

Review

Snake Venom: Any Clue for Antibiotics and CAM?

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Lately several naturally occurring peptides presenting antimicrobial activity have been described in the literature. However, snake venoms, which are an enormous source of peptides, have not been fully explored for searching such molecules. The aim of this work is to review the basis of antimicrobial mechanisms revealing snake venom as a feasible source for searching an antibiotic prototype. Therefore, it includes (i) a description of the constituents of the snake venoms involved in their main biological effects during the envenomation process; (ii) examples of snake venom molecules of commercial use; (iii) mechanisms of action of known antibiotics; and (iv) how the microorganisms can be resistant to antibiotics. This review also shows that snake venoms are not totally unexplored sources for antibiotics and complementary and alternative medicine (CAM).

Keywords: antibacterial – resistance – mechanism – peptide – reptiles

Introduction

Snake venoms contain a large number of biologically active proteins and peptides that are usually similar in structure but not identical to that of prey physiological systems. These molecules are produced by specialized glands, which are evolutionarily related to salivary glands, and are toxic to the prey (1). Interestingly, more than 100 million years ago, snakes evolved from lizards and since then, they independently evolved their own venom apparatus in ophidian evolution, at the base of the *Colubroidea* radiation (1–3).

In an effort to show snake venoms as a promising source for antibiotics, this work briefly discusses the known biological

activities of snake venoms, using snake venom molecules from the Viperidae family as examples, and concepts about antibiotics such as their mechanism and resistance. We also highlight the data about the antibacterial activity of some snake venoms described in the literature to date.

Snake Venom and its Constituents

All the known advanced snake species are venomous. Most of these snakes are found in the superfamily *Colubroidea* that also includes the families Elapidae (incl. Hydrophiidae; Cobras, Kraits, Coral Snakes, Sea Snakes) and Viperidae (Vipers and Pit Vipers) (3). Their venoms are a wide mixture of proteins and peptides (90–95%), also including amino acids, nucleotides, free lipids, carbohydrates and metallic elements bound to proteins (5%) (2–5).

Snake venom protein constituents may present different biological activities that affect physiological processes such as neurotransmission, the complement system and homeostasis (6–8) (Fig. 1). These venoms can act in more than one system

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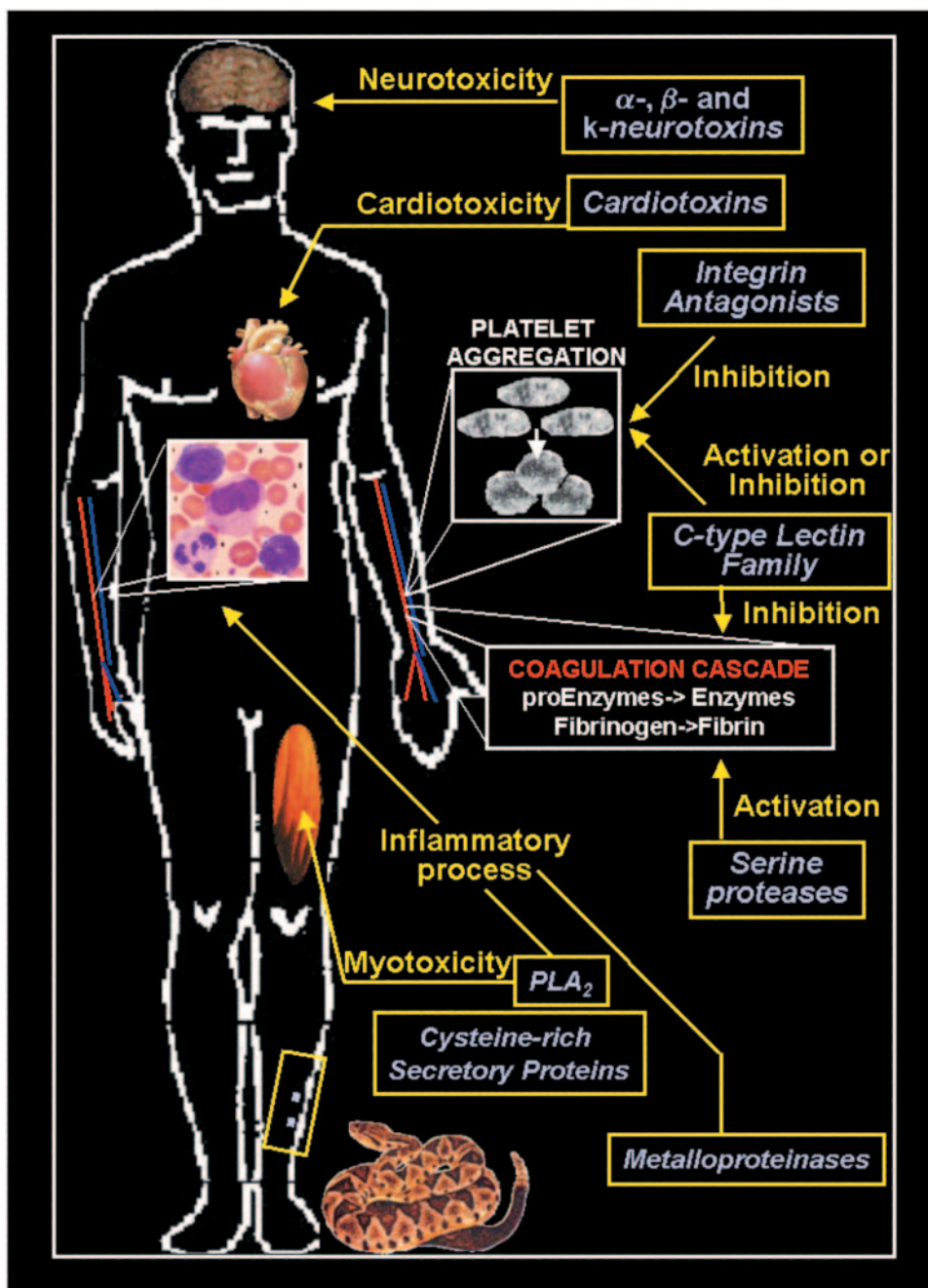


Figure 1. The biological effects of some snake venoms during the envenomation process.

at the same time and they may present antigenic effects (8–10). Viperidae family venom molecules are good examples, such as in homeostasis, where they act as pro- and anticoagulant factors, and also as inducers and inhibitors of platelet aggregation (8,10–12).

Due to their diversity, the proteins from the Viperidae family members are classified (11) in the following categories: serine proteases, metalloproteinases, phospholipases A₂ (PLA₂), C-type lectins and disintegrins. Only the initial three groups display enzymatic activity (4,11).

Among the Viperidae serine proteases are (i) thrombin-like enzymes that convert fibrinogen into fibrin (13–15), such as

batroxobin in *Bothrops atrox* (16), crotalase and gyroxin in *Crotalus durissus terrificus* (15,17), and LMTL in *Lachesis muta* venoms (18); (ii) factor X or factor V activators such as that described in *Bothrops jararaca* and *B.atrox* venoms (19,20); (iii) prothrombin activators present in bothropic venoms (21); and (iv) platelet activators such as that found in *B.atrox* and *B.jararaca* venoms (22,23) (Fig. 1).

Snake venom metalloproteinases are zinc-dependent enzymes that induce hemorrhaging by directly affecting capillary blood vessels and their interaction with endothelial cells (24). They cleave basement membranes, leading to blood extravasation that occurs through gaps formed in endothelial

cells. Therefore, these metalloproteinases present a hemorrhagic effect such as BaH1 and BaP1 from the venom of the snake *Bothrops asper* (25). This ability also induces myonecrosis and plays a vital role in the significant local inflammatory response of the envenomation (21,24,26) (Fig. 1).

Phospholipases A₂ (PLA₂s) are enzymes that cleave phospholipids at the A₂ position, and >150 have been identified in several snake venoms (27,28). They are described as responsible for some of the envenomation symptoms, which involve not only the hemostatic system, with an anticoagulant and an antiplatelet profile (29–32), but also inflammatory and myotoxic effects (33,34) (Fig. 1). Local inflammation and pain are important features of Viperidae and Elapidae snakebite envenomations that are rich in myotoxic nociceptive events induced by PLA₂ (27,34,35). Interestingly, the elapid and viper PLA₂ toxins belong to different groups (“pancreatic-type”-group I and “synovial-type”-group II, respectively). They represent independent use of PLA₂ as toxins and are thus not homologous to each other as result from separate recruitment events. The snake presynaptic neurotoxins can also present PLA₂ activity, which leads to the release of acetylcholine followed by impairment of synaptic functions. These neurotoxins are spread through several families of Colubroidea superfamily (8,36,37). β -bungarotoxin is a basic protein from *Bungarus multicinctus* that partially paralyzed mouse hemi-diaphragm nerve-muscle preparations also due to the PLA₂-mediated destruction of membrane phospholipids in motor nerve terminals (38) (Fig. 1).

The C-type lectin family from Viperidae is one of the most fully characterized lectin groups described in the literature (39,40). These calcium-dependent proteins are divided into two groups (I and II), those with a complete (I) or an incomplete (II) carbohydrate recognition domain (CRD) (39,41). The first group is involved in cell recognition such as adhesion, endocytosis and pathogen neutralization usually by using the CRD (42,43). Meanwhile, while conserving most of the primary structure, the incomplete CRD protein group displays different biological activities (40). These molecules are not able to bind carbohydrates but, by using different mechanisms, they induce or inhibit different steps of the same physiological system or even of different systems (39,40,44). These molecules can be found in several venoms such as botrocetin, a platelet-agglutinating protein (45), and bothrojaracin, a thrombin inhibitor, in *B. jararaca* venom (46,47); and convulxin, a pro-aggregating protein that binds to platelet GPVI receptor, in *C. durissus terrificus* venom (44,48,49) (Fig. 1).

Snake venoms also contain several peptides. They may vary from presenting neurotoxic (8,50,51) cardiotoxic (52,53) or even an inhibitory platelet profile (3,4,6,26,54,55). They also may present cytotoxic effects characterized by the cytolytic effects that present a cationic site flanked by a hydrophobic surface (56). In the group of peptides with inhibitory platelet activity, the disintegrins, also known as RGD peptides (molecules containing the Arg–Gly–Asp sequence), are integrin antagonists (Fig. 1). They act as potent inhibitors of platelet aggregation by binding specifically to integrins present on cell membranes

of not only platelets (44,55,57) but also metastatic cells (54,58) (Fig. 1).

Finally, it is possible to observe the presence of other protein compounds with an enzymatic profile in snake venoms. Those include cysteine-rich secretory proteins, which inhibit smooth muscle contraction and cyclic nucleotide-gated ion channels (59) (Fig. 1). There are also phosphomonoesterases, phosphodiesterases, arginine esterases, hyaluronidases, L-aminooxidases, 5' and NAD nucleotidases, and acetylcholinesterases in snake venoms (3,4,60,61). Interestingly, the concentration and distribution of all snake venom proteins and peptides vary from individual to individual, species to species, genus to genus and family to family, probably due to their features, feeding and environmental conditions (12,47,62–64).

Snake Venom Molecules of Commercial use

At the end of the last millennium, the development of therapeutic drugs made a significant improvement to the understanding of the mechanisms of action and structure–function relationship of important biological molecules (40,65,66). The broad spectrum of snake venom activities, including their biochemical, toxicological, physiological and pharmacological profiles, results from the action of their constituents. Therefore, snake venom are of biological interest as a potential source of active compounds. These molecules could act as (or be used as a prototype for) (i) therapeutic agents (67,68); (ii) research tools for use in the diagnosis of several diseases (68–70); and/or (iii) in basic research for understanding physiological and pathological processes (70–73). One of the most successful examples of using snake venom as a source for searching for drug prototypes also involved venom from the Viperidae family. In the 1960s, Ferreira, a PhD student at the time, and co-workers found a peptide presenting an angiotensin-converting enzyme inhibitory activity in *B. jararaca* venom. This molecule was named nonapeptide bradykinin potentiator (BPP9a) and was able to decrease arterial pressure using this mechanism (75,76). Based on BPP9a, several drugs were developed and protected by patents, now used by the international industry systems. These drugs, symbolized by captopril, represent a world market of billions of dollars annually. BPP9a is a good example where the use of a natural prototype found in a biological source can generate a medicine for worldwide use. In fact, there are many peptides from several natural sources, other than snake venom, described as potential prototypes for drug development. One of them is hirudin, a thrombin inhibitor from *Hirudo medicinalis* saliva, studied for its potential as an antithrombotic molecule (77–79).

Antimicrobial Peptides Versus Enzymes

Clearly snake venom peptides have the potential for practical and therapeutic use. However, enzymes and proteins are also very important as some of them are described as laboratory diagnosis reagents. Russel viper venom (RVV) X and V

enzymes and ecarin from *Echis carinatus* venom are proteins used for factors X and V, and prothrombin determination in blood, respectively (19,20). Due to their characteristics, RVV enzymes have been used for the improvement of the detection of von Willebrand disease (6,15). Similarly, snake venom thrombin-like enzymes (SVTLEs) are very useful for blood measurements of several parameters of heparin-treated patients since they are not affected by heparin in the same way as thrombin, a key enzyme of the coagulation cascade (80). SVTLEs and snake venom proteases presenting fibrinolytic activity acting on coagulation contributed to the study of the treatment of vascular thrombosis. Included in this group are batroxobin (Defibrinase R), from *B.atrox* venom, and ancrod (Arvin R), from *Calloselasma rhodostoma* venom, currently used for controlled depletion of fibrinogen (80–82). They act as selective antithrombotic agents on deep vein thrombosis peripheral arterial diseases and on vascular surgery (69,80,82).

In the last decade, several snake venom compounds were used as important tools for the understanding of human physiological systems (83,84). Due to their similarity to physiological molecules, studies on myoblast fusion and fertilization, and matrix metalloproteinase (ADAMs)–cell interactions have been performed using the homologous snake venom metalloproteinases and peptide neurotoxins in order to characterize human cancers and small lung carcinoma. These studies are good examples of the use of snake venom molecules in basic research (25,26,70,83,84).

Antibiotics? What are they?

Antibiotics are a heterogeneous group of molecules produced by several organisms, including bacteria and fungi, presenting an antibacterial profile (85,86). At the present time, synthetic antimicrobials, known as chemotherapics, display different mechanisms of action and a broad antibacterial spectrum. The antimicrobials are produced by the international pharmaceutical industry and used worldwide. In fact, the control of the deleterious effects of microorganisms was significantly increased by the introduction of the sulfonamides (chemotherapics) and of penicillin (antibiotic) in 1936 and 1941, respectively (85–87). These drugs were crucial for the reduction of the incidence of several bacterial infections such as meningitis, endocarditis, pneumonia and gonorrhoea (85,86).

The main effects of antibiotics are: (i) inducing the death of the agent (bactericidal effect); and/or (ii) inhibition of bacterial growth (bacteriostatic effect). Their targets are the essential biosynthetic process or routes of these microorganisms (85,87). Among them, the inhibition of the synthesis of cell membrane, nucleotides and peptide bonds interferes directly with survival, chromosome replication and protein synthesis, respectively, of the bacteria (Fig. 2). They can also act by increasing cell permeability, or inhibiting through binding to ribosomes, which prevents nucleotide polymerization (85,87).

The main characteristic of antimicrobials (synthetic or natural) is their selective toxicity. This feature is based on the presence of the target only or mainly on the infectious agents,

which allows their systemic administration without deleterious effects to the host cells (85,87).

Interestingly, antibiotics are usually produced by water- or soil-dwelling bacteria, where the absence or low concentrations of supplies turns the competition into an important issue for survival (88). The production of an antibiotic at the bacterial stationary phase probably reproduces the bacteria's behavior at a low nutrition environment, where these molecules are necessary for eliminating competitors and guarantee 'food supplies' (89). On the other hand, microorganisms that grow without food restriction, such as those of the intestinal flora (enterobacterias) or of an animal's oral cavity, generally produce bacteriocins, which are proteins presenting an antibacterial profile (90,91). These proteins are very different from antibiotics, clearly obvious by their chemical structure or non-metabolic characteristics, but mostly because they are produced during the exponential phase of Gram-positive and Gram-negative, pathogenic or non-pathogenic bacteria (e.g. *Escherichia*, *Acetobacter*, *Actinobacillus*, *Bacillus*, *Clostridium*, *Lactobacillus*, *Streptococcus* and *Staphylococcus*) (90,91). Colicin is a bacteriocin produced by *E.coli* against other homologous species. Similar to other bacteriocins, colicin's main effect is bactericidal (92). The inhibitory mechanism of these proteins is not fully characterized, but for colicin three steps are already confirmed: (i) binding of colicin to the receptor; (ii) its transport through the cell membrane; finally (iii) death of the agent (90–92).

The use of bacteriocins as therapeutic tools is very restricted since they can be destroyed due to their protein structure, and/or induce an immune response by the patient treated because of their antigenic profile (90,91). These proteins are mainly useful when present in food produced by using microorganisms such as yoghurt. In this specific case, these microorganisms, such as *Lactobacillus*, produce bacteriocins, which restrict the cell growth of other potential contaminants (93).

Still Searching for Antibiotics? What for?

Although extremely effective, antibiotics are able to induce resistance in bacteria. For >50 years, bacterial resistance has been the main factor responsible for the increase of morbidity, mortality and health care costs of bacterial infections (94). This bacterial defense mechanism is widely present in bacteria (e.g. *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Acinetobacter*, *Salmonella*, *Staphylococcus*, *Enterococcus* and *Streptococcus*) and became a world health problem worsened by developments in human, animal and plant transportation (94–96). The airlines facilitated the rapid dissemination of resistant microorganisms through different countries and, as a consequence, the monitoring of those agents by the government became more and more difficult (94–96).

According to genetic studies, resistant bacteria are always present in a small number in any bacterial colony. This number of bacteria can increase by selective pressure induced by the presence of the drug used (94–96). The most common causes for the appearance of multiresistant bacteria are the

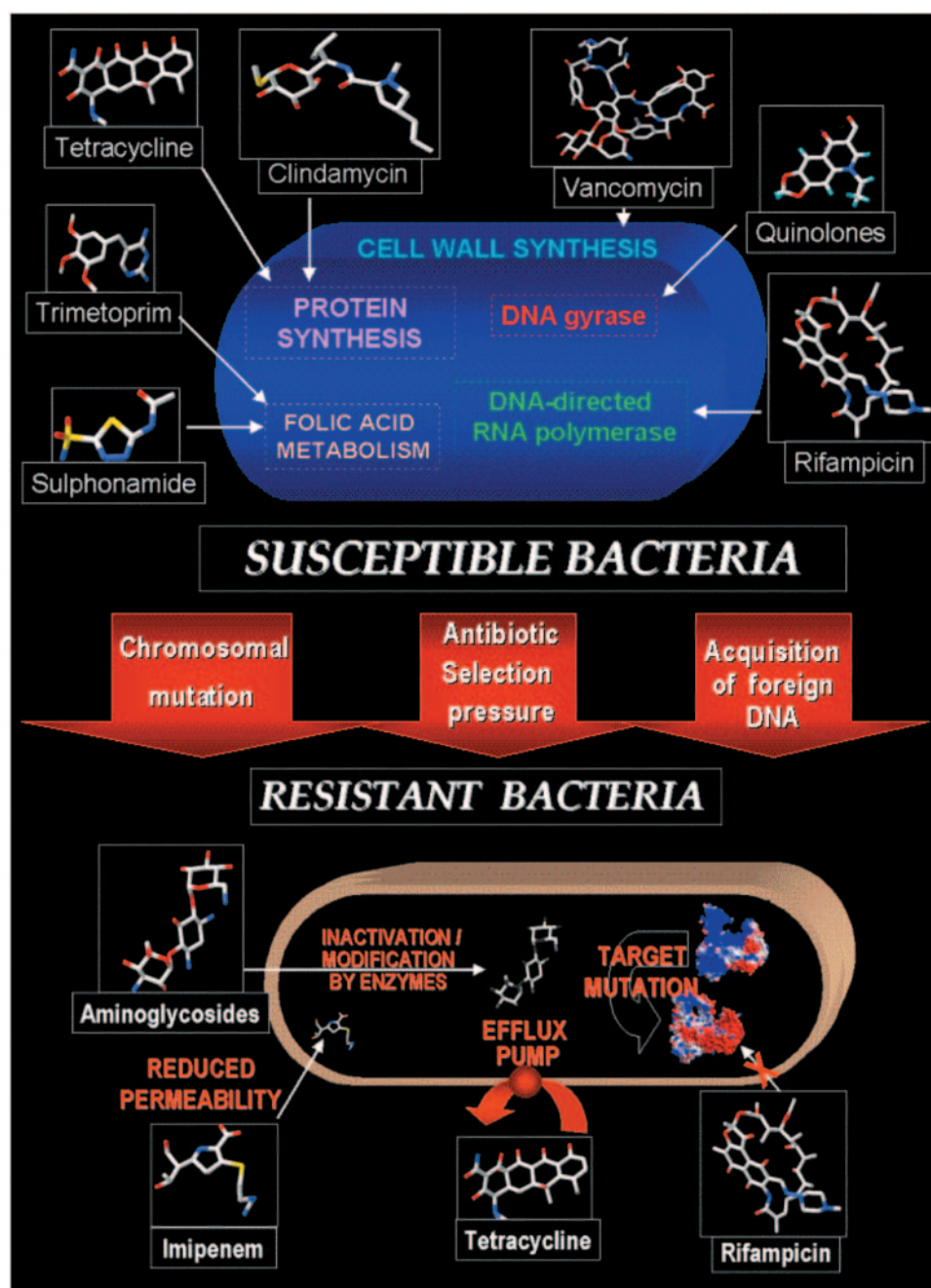


Figure 2. Schematic representation of the emergence of resistant bacteria.

inadequate or excessive use of antibiotics. In some countries, prescriptions are still determined empirically without previous identification of the pathogenic agents (94–96). In addition, inappropriate use such as (i) not following the intake schedule; (ii) giving up the treatment; (iii) the low quality of the medicines; (iv) self-medication; and (v) incorrect drug storage can lead to a selective pressure, which contributes to the selection of these multiresistant microorganisms. Other problems also include the amount of antimicrobials used in agribusiness and agriculture for protecting animal and plant growth. Environmental changes, and the increase of agro

products and animal migration, contribute to the spreading of multiresistant agents (94–96).

The microorganisms can be resistant to antibiotics through an intrinsic resistance, which is determined by the original cell genes and is displayed by all individuals of the species (97). A good example is *Lactobacillus* that, similarly to mammalian cells, synthesizes tetrahydrofolate from *p*-aminobenzoic acid. Therefore, this microorganism is resistant to sulfas such as the mammal's cells (98).

The microorganisms can also become resistant through acquired resistance, which is represented by specific mutations

on antibiotic targets acquired by plasmidial or transposon genes (94–97,99) (Fig. 2). This event leads to a new generation of insensitive cells. There are four known acquired resistance mechanisms.

Production of Enzymes or Isoenzymes

Enzymes and isoenzymes are usually produced by microorganisms as instruments for protection against antibiotics. A classical model is observed for streptomycin-resistant bacteria, which synthesize enzymes that phosphorylate, adenylate or acetylate hydroxyl or amino groups of amino glycosides, acquiring this ability through plasmids (94–96). The use of β -lactamases by resistant Gram-positive or Gram-negative bacteria in order to cleave the β -lactamic ring of penicillin. This cleavage leads to the formation of the penicillinoic acid that is devoid of antimicrobial activity. Using β -lactamases, these microorganisms are resistant not only penicillin to, but also to cephalosporin (100,101) (Fig. 2).

Because of this, the literature describes effort to synthesize new penicillin-like drugs by adding different chemical groups to the original penicillin, to modify the affinity between these drugs and this enzyme. However, these modifications also decrease absorption of these molecules and also induce a compensation system where the microorganisms increase the synthesis of the enzyme (1–2% of total proteins), which guarantees the resistance level (101).

Chloramphenicol acetyltransferase is another important enzyme directly involved in acquired resistance. This protein is able to inactivate chloramphenicol through the addition of an acetyl group from acetyl coenzyme-A, and its presence can be intrinsic or induced (102).

The synthesis of isoenzymes is also an induced resistant system. In sulfonamide resistance, the isoenzyme dihydropteroate synthase, acquired by plasmid genes, presents the same affinity for the substrate (*p*-aminobenzoate) but a 10 000 times lower K_d for the drug compared with the original enzyme (103).

Target Mutation

This acquired resistance is characterized by a specific mutation on the antibiotic target, which will result in drug-insensitive bacteria. In the case of streptomycin-resistant bacteria, mutation of the S12 protein prevents binding of the bacterial ribosome to this drug (104). Similarly, rifampicin-resistant bacteria present a mutation on the DNA polymerase β -subunit, which is sufficient to make this microorganism insensitive to rifampicin treatment (105–107) (Fig. 2).

Changes in Membrane Permeability

This acquired resistance can result from: (i) changes to the antibiotic structure, which make its passage difficult through bacterial permease, or to the cell membrane constitution, such as changing lipopolysaccharides; (ii) permease mutations, which decrease amino acid and antibiotic transport; and (iii) the efflux process that pumps the drug out of the cell (106–109) (Fig. 2).

Increase of Metabolic Molecules

Resistance can be acquired through an increase of a metabolic molecule when the drug's mechanism is in direct competition with this molecule (competitive antagonism), e.g. by increasing *p*-aminobenzoate production, sulfur-resistant bacteria are able to avoid antibiotic effects (110,111).

Are Snake Venoms Totally Unexplored Sources for Antibiotics? Not Really

More than 700 antimicrobial peptides have already been identified in all living species (112,113,114), including bacteria (86), fungi (115), amphibians (116), fish (117), insects (118) and mammals (119,120). These molecules are 5 kDa peptides with a high level of basic and hydrophobic amino acids. They present a broad antimicrobial spectrum against bacteria, fungi or parasites, by acting through insertion into the cell membrane or binding to receptors. These molecules are promising for development of antibiotics, especially for treatment of multiresistant microorganisms (112,113).

In the case of snake venoms, despite heavy snake oral and fang contamination with a wide variety of potentially pathogenic bacteria, envenomation is a process associated with a low incidence of bacterial infection (120,121). Therefore, this feature could indicate the presence of antibacterial molecules in the snake venoms that would protect the snakes during feeding. Some of the first reports about antibacterial activity in snake venoms were in 1948, and in 1968, involving Elapidae and Viperidae venoms (122,123). Viperidae were described as having antimicrobials against the *Sarcina* species, while in the Elapidae family, a lytic factor or cytotoxin composed of a basic, low molecular weight protein was found in *Naja* sp. and *H. haemachatus*. They were able to disrupt *Staphylococcus aureus* and *E. coli* phospholipid membranes respectively (122,123). Not only peptides but also enzymes were involved in the antimicrobial activity of snake venoms as described by Skarnes in 1970 (124). *Crotalus adamanteus* L-aminooxidase affects Gram-positive bacteria, while those from *Agkistrodon halys pallas*, *Bothrops alternatus* and *Trimerus jerdoni* have an inhibitory activity against *E. coli*, and *S. aureus*, *Pseudomonas aeruginosa* and *Bacillus megaterium*, respectively (124–127). Interestingly, LAO1, an L-aminooxidase from *Pseudechis australis*, was 70 times more effective than tetracycline against *Aeromonas* (128).

Several antimicrobial studies involving many snake venoms have already been described in the literature. For example, Stocker and Traynor in 1986 wrote about the inhibitory effects of *Naja naja soutratrix*, *Vipera russelli* and *C. adamanteus* in *E. coli* (129); in 1991 Stiles described the antibacterial properties of 30 different snake venom where the Asian and African snakes (*Naja* sp.), Australian elapids (*Notechis scutatus* and *Pseudechis australis*) and North American snakes (*Crotalus* sp.) presented the highest activity and Talan and co-workers using Crotalid venoms against Gram-negative and Gram-positive bacteria (128). Recently, Blaylock studied

Kwazulu Natal snake venoms in South Africa and showed that the eight venoms tested presented antibacterial activities. Adders showed most activity against aerobes, while cobras showed no distinct activity against aerobes or anaerobes (130). In this study, snake venoms from *Causus rhombeatus*, *Bitis gabonica*, *Bitis arietans*, *Dendroaspis polylepsis*, *Dendroaspis augusticeps*, *Naja melanoleuca*, *Naja annulifera* and *Naja mossambica* were detected presenting antibacterial activity against *S.aureus*, *E.coli*, *Paeruginosa*, *Bacterioides fragilis*, *Bacterioides intermedius*, *Clostridium sordellii* and *Clostridium perfringes* (130). More recently, Xie and co-workers described peptides from *Naja atra* venom that act against multiresistant *Mycobacterium tuberculosis* in vitro (131).

Is There a Chance?

Currently certain bacterial infections are multidrug resistant. However, this worldwide problem may decrease if some attitudes can be adopted in a global perspective. Among them, the most important is still a reduction of the inappropriate and/or excessive use of antibiotics (132,133).

Despite reaching future positive statistics on antibiotic use, new antimicrobials will always be necessary to fight against multidrug-resistant microorganisms (132,133). Therefore, these drugs will be very important, particularly for treatment of the elderly, children and immune compromised patients (134–136). Thus, investment in antibiotic research and in finding new sources of new drugs or prototypes is of major interest to CAM.

This minireview does not intend to cover all data about snake venoms or antibiotics. Its main objective is to reinforce that both proteins and peptides from snake venoms can be good candidates for testing in antibiotic screening assays using multiresistant microorganisms. Compared with other snake venom biological activities, the antibacterial profile of these natural sources has not been fully investigated despite the positive results found to date. Although snake venom peptides and proteins have a direct therapeutic use limited by their antigenic and 'digestible' structure, their usefulness as prototypes has clear potential. These molecules could also be of interest for the food industry, since they can be easily degraded by the human digestive system and therefore could be useful to protect against contamination by food microorganism.

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