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MEDICAL AND DIAGNOSTIC APPLICATIONS OF SNAKE VENOM PROTEOMES

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Abstract: Snake venom is a highly toxic secretion produced and stored in specialized salivary glands of snakes which constitutes a vast array of biologically-active compounds, such as enzymes, proteins, peptides and low molecular weight compounds. These substances target an immense number of receptors and membrane proteins as well as coagulation proteins with high affinity, selectivity and potency, and can serve as potential drugs or scaffolds for drug design. During the recent years, much attention has been given to understand the mechanism of action of complex venom proteins for the development of novel drugs and therapeutic agents to treat life-threatening diseases such as cardiovascular diseases, cancer, thrombosis, arthritis, microbial infections and hypertension etc. Further, snake venom components have found uses in the diagnosis of haemostatic disorders. This paper reviews the various biomedical applications of snake venom proteins in terms of therapeutic and diagnostic values.

Key words: anticancer drug; antimicrobial agent; diagnostic reagents from snake venom; medical application of venom toxins; thrombolytic agents.

Introduction

The Indian subcontinent apart from being a rich source of flora and fauna is also home to a rich diversity of ophidian species. Only 242 species of snakes have been identified in India so far which includes 57 poisonous or harmful species. Snake venom is an evolutionary adaptation to immobilize, incapacitate as well as to digest their prey, which is also used as a defense mechanism by the snakes when encountered by enemies. In the recent years, snake venom and its components has gathered enormous interest by researchers across the globe. Scientists over the past few decades have postulated that despite of the harmful and life-threatening affects of snake venom, its component may provide highly specific research tools for the development of novel life-saving medicines and drugs against some common and life threatening diseases (Stocker, 1990) besides providing insight to basic coagulation process.

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A Brief Account of Snake Venom Composition

Over 90% of the solid snake venom components are pharmacologically active proteins and polypeptides, responsible for exerting pharmacological effects in victims (Sarkar and Devi, 1968; Stocker, 1990). Non-protein ingredients of venom include carbohydrate and metals (often in the form of glycoprotein and metalloprotein enzymes), lipids, free amino acids, nucleotides and biogenic amines.

The proteins/ polypeptides constituting snake venom can further be divided into enzymatic and non-enzymatic toxins, which in turn may be coagulant, anticoagulant or fibrinolytic in nature. Non enzymatic toxins are found to be predominant in cobra venom, whereas viper venom is found to be composed mainly of enzymatic proteins (Sarkar and Devi, 1968; Stocker, 1990). Snake venom toxins may have more than one specific activity, and therefore they may play multiple roles in the overall effect of envenoming. Considering this fact, the isolation and characterization of individual venom

components constitutes the mainstay of toxinology, as a key strategy to scrutinize and to analyze the complex series of events involved in envenoming. The number of enzymes and their specific activities varies from venom to venom and about 26 such enzymes have been identified. Although no single venom contains all of them, at least 10 of these enzymes are present in every snake venom, while others are found in several combinations in different varieties of snakes (Stocker, 1990; Sarkar and Devi, 1968; Mebs, 1970). A comparative study on the activities of enzymes in venom of 42 species comprising Colubridae, Elapidae, Viperidae and Crotalidae snake families led to the conclusion that Elapidae venoms are rich in phospholipases and phosphodiesterase (Mukherjee *et al.*, 2000; Mukherjee and Maity, 1998a; Mukherjee and Maity, 1998b; Doley and Mukherjee, 2003; Doley *et al.*, 2004; Mukherjee, 2007), whereas Viperidae venom contain proteases, coagulant, kinin-releasing and arginine ester hydrolyzing enzymes (Stocker, 1990; Kini 1997; Mukherjee *et al.*, 2000).

The variation in venom composition is a common phenomenon and plays an important role in pathophysiological symptoms following envenomation and therefore deserves dedicated medical concern. The venom composition varies greatly due to variation in individual, geographical origin, age and diet of the snakes (Mukherjee and Maity, 1998a; Daltry *et al.*, 1996; Tsai *et al.*, 1996). The mutational changes in the gene which is the primary basis of evolution also contributes significantly to the venom variation that occurs between closely related species or even within a species (Daltry *et al.*, 1996; Assakura *et al.*, 1992; Fry *et al.*, 2003).

Biomedical and Therapeutic Application of Snake Venom Components

Investigations over the past few decades, have shown that the myriad of proteins found in venoms of different snakes have the potential to be developed as a drug for the treatment of a number of medical concerns such as cardiovascular ailments, thrombosis, arthritis, cancer and many other diseases (Stocker, 1990; Lipps, 1999; Toombs, 2001; Marsh and Williams, 2005). Besides, many of them are now successfully employed as conventional diagnostic tools for the assessment of different coagulation factors and proteins involved in the coagulation cascade. Venom toxins have developed highly specific molecular targets, which make them valuable for drug usage in terms of limiting potential side effects. Studies about these protein toxins and their mechanism of action have contributed to the knowledge about the various molecular mechanisms involved in the physiological processes and in the development of novel therapeutic agents for the treatment of various life threatening diseases.

The search for lead compounds for the development of new therapeutic agents has long included a focus on snake venoms. The descriptions below and Table 1 lists some of the snake venom proteins which have found a place in the biomedical industry.

(a) Novel Drugs from Snake Venom Showing Anticancer and Anti-tumor Activity

Cancer, which has long been known to the human as a life threatening disease is characterized by uncontrolled growth and spread of abnormal cells. Medical science despite of its constant research for the development of effective drugs against cancer has not been fully successful in dealing with this deadly disease.

A major problem faced by the physicians is various drugs and chemotherapeutic agents usually used for the treatment of cancer are unable to distinguish between tumor cells and other healthy cells, causing undesirable side effects, sometimes leading to death. The venom-derived toxins seem to act only on certain types of cells and have shown differential lytic activity against various cell lines and subcellular organelles (Doley *et al.*, 2004). Recent research has been directed with special emphasis on potential application of purified proteins and crude snake venoms that might disrupt the normal sequence of events leading to the spread of tumor. In 1933, Calmette and his colleagues first time reported the anti-carcinogenic activity of cobra (*N. naja*) venom (Iwaguchi *et al.*, 1985) and thereafter, many reports have established the anticancer potential of different species of Elapidae, Viperidae and Crotalidae snake venoms

Table 1
Therapeutic Applications of Snake Venom and Snake Venom Components

Snake venom component	*Example*	*Source*	*Biological functions*	*Applications*	*Reference*	*Comments*
Snake venom thrombin-like enzymes	Ancrod	*Agkistrodon rhodostoma*	Converts fibrinogen into non-clottable form of fibrin i.e. Therapeutic difibrination	Treatment of ischaemic stroke, HATT syndrome, deep vein thrombosis and peripheral occlusive diseases, alternative to heparin in cardiopulmonary bypass	[2]	Marketed as Viprinex ™
Plasminogen activating enzymes	TSV-PA	*Trimeresurus stejnegeri*	Dissolution of fibrin clot via activation of plasminogen to plasmin	Treatment of vascular diseases such as myocardial infarction, pulmonary embolism, stroke, deep vein thrombosis and other vascular thromboses, cancer	[39] [50]	Potential to be developed as a fibrinolytic drug In Phase II clinical trials
Direct fibrinolytic enzymes	Alfimeprase (recombinant form of Fibrolase)	*Agkistrodon contortrix contortrix*	Dissolution of fibrin/ whole blood clot directly (plasmin-like activity)			
Disintegrins	Contortrostatin	*A.contortrix contortrix*	Blocks integrins during tumor progression	Applicable as antitumor agents	[51]	Not yet available in market
	Rhodostomin	*Calloselesma rhodostoma*	Inhibits angiogenesis		[55]	-do-
	Salmosin	*A. hays brevicaudus*			[21]	-do-
Platelet glycoprotein IIb/IIIa antagonists	Integrilin (Eptifibatide)	*Sistrurus miliarius barbouri*	Inhibits platelet aggregation	For reducing the risk of acute cardiac ischemic events in patients with unstable angina	[57]	Marketed as Integrilin® by Millenium Pharmaceuticals
Thrombin inhibitors	Bothrojaracin	*Bothrops jaraca*	Anticoagulant		[58]	Not yet marketed
Plasmin inhibitors	Textilinin-1	Pseudonaja textilis	inhibitor of plasmin-catalysed fibrinolysis	Anti-bleeding agent in the treatment of chronic inflammatory diseases such as rheumatoid arthritis and arteriosclerosis. Restriction of cell proliferation during cancer.	[9]	Marketed by QRXPHARMA, but withdrawn due to concerns over side effects
Angiotensin-converting enzyme (ACE) inhibitor	Captropril	Bothrops jaraca	blocks the conversion of angiotensin I to angiotensin II of the RAAS system	Treatment of hypertension, congestive heart failure, also investigated for use in treatment of cancer	[45]	Marketed by Bristol-Myers Squibb under the trade name Capoten.

(Iwaguchi *et al.*, 1985; Yang *et al.*, 2005a; Debnath *et al.*, 2007; Gomes *et al.*, 2007).

It has been suggested that fibrin deposition plays an important and distinct roles at different stages of tumor growth and dissemination. Blood vessel thrombus due to fibrin accumulation may lead to myocardial infarction or other cardiovascular diseases. Moreover, occurrence of venous thromboembolism is a common complication in cancer patients. Although the degree of malignancy is not related to the extent of fibrin deposition, but the amount of fibrin deposited is a characteristic of a particular type of tumor (Markland, 1990). In addition to fibrin, there are strong evidences to show that platelets also play an important role in spreading the tumor metastasis and this is probably by shielding tumor cells in platelet thrombi (Markland, 1990). Fortunately, many of snake venoms are enriched in fibrinolytic enzymes (Trikha *et al.*, 1994; Leonardi *et al.*, 2007); therefore, attempts have been made to inhibit the tumor growth and invasion by interfering with fibrin deposition and/ or platelet aggregation with the help of fibrinolytic enzymes.

Infusion of a purified 33.7 kDa monomeric fibrinogen clotting snake venom enzyme crotalase to different animals resulted in a benign state of defibrinogenation (Markland, 1990). There was neither fibrin deposition in the internal organs nor any intravascular coagulation was observed. In order to suppress the tumerogenecity of mouse B16 melanoma cell lines, the cells were treated with purified crotalase *in vitro* and then subcutaneously transplanted into C57 BL/6 mice (Markland, 1990). Interestingly, although crotalase did not show cytotoxicity against B16 melanoma cells, but treatment with crotalase resulted in inhibition of the growth of cancer cells. It has been suggested that by inhibiting the fibrin gel formation around a tumor cell, crotalase could create a more favorable condition for immunogenic attack of cancer cells by macrophages or other effector cells. Thrombin-like venom enzymes may help to stop metastasis of breast cancer as well as ovarian cancer as it can inhibit tumor dissemination and angiogenesis (Trikha *et al.*, 1994).

Osteosarcoma is a very malignant bone tumor which has a high metastatic potential and usually lead to poor prognosis. In the metabolic cascade, the adhesion of tumor cells to the endothelium or, extracellular matrix is an essential step. Rhodostomin, a peptide isolated from the venom of *Calloselesma rhodostoma*, can block the osteosarcoma cell line (ROS 17/2.8) of rat (Yang *et al.*, 2005a). It is known that Rhodostomin may serve as a potent anti-metastatic agent in blocking the thrombin enhanced cell adhesion potential. Further investigation of anti-adhesion therapy with venom components perhaps leads to the prevention of potential metastasis in certain cancer patients (Yang *et al.*, 2005a). Salmosin, is yet another example of a peptide isolated from the venom of *Agkistrodon hays brevicaudus*, which exhibits anti-tumor and anti-angiogenic effects (Kang *et al.*, 1999).

Among the various toxins of the venom, cytotoxins, the primary target for which is the cell membrane, are among the most prominent components of cobra venom (Mukherjee and Maity, 1998a; Mukherjee and Maity, 2002; Feofanov *et al.*, 2005; Izidoro *et al.*, 2006) .For example, cytotoxins CT1 and CT2 from *Naja oxiana*, CT3 from *N. kaouthia* and CT1 from *N. haje* are demonstrated to possess this property with respect to human lung adenocarcinoma A_{549} and promyelocytic leukemia HL60 cells (Feofanov *et al.*, 2005). It was demonstrated further that an L-amino acid oxidase isolated from *Bothrops pirajai* snake venom possessed cytotoxic activity and in *in vitro* condition it showed antitumor activity against S180 tumor, human breast (SKBR-3), acute T cell leukemia (Jurkat) cancer, and Erlich ascitic tumor (EAT) cell lines (Izidoro *et al.*, 2006). Similarly, in a study done by Debnath and his colleagues, it has been shown that venoms of Indian monocellate cobra (*N. kaouthia*) and Russell's viper (*Daboia russelli*) possessed anticancer activity and in *in vitro* condition prevented the proliferation of malignant cells, at non-toxic concentrations (Debnath et al, 2007). Similarly, Yang and his colleagues in 2005 also demonstrated the induction of apoptosis in human leukemia K562 cells by cardiotoxin III isolated from *N. n. atra* venom. In fact, this was the first report on the mechanism of the anticancer effect of CTX III on human leukaemia K562 cells

through a mitochondrial mediated pathway (Yang *et al.* 2005b).

(b) Snake Venom Proteins for the Prevention/ treatment of Thrombosis and Vascular Occlusive Diseases

Therapeutic defibrinogenation with snake venom enzymes has been attempted to treat peripheral arterial occlusion diseases in which surgical revascularization could not be satisfactorily achieved (Furukawa and Ishimaru, 1990). Batroxobin (Funk *et al.*, 1971) and Ancrod (Burkhart *et al.*, 1992) are thrombin-like enzymes; purified from the venom of *Bothrops moojeni* and *Agkistrodon rhodostoma,* respectively, have found tremendous application in this aspect. Intravenous or subcutaneous injection of Arvin®, (a commercial preparation of Ancrod) into human causes a specific, dose-dependent reduction of the plasma fibrinogen level; therefore, Arvin is used in the treatment and prevention of vascular occlusive diseases (Furukawa and Ishimaru, 1990). Similarly, batroxobin (Defibrinase R) is currently used for controlled depletion of fibrinogen and is a selective antithrombic agent on deep vein thrombosis peripheral arterial diseases (Itoh *et al.*, 1988).

Unlike snake venom thrombin like enzymes, fibrinolytic enzymes isolated from snake venom do not lead to defibrinogenation but can digest fibrin clots directly; thereby suggesting potential application of fibrinolytic enzymes for the treatment of thrombotic ailments such as strokes, heart attacks and other diseases associated with the formation of thrombus. Of the many fibrinolytic enzymes identified so far, Fibrolase, a direct-acting fibrinolytic metalloproteinase enzyme isolated from southern copperhead venom (*Agkistrodon contortrix contortrix*), has been used to digest occlusive blood clots in animal models (Retzios and Markland, 1988). In 1997, Sanchez and colleagues reported the preparation of a superior thrombolytic agent possessing both thrombolytic and antiplatelet properties, by conjugating fibrolase to a peptide which inhibits platelet aggregation (Sanchez *et al.*, 1997). Recombinant fibrolase called Alfimeprase was also been produced which seemed to be a potent thrombolytic agent (Toombs, 2001). However, its failure at the phase III clinical trials lead to the abolishment of its further implications as a thrombolytic drug. We have also isolated a low molecular weight (~12 kDa) direct fibrinolytic, non-toxic enzyme from venom of *Daboia russelli*. *In vitro* studies demonstrated that this non-cytotoxic peptide has tremendous potential to dissolve fibrin as well as artificial blood clot thus advocating the future application of this peptide as thrombolytic agent (Mukherjee, A. K. and Thakur, R., unpublished observation). Plasminogen activators are yet another class of fibrinolytic enzymes which result in the breakdown of thrombus by activating inactive plasminogen to plasmin which in turn degrades fibrin. TSV-PA is once such enzyme purified from *Trimeresurus stejnegeri* (Parry *et al.*, 1998).

(c) Other Biomedical Application of Venom Proteins

Besides having potential therapeutic importance in handling diseases associated with thrombosis such as cardiovascular diseases, and cancer, snake venom proteins are also found to have other therapeutic applications. For example, snake venom neurotoxins, mainly α–Btx, played a leading role in myasthenia gravis research (Samson *et al.*, 2001). Crotamine, myotoxin a, and homologous peptides are also seem to be useful candidates to study the muscle cell membrane and their sodium channels (Hong and Chang, 1985). Dendrotoxin (a toxin derived from *Dendroaspic* venom) and their homologues, are becoming extremely valuable tools to characterize K^+ channels which are involved in the regulation mechanisms of cell excitability and synaptic transmission (Harvey and Anderson, 2004).

Antagonists and inhibitors of different coagulation and membrane proteins isolated from snake venom, has hypothesized for its further biomedical applications in the field of hemastatic disorders and ischemic ailments. For example, Inhibitors of angiotensin converting enzymes such as captropril isolated from *B. jararaca* and marketed as captopen, serves for the control of hypertension and congestive heart failure (Smith and Vane, 2003). Another example is of bothrojaracin, which is a potent thrombin inhibitor and as such a strong anticoagulant

(Zingali *et al.*, 2003). Textilin-1 inhibits plasmin catalysed fibrinolysis, thereby suggesting its application as a potent anti-bleeding agent (Flight *et al.*, 2009). Platelet glycoprotein IIb/IIIa antagonists such as Integrilin which inhibits platelet aggregation, is widely used for reducing the risk of acute cardiac ischemic events in patients with unstable angina (Zeymer, 2007).

Recent studies have shown that snake venom can be explored as a promising source for analgesics (Mancin *et al.*, 1998; Pu *et al.*, 1995). Crotamine (~5kDa) from *Crotalus durissus terrificus* is 30 fold more potent than morphine as analgesic (Mancin *et al.*, 1998). Hannalgesin from *Ophiophagus Hannah* (Pu *et al.*, 1995) and Cobrotoxin b from *Naja naja atra* (Chang *et al.*, 1997) are potent analgesics isolated, that do not cause neurological or muscular deficit.

Diagnostic Applications of Snake Venom and its Components

As shown in Table 2, Snake venoms contain a vast array of components, many of which have found extensive applications in the diagnosis of haemostatic disorders (Marsh and Williams, 2005). For example, Russell's viper venom contains toxins which are used to assay blood clotting factors V, VII, X, platelet factor 3 and lupus anticoagulants as well as coagulation proteins such as fibrinogen and prothrombin.

RVV contains a potent activator of factor X (RVV-X) and RVV-X® (Pentapharm) has been employed in a number of clotting assays, notably for the measurement of factor X itself, for distinguishing between factor VII and factor X and in lupus anticoagulant (LA) assay (Takeya *et al.*, 1992). RVV-X enzyme has also been used to assay platelet factor 3 (Hardisty and Hutton, 1966). Due to their unique characteristics, RVV enzymes have been used for the improvement of the detection of Von-Willebrand disease as well. The dilute Russell's viper venom time (dRVVT) occupies a particular niche in the assay of LA as it is quick, sensitive and inexpensive. RVV-V, an enzyme which is commercially marketed by pentapharm for the measurement of Factor V and study of factor V activation was also isolated and purified from *Daboia russelli* venom (Kisiel, 1979).

Yet another diagnostically important compound is the Reptilase® reagent which is the trademark for a lyophilized preparation of pure Batroxobin from *Bothrops atrox* venom, used for measuring "reptilase time", a very popular screening test which is always performed in parallel with thrombin time (Funk *et al.*, 1971). Batroxobin and possibly several other fibrinogen coagulant snake venom enzymes may be used for quantitative determination of fibrinogen in plasma (Funk *et al.*, 1971).

A potent, fast acting Protein C activator (Protac®, Pentapharm) from the venom of the Southern copperhead snake, *Agkistrodon c. contortrix* (Stocker *et al.*, 1987) has been identified which is extremely useful in activating Protein C for assay by direct chromogenic method (Nathan *et al.*, 1987), an indirect chromogenic activated partial thromboplastin time (Stocker *et al.*, 1988); and a functional clotting assay (Martinoli and Stocker, 1986). Many other proteins isolated serve for measuring vWF level or prothrombin levels in plasma as well (Usami *et al.*, 1993; Weigner *et al.*, 1980).

Disintegrins are yet another diagnostically important component from snake venom which prevent platelet aggregation via inhibition of surface glycoprotein receptor activity (Ouyang *et al.*, 1992). Because of the presence of this property, disintegrins offer a unique opportunity for the study of platelet-platelet and platelet-endothelium interactions (Marsh and Williams, 2005). One of the most studied disintegrins include Triflavin from *Trimeresurus flavoviridis* (Huang *et al.*, 1991). Disintegrins may also find a role in imaging both thrombi and emboli and it has been shown that Bitistatin from *Bitis arietans* venom is the most promising in this regard (Knight *et al.*, 1996).

Conclusion

It is obvious that these proteins or peptides, derived from snake venom could produce potentially huge medical benefits for mankind. During the last decade, many of the snake venom proteins and toxins showing great promise for medical application have been isolated, purified and characterized; however, further studies for many of the isolated proteins are required for

Table 2
Diagnostic Applications of Snake Venom and Snake Venom Components

Snake venom component	*Example*	*Source*	*Biological functions*	*Applications*	*Reference*	*Comments*
Prothrombin activators	Ecarin	*Echis carinatus*	Activation of prothrombin	Detection of abnormal type of prothrombin	[54]	Marketed by Pentapharm as Ecarin
Thrombin like enzymes	Batroxobin	*Bothrops moojeni*	Release of fibrinopeptide A of fibrinogen	Deplete fibrinogen and make plasma unclottable	[11]	Commercially available as Reptilase
Factor X activators	RVV-X	*Daboia russelli*	Activation of Factor X	Measurement of Factor X	[49]	Marketed as Pefachrome® FXa by Pentapharm
Factor V activators	RVV-V	*Daboia russelli*	Activation of factor V	Measurement of Factor V and study of factor V activation	[23]	Marketed by Pentapharm
C-type lectin	Botrocetin	*Bothrops jaraca*	Agglutination of platelets in the presence of vWF	Used as a diagnostic of von Willebrand disease and Bernard-Soulier syndrome	[53]	Botrocetin
Protein C activators	ACC-C	*Agkistrodon c. contortrix*	Activation of Protein C	Quantitative determination of Protein C and S in human plasma	[47]	Protac®

better understanding of the structure-function relationship and mechanism of action of these venom proteins in order to develop novel drugs and diagnostic reagents.

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