] does not occur in this population, as venoms analyzed here do show age-related changes in composition. It should be noted, however, that total SVMP activity of venoms from this population of *C. v. viridis* are not particularly high when compared with several type I venoms [7, 10].

Our antivenomics results show that CroFab®, developed against venom of three *Crotalus* and one *Agkistrodon* species, efficiently immunodepleted many of the major compounds present in *C. v. viridis* venom. Our antivenomics results show that CroFab®, developed against venom of three *Crotalus* and one *Agkistrodon* species, efficiently immunodepleted many of the major compounds present in *C. v. viridis* venom. Myotoxin a, abundant in both adult and neonate *C. v. viridis* venoms, did not appear to be efficiently immunocaptured during the antivenomics experiment, but Western blot analysis indicated that it is recognized by CroFab® as well as by the specific myotoxin a antibody. Considering the high efficacy of CroFab® in treating *C. v. viridis* snakebites, it appears that the relatively low immunoreactivity of CroFab® to myotoxin a is indeed sufficient for effective treatment of snakebite. The current study defines the venom

proteome of a discrete population of *C. v. viridis* from Colorado, but a more detailed population venomics study evaluating venom composition, and antivenom reactivity, of this species throughout its entire range (spanning 22° of latitude) may demonstrate distinct regional differences in venom protein family distribution, concentration, and immunoreactivity against existing antivenoms.

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Table

Table 7.1: Percent Abundance of Protein Families in *C. v. viridis* Venom. --, not detected; M ± SD, mean ± standard deviation

	Adult			Neonate		
	Male	Female	M (±SD)	Male	Female	M (±SD)
Protein Family	% of total venom proteins					
BPP	8.2	6.5	7.4 (0.8)	6.4	11.2	8.8 (2.4)
Disintegrin	0.1	0.1	0.1 (0.0)	0.8	0.7	0.7 (0.1)
CRISP	3.9	2.1	3.0 (0.9)	4.0	4.8	4.4 (0.4)
C-type lectin	1.8	3.3	2.6 (0.7)	7.3	1.9	4.6 (2.7)
PLA_2	7.7	10.6	9.2 (1.4)	10.9	16.3	13.6 (2.7)
• D49 PLA_2	7.7	10.2	9.0 (1.3)	10.9	16.3	13.6 (2.7)
• Acidic PLA_2	--	0.4	0.2 (0.2)	--	--	--
Ohanin-like Toxin	0.5	0.6	0.5 (0.1)	--	0.2	0.1 (0.1)
Myotoxin	38.1	35.6	36.9 (1.2)	25.2	5.7	15.5 (9.7)
• Myotoxin a	37.5	35.6	36.6 (1.0)	25.2	5.7	15.5 (9.7)
• Myotoxin 2	0.6	--	0.3 (0.3)	--	--	--
Serine Proteinase	26.8	26.9	26.8 (0.1)	18.2	20.6	19.4 (1.2)
LAAO	1.9	2.5	2.2 (0.3)	7.6	11.9	9.8 (2.1)
SVMP	11.0	11.4	11.2 (0.2)	14.2	18.0	16.1 (1.9)
• PIII SVMP	3.1	4.9	4.0 (0.9)	8.4	8.8	8.6 (0.2)
• PII SVMP	0.9	3.7	2.3 (1.4)	1.7	6.9	4.3 (2.6)
• PI SVMP	0.2	--	0.1 (0.1)	0.8	--	0.4 (0.4)
• PI SVMP fragments	6.6	2.9	4.8 (1.9)	3.4	2.3	2.9 (0.6)
Glutaminyl Cyclase	0.1	0.1	0.1 (0.0)	0.8	0.1	0.5 (0.4)
Phospholipase B	--	0.1	0.1 (0.1)	0.3	0.1	0.2 (0.1)
SVMP Inhibitor	--	< 0.10	0.1 (0.1)	4.5	8.5	6.5 (2.0)
• ZNW	--	--	--	3.0	5.7	4.4 (0.3)
• ZQW	--	< 0.10	0.1 (0.1)	1.5	2.8	2.2 (0.6)

Figures

Figure 7.1: Geographic Distribution of *C. v. viridis*. Venoms from *C. v. viridis* used in the proteomic characterizations reported here were collected from Weld County, Colorado (indicated by the black dot).

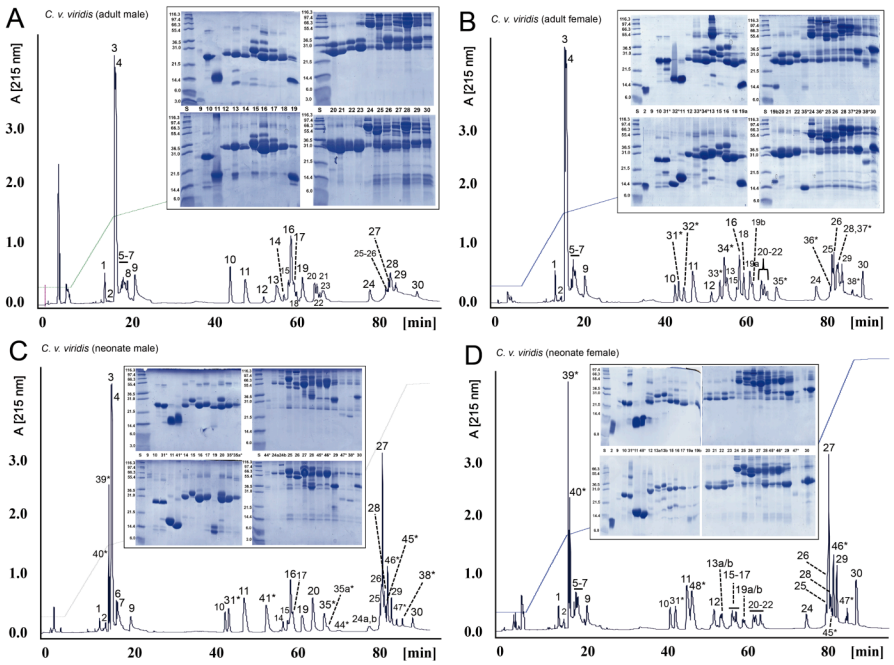

Figure 7.2: Characterization of the Venom Proteomes of *C. v. viridis*. Panels A-D display reverse-phase HPLC separations of the venom proteins from an adult male, adult female, neonate male and neonate female snake, respectively. Fractions were collected manually and analyzed by SDS-PAGE (insets) under non-reduced (top gel panel) and β-mercaptoethanol-reduced (bottom gel panel) conditions.

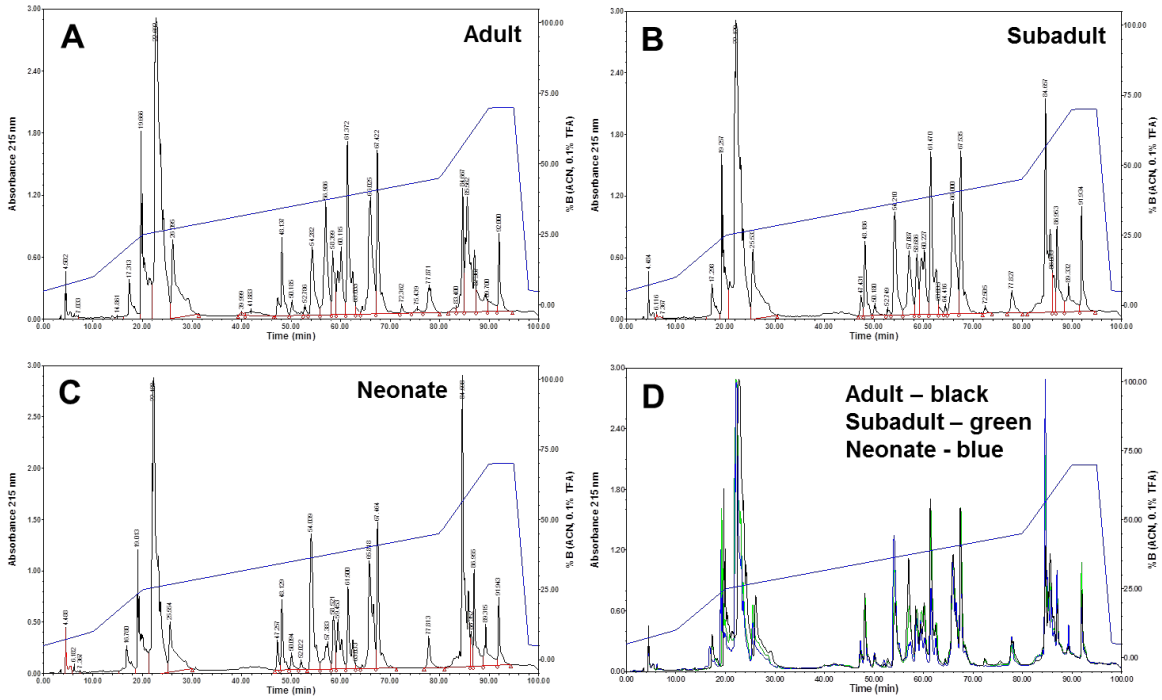

Figure 7.3: Combined Samples Representing Three Age Classes of *C. v. viridis*. These chromatograms essentially represent a graphical average of 12 individual venoms for each age class. A. Adult venoms. B. Subadult venoms. C. Neonate venoms. D. Overlay of chromatograms A-C; adult – black line; subadult - green; neonate - blue. Note that significant differences exist between adults and neonates, in particular the myotoxin a (myo a) and metalloproteinase (SVMP) peaks.

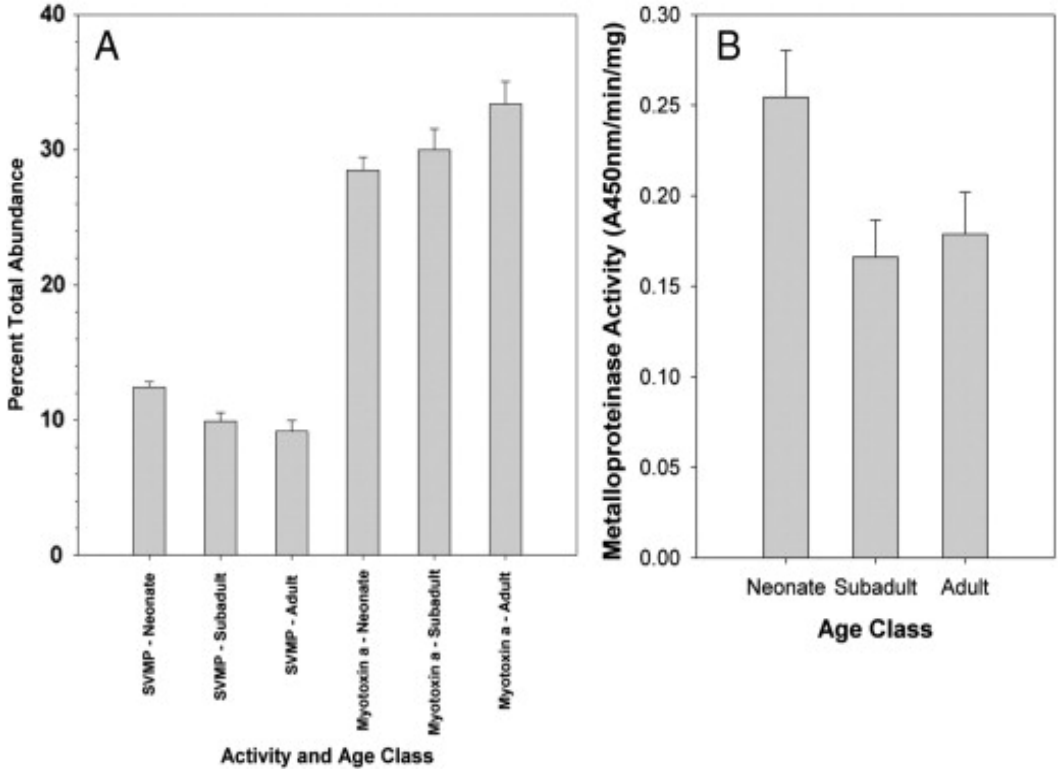

Figure 7.4: Age-related Changes in SVMP and Myotoxin a abundance in *C. v. viridis* venoms. A. SVMP and myotoxin a content of all 38 venoms analyzed (12 adult and subadult, 14 neonate) by RP-HPLC. Adult and neonate venoms differ in SVMP ($p = 0.002$) and myotoxin a ($p = 0.05$) content; SVMP content of subadult venoms also significantly differed when compared to neonate venoms ($p = 0.02$), however there was no difference between subadult and adult venoms for myotoxin a or SVMP content (p's = 0.23 and 0.69, respectively). B. SVMP activity toward azocasein substrate. Consistent with the RP-HPLC-based content differences, neonate venom activity levels also differ statistically when compared to subadult and adult venoms ($p < 0.001$). SVMP activity was not significantly different between subadult and adult venoms ($p = 0.61$).

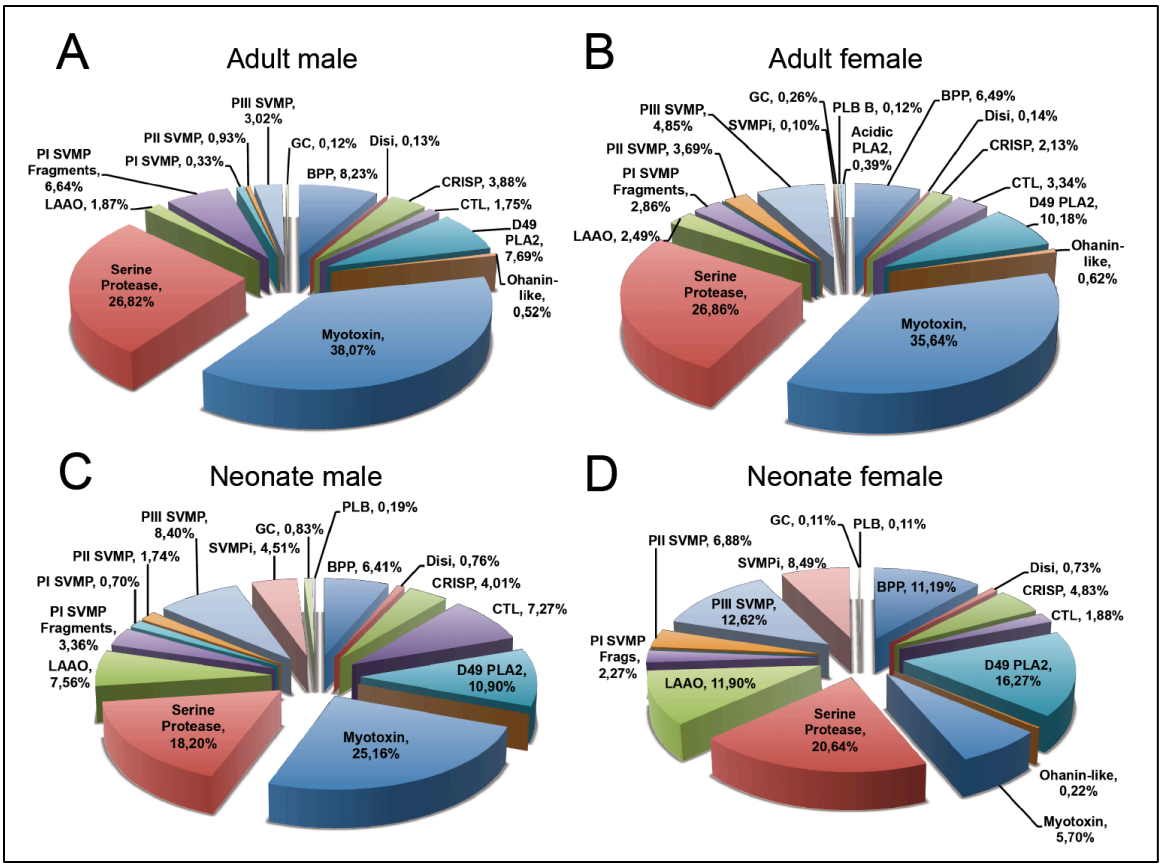

Figure 7.5: Protein Family Composition of Primary *C. v. viridis* venoms (adult male 281; adult female 288; neonate male 280; and neonate female 249). Pie charts represent the relative occurrence of proteins from the different toxin families as identified in the current work. Percentages below protein families represent the percent of the total RP-HPLC-separated components found in *C. v. viridis* venom. BPP, bradykinin-potentiating peptide; Disi, disintegrin; CRISP, cysteine-rich secretory proteins; CTL, C-type lectin-like; PLA_2, phospholipase A2; LAAO, L-amino acid oxidase; SVMP, snake venom metalloproteinase; GC, glutaminyl cyclase; PLB, phospholipase B.

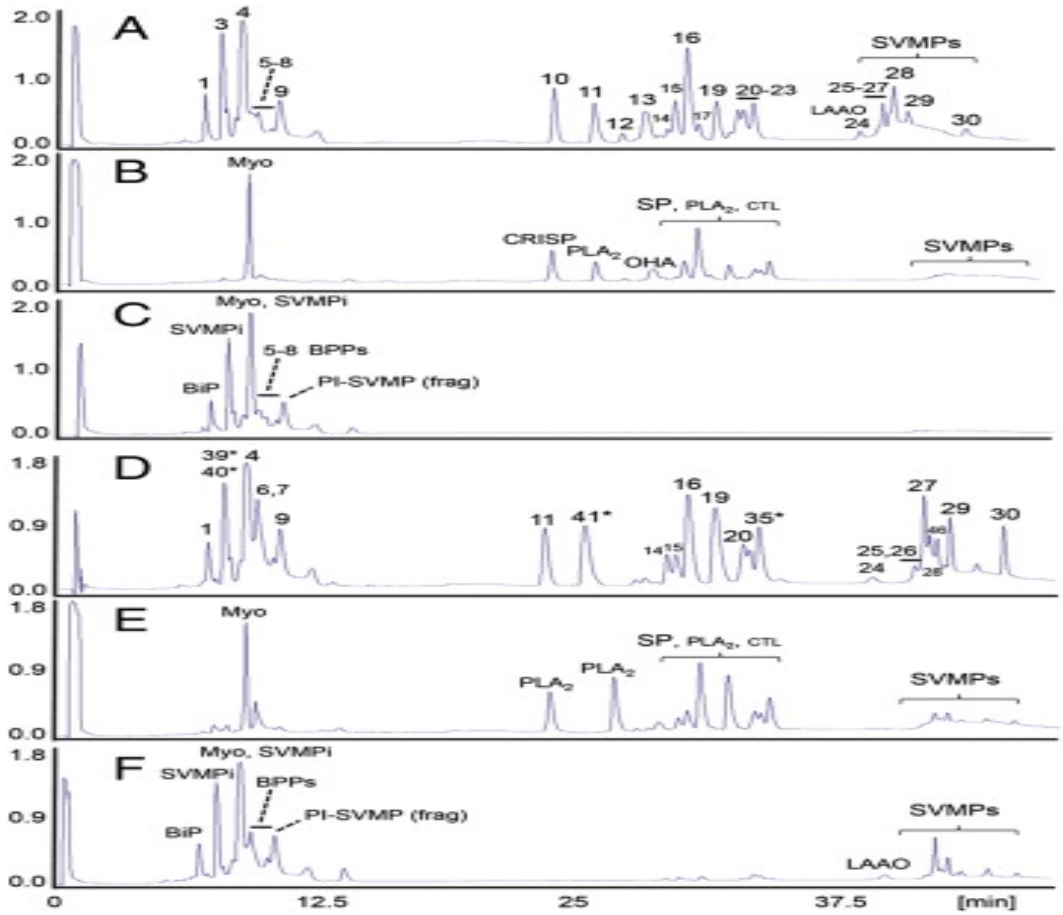

Figure 7.6: Antivenomic Analysis on a CroFab® Affinity Column. Panels A and D, RP-HPLC separation of the venom proteins of one adult and one neonate male *C. v. viridis*. Panels B and C show, respectively, reverse-phase HPLC separations of the components of adult male *C. v. viridis* recovered in the bound and the flow-through fractions of the affinity column. Panels E and F show the affinity column immunocaptured and non-retained protein fractions of neonate *C. v. viridis* venom, respectively. Protein peaks are labeled as in panels A (adult male) and C (neonate male) of Fig.2. Supplemental Table S1 lists the proteins found in each chromatographic fraction. BiP, bradykinin inhibitory peptide; OHA, ohanin-like protein. Other acronyms as in the legend of Fig.3.

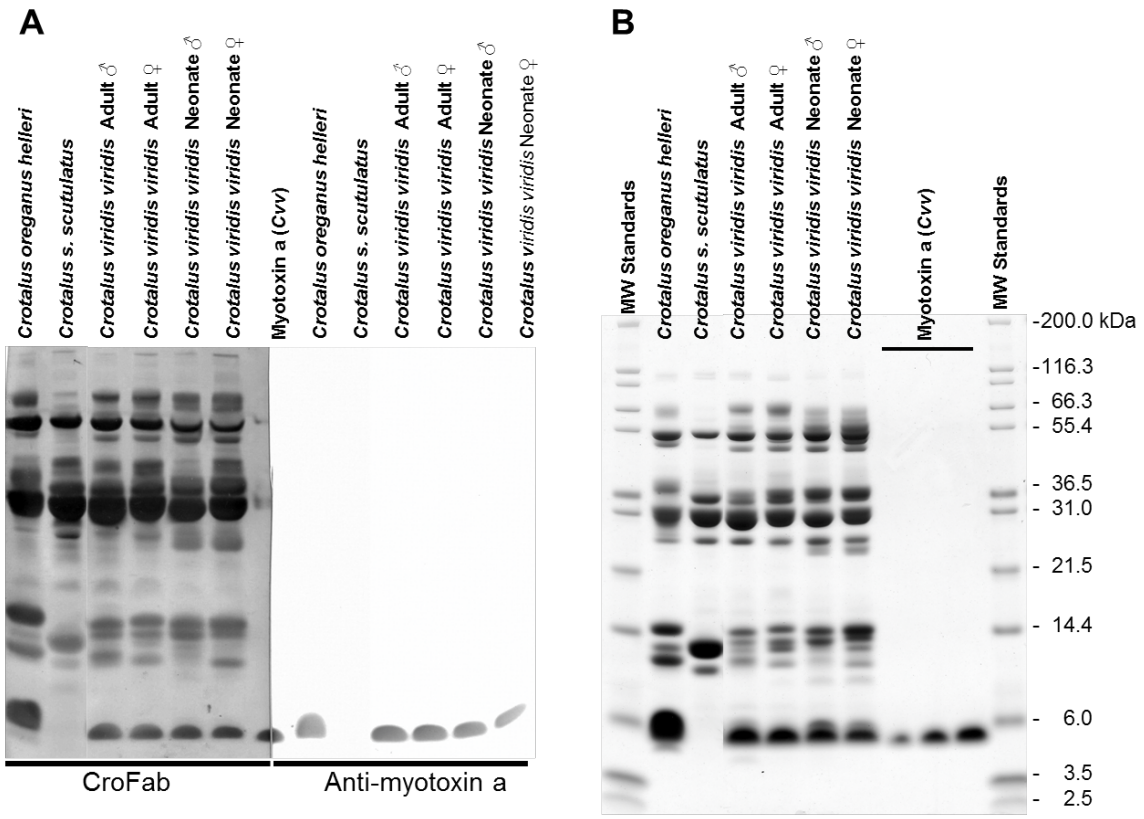

Figure 7.7: Western blot and SDS-PAGE Analysis.Panel A, venoms and myotoxin a on nitrocellulose were detected with either CroFab or specific anti-myotoxin a antibodies (rabbit). Note that myotoxin a is detected by both CroFab® and specific anti-myotoxin a antibodies. Panel B, SDS-PAGE analysis of the same venoms (16 µg/lane) and myotoxin a (1, 3 and 5 µg/lane) as in A. For both panels A and B, *C. o. helleri* and *C. s. scutulatus* venoms were included as myotoxin a-positive and negative controls, respectively.

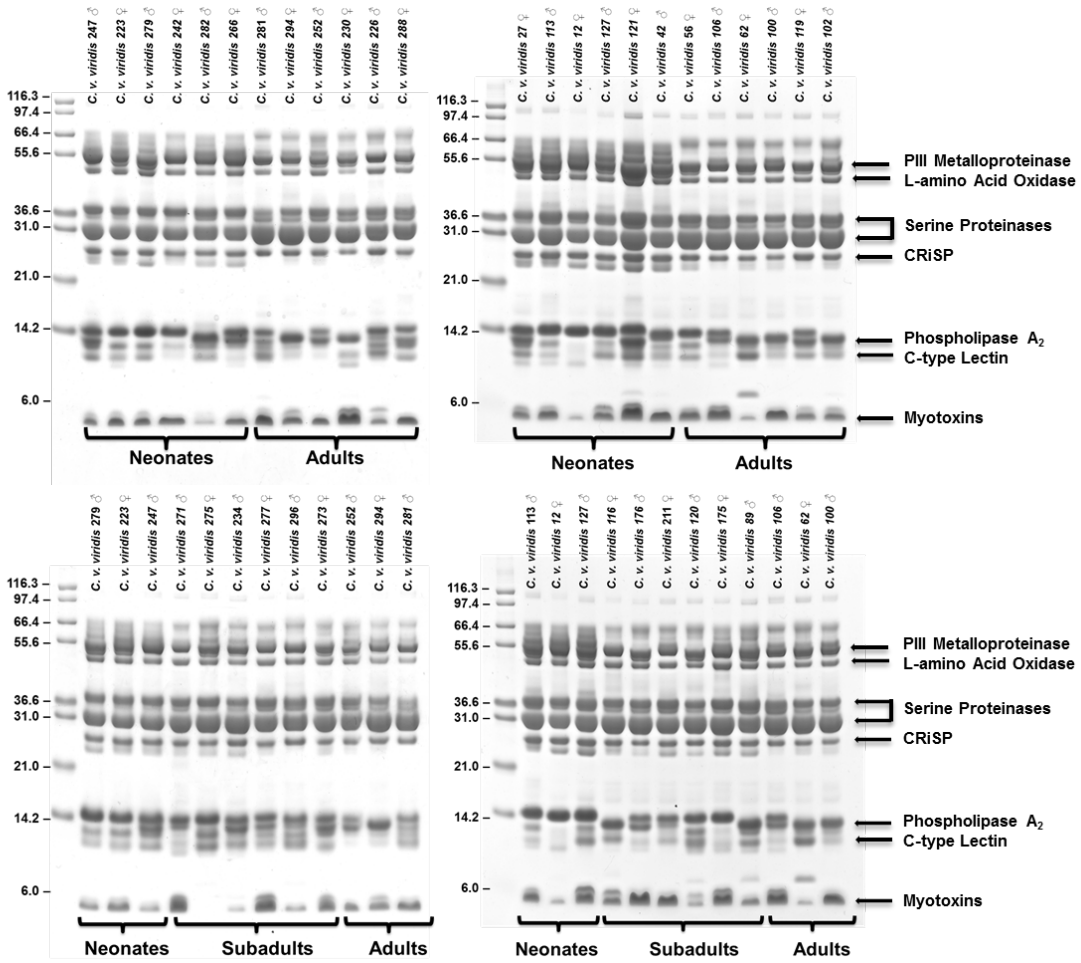

Figure 7.8: Reducing SDS-PAGE of Secondary Venom Samples - 16 µg/lane. Protein families found in bands of specific masses [1,7] are indicated on the right. Note that although most bands are shared between all individuals, differences in intensities (representing differing concentrations) exist, particularly among P-III metalloproteinases, PLA_2s and myotoxin a bands.

CHAPTER VII

CONCLUSION

As with so many disciplines in biological sciences, the fields of herpetology and toxinology have grown tremendously over the last few decades, largely due to advances in laboratory techniques. As venoms are shaped by numerous biological factors, most importantly diet, significant venom variation may be documented, at times even between individuals of the same species as they mature. These ontogenetic shifts in venom composition often accompany changes in diet and may correlate with a shift to more complex prey-handling behaviors (Mackessy 1988; Hammerson 1999; Hayes 1992). Chapter II of this dissertation clearly shows that chemosensory responses to prey-derived cues shift with snake age in *C. v. viridis*. In neonates, rates of tongue flicking were significantly higher to natural lizard stimuli when compared to tongue flick rates in sub-adult or adult snakes. This response declined as snakes increased in size, however, and tongue flicking to natural rodent cues was significantly higher when compared to those of neonates. Chapter III provides additional evidence that these chemosensory behaviors are innate, and although responses to prey cues may be learned over time, the sample of stunted *C. v. viridis* analyzed, which never encountered natural prey items, responded significantly to natural prey stimuli. Being gape-limited predators, neonate rattlesnakes are limited primarily to taking small ectothermic prey, which they often obtain by a strike -and-hold predatory behavior. However, it is likely that small ectothermic prey are not energetically favorable prey items for adult Prairie Rattlesnakes, and larger

endothermic prey would be more metabolically advantageous for these snakes to consume. Yet, predatory encounters between adult rattlesnakes and larger endothermic prey present the risk of prey retaliation, which may be threatening to the snake. Therefore, adult rattlesnakes often utilize a strike-and-release mode of prey envenomation, which minimizes contact with potentially dangerous prey, yet creates the additional task of locating prey that has wandered from the attack site. Although it is reasonable to assume that snakes are opportunistic feeders in the wild, the ability to discriminate between chemical stimuli not only leads to location and capture of preferred prey, it also assists in the location of envenomated prey following the predatory strike.

This ability to release potentially dangerous prey during predatory episodes, and then relocate this prey once it has succumbed to venom, is a remarkable behavioral adaptation seen among adult viperid snakes. In Chapter IV, I identify crotatroxin 1 and 2 as the molecular components, disintegrins, of venom allowing for prey relocation in rattlesnakes. The downstream signaling that is the result of disintegrin-integrin interactions, ultimately leading to the release of chemical odor(s) detectable by rattlesnakes during prey relocation, deserves further attention. It is possible that upon disintegrin binding, a downstream signaling cascade causes a release of novel odors, or increase of already present odors, that are detectable by the snakes. Since disintegrins appear to be present in all viperid snakes, but absent from venoms of all other advanced snakes, it is hypothesized that the presence of this compound in venom has led to the evolution of the complex strike-and-release behavior. Further, as several species of vipers contain non-RGD and dimeric disintegrins, further studies should examine if these

compounds exhibit the same prey-relocation response as the monomeric disintegrins present in *C. atrox*.

Disintegrins function by blocking an array of integrin receptors (Scarborough et al. 1993; Calvete et al. 2009), and the roles of integrins in numerous human pathologies is being examined continuously (Wehrle-Haller and Imhof 2003; Desgrosellier and Cheresh 2010). The recognition that disintegrins have unique anti-cancer activity (e.g., Trikha et al. 1994) has led to a plethora of studies examining the therapeutic potential of these compounds (Zhou et al. 1999, 2000; Sánchez et al. 2009; Galan et al. 2008; Lucena et al. 2011, 2012, 2015). In spite of these numerous studies, significant differences with regards to disintegrin binding affinity towards specific integrin receptors provides a strong motivation to continue to examine snake venoms for novel disintegrins that may have application in biomedical research. In Chapter V, I use a combination of molecular and proteomics techniques to screen the venom of *C. s. tzabcan* for disintegrins, identifying six novel disintegrin sequences and isolating and characterizing the dominant isoform, named tzabcanin. Tzabcanin, a 7.1 kDa RGDN-containing disintegrin, showed only slight cytotoxicity toward human colon cancer (Colo-205) cells, but it inhibited cell adhesion of both breast (MCF-7) and colon (Colo-205) cancer cells to fibronectin and vitronectin. Although I was unable to identify the specific integrin(s) tzabcanin was binding to in these two cell lines, in Chapter VI integrin $\alpha_v\beta_3$ was identified as a binding site for tzabcanin in human melanoma (A-375) and lung (A-549) cancer cell lines. Further, by binding $\alpha_v\beta_3$, tzabcanin inhibited adhesion of both cell lines to the extracellular matrix protein vitronectin, and it inhibited cell migration over 24 hrs in A-375 and 72 hrs in A-549 cells. These results support the potential for tzabcanin, and

perhaps other *C. s. tzabcan* venom disintegrins, to provide useful information toward the development of novel drug therapies.

Venomics (venom proteomics) is a relatively new method for probing venom proteomes, and it provides in-depth analyses of venom composition which is a necessary early step toward identifying compounds that have potential medicinal value, and by coupling this with antivenomic methods, one can gain significant insight into antivenom efficacy and more effective methods for antivenom production (Calvete et al. 2009; Calvete 2010). The final research chapter of my dissertation (Chapter VII) examined the venom proteome of a very wide-spread species, *C. v. viridis*, revealing a unique pattern of ontogenetic shift in venom composition. In rattlesnakes, venom compositions often shift from neonates containing high toxicity and low metalloproteinase activity, to a lower toxicity and higher metalloproteinase activity seen in adults. However, venomic analyses of the current *C. v. viridis* samples indicate the opposite relationship, with overall metalloproteinase concentrations being lower in venoms from adult snakes, and myotoxin a and 2 (two potent rodent-specific toxins) concentrations higher in adult venom samples. Further, some unusual venom compounds were identified in the *C. v. viridis* venom proteome, such as phospholipase B and ohanin-like toxin. Although the biological roles of these two compounds remains largely unknown, the presence of these two proteins, combined with the novel ontogenetic shift in overall composition, may be correlated with prey preference for this population of *C. v. viridis*. Toxins with prey specificity have been documented (Pawlak et al. 2009), and it is hypothesized that the presence of specific compounds in the venom proteome of *C. v. viridis* are there to target distinct prey items. Further, antivenomics results suggested that CroFab® does not appear to immunocapture

myotoxins and other small peptides efficiently; however, Western blot analysis does indicate that these compounds are recognized by CroFab®. When considering the reasonably high efficacy of CroFab® in treating viperid snakebites, including those by *C. v. viridis*, it is likely that the relatively low immunoreactivity of CroFab® to myotoxin is indeed sufficient enough for effective clinical treatment following envenomation.

In conclusion, the results from this dissertation provide a unique look into numerous facets of biological and toxinological research, from the feeding ecology and behavior of rattlesnakes, to the individual biological roles of venom components (disintegrins), to the anti-cancer effects of a single venom protein. Due to the presence of disintegrins in venom, rattlesnakes can use their exquisite chemosensory recognition abilities to distinguish between envenomated and non-envenomated prey. Interestingly, the same protein family that allows for prey relocation in rattlesnakes also exhibits unique anti-cancer functions by blocking integrin receptors, and disintegrins may provide a novel therapy for cancer treatment. Finally, proteomic analyses of *C. v. viridis* venom demonstrated a novel ontogenetic shift in venom composition and identified several less well known and less abundant venom compounds. Advancements in laboratory techniques will continue to unravel the mysteries of the many venom compounds, which still exhibit unknown functions, and may provide greater insights on the biological roles and potential medicinal values of this highly complex mixture of proteins and peptides.

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APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

All animal research conducted was carried out under protocol #1302D-SM-S-16 (S.P.M) approved by the Institutional Animal Care and Use Committee of the University of Northern Colorado.