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Cloning and characterisation of three novel disintegrin precursors from the venoms of three *Atheris* species: *Atheris chlorechis*, *Atheris nitschei* and *Atheris squamigera*

He Wang [a,1], Xiaole Chen [a,b,1], Lei Wang [a], Wei Chen [a,*], Mei Zhou [a,*], Tianbao Chen [a], Chris Shaw [a]

[a] *Natural Drug Discovery Group, School of Pharmacy, Queen's University Belfast, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland, UK*
[b] *School of Pharmacy, Fujian Medical University, Fuzhou 350004, Fujian, China*

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ABSTRACT

Snake venom constitutes one of the most complex mixtures of naturally-occurring toxic proteins/polypeptides and a large number of these possess very profound biological activities. Disintegrins, that are commonly found in viper venoms, are low molecular weight proteins that usually contain an -Arg-Gly-Asp- (-RGD-) motif that is known to be involved in cell adhesion ligand recognition, binding specifically to cell surface integrin receptors and also exhibiting platelet anti-aggregation activity.

Here, we report for the first time, the successful cloning of three cDNAs encoding disintegrin precursors from lyophilised venom-derived libraries of *Atheris chlorechis*, *Atheris nitschei* and *Atheris squamigera*, respectively. All of these disintegrins belong to the short-coding class and all exhibit high degrees of structural identity, both in their amino acid sequences and in the arrangement of their functional domains. Mass spectrometric analyses of the HPLC-separated/in-gel digested venom proteins was performed to characterise the mature disintegrins as expressed in the venom proteome. Studies on both the structures and conserved sites within these disintegrins are of considerable theoretical interest in the field of biological evolution and in the development of new research tools or novel templates for drug design.

1. Introduction

Snake venoms are complex mixtures of notoriously toxic and often spectacularly biologically-active components (Markland, 1997). Proteins and polypeptides are predominant in these mixtures and are invariably the most toxic and debilitating agents (Doley and Kini, 2009). Though the complexity of components is one of the major factors leading to an incomplete understanding of snake envenomation and to difficulties in clinical therapy, many snake venoms possess powerful therapeutic properties (Kocholaty et al., 1970; Harvey et al., 1998). Hence, in the past years, the level of research into snake venoms has intensified, particularly towards the engineering of highly-active agents, the understanding of their structures, functions and putative applications.

In snake venoms, diverse toxins play a number of roles, primarily in immobilising, killing and digesting prey or are often employed in defence. These toxins often possess novel, highly-specific biological or pharmacological activities (Mackessy and Baxter, 2006). A large proportion of snake venom research is aimed at the discovery and molecular characterisation of novel agents that could provide templates for drug design. The best-known example are the

* Corresponding authors. Tel.: +44 (0) 2890 972129; fax: +44 (0) 2890 247794.
E-mail addresses: wchen04@qub.ac.uk (W. Chen), m.zhou@qub.ac.uk (M. Zhou).
[1] These authors contributed equally to this work.

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bradykinin-potentiating peptides (BPPs) as leads in angiotensin-converting enzyme (ACE) inhibitor innovation, and these contributed to the development of clinically-important drugs with ACE inhibition activity, that play a fundamental role in the clinical therapy of hypertension (Bryan, 2009). Due to the enormous variety of venomous snakes and the fact that there is an incomplete understanding of many toxins, there are still numerous components that remain to be discovered which might have major therapeutic efficacy. Many of them (mainly the proteins and peptides), may serve as future interesting leads for the development of novel drugs (Koh and Kini, 2012).

Since the first disintegrin, trigramin, was described in the 1990s, there has been great interest in these proteins from viper venoms (Niewiarowski et al., 1994). Disintegrins are a family of non-enzymatic proteins found in the venoms of viperid and crotalid snakes, which usually are of relatively low molecular mass (4–14 kDa). The structures of disintegrins are highly-conserved and the conserved motif sequence, –RGD–, (arginine-glycine-aspartic acid) is usually present (McLane et al., 1998; Reiss et al., 2006). This motif is interactive with cell surface integrins and acts as a competitive inhibitor of ligand-integrin receptor binding. Disintegrins are typically rich in cysteine residues that form intramolecular disulphide bonds, but which also may contribute to intermolecular dimer formation in some instances (Park et al., 1998; Okuda et al., 2002).

Besides the single domain-encoding precursors characterised here, disintegrin precursors often also encode an SVMP (snake venom metalloproteinase) and release both proteins through targeted post-translation proteolytic processing (Kini and Evans, 1992). This could explain the existence of a typical disintegrin region in general metalloproteinase precursors and its generation after hydrolysis from Type II SVMPs (PII). Also, the disintegrin-like domains are contained in the PIII SVMPs in addition to the zinc metalloproteinase regions, but normally without the conserved –RGD– sequence (Hite et al., 1994; Singhamatr and Rojnuckarin, 2007). Moreover, a relatively new class of proteins, the ADAMs (a disintegrin and metalloproteinase domain), are multidomain molecules consisting of metalloproteinase and disintegrin-like domains, which are thought to participate in blood coagulation processes and in cell–cell fusion events (Llamazares et al., 2003; Mochizuki and Okada, 2007).

Functional analysis of disintegrins has demonstrated their participation in a wide range of processes. Disintegrins possess a considerable variability in potency and selectivity of their cell-matrix interactive effects (McLane et al., 2004). This family of proteins obtained its name for effects on integrates: dispensing with the connections between receptor proteins and disintegrating cells and tissues and also serving as antagonists of cell adhesion. The conserved internal tripeptide sequence, Arg-Gly-Asp, which is also present on cell matrix proteins involved in adhesion, acts as an integrin recognition motif; disintegrins can thus effectively block cell adhesive actions due to their action of competitively-binding to integrins (Scarborough et al., 1993; Du et al., 2006).

The integrin family is composed of a large number of cell surface adhesion receptors that bind to ligands such as collagen, fibrinogen, and von Willebrand factor receptors that work for different cell types in distinct tissues (Huang, 1998). Disintegrins possess the ability to bind to these transmembrane integrins selectively, playing roles in inhibiting the diverse integrin-ligand interactions. They block the adhesive ligand fibrinogen's interaction with its cognate integrin receptor $\alpha_{IIB}\beta_3$ on platelets, hence counteracting blood clotting (Du et al., 2006; Lambert et al., 2008); they affect extracellular matrix interactions mediated by integrin $\alpha_5\beta_1$, $\alpha_v\beta_3$ on human umbilical vein endothelial cells, osteoclasts or some types of cancer cells such as melanoma cells, thus inhibiting their adhesion to endothelium, basement membranes or the resorption of bone (Lu et al., 2006).

Also, snake venom disintegrins have facilitated investigations into the molecular mechanisms at work in cell-extracellular matrix interactions and the function of the adhesion motif. Intracellular signalling events have been elucidated through the use of venom disintegrins, including events related to programmed cell death, motility and cell proliferation (Clark et al., 1994; Zigrino et al., 2002). They are of great importance as drug templates for the treatment of heart disease for their simple structural property of interacting with cell matrix proteins. Several disintegrins have been undergoing clinical trials as antithrombotic agents due to their function in blocking platelet aggregation (Kini, 2011). Moreover, investigators have evaluated disintegrin applications in therapies for asthma, osteopenia and inappropriate angiogenesis, that are all closely-related to the induction of cancers (Swenson et al., 2004; McLane et al., 2008).

Due to their functions in affecting cell adhesion and in anti-clotting, disintegrins work in the capturing and digesting of prey for viperid snakes. Following injection into a victim's tissues, disintegrins destroy cell-matrix interactions and separate cells in epithelia, inhibit the clumping of platelets and induce a persistent bleeding (Matsui et al., 2000). In addition, following the disintegration of cells, disintegrins combine with other enzymes or non-enzymatic toxins, to flow through the blood vessels of the prey causing more widespread damage.

The symptoms described above can be found in the snakebite case reports of *Atheris* species envenomation. The venoms of these small African vipers contain various enzymes and non-enzymatic toxins, but generally they have been poorly-studied, probably because they do not represent a major danger for humans (Spawls and Branch, 1995; Mallow et al., 2003). However, the severe coagulopathy observed after a bite by these snakes could be fatal or cause serious problems (Top et al., 2006). The documented clinical manifestations provide preliminary information on the venom's activity on platelet aggregation and haemostasis, which indicate the probability of components existing that attack the haemostatic system (Mebs et al., 1997, 1998).

Thus, to understand the haemotoxic venom components of *Atheris* snake species and the molecular mechanisms underlying their structural diversification, we initiated a study of to clone cDNAs encoding disintegrin precursors from lyophilised venom sample libraries of *Atheris chlorechis*, *Atheris nitschei* and *Atheris squamigera*.