Antimicrobial properties of the skin secretions of frogs

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Antimicrobial resistance results in increased morbidity and mortality, and increased health-care costs. Therefore the need to develop new classes of antibiotics is indispensable. Antimicrobial peptides are a relatively new class of potential antibiotics which are fast acting, possess broad-spectrum activity and are able to escape many of the currently known mechanisms of drug resistance. They have been shown to be active against Gram-negative and Gram-positive bacteria, fungi, enveloped viruses and even cancer cells. However, toxicity to healthy host cells remains a concern and has affected the clinical development of therapeutics based on antimicrobial peptides. The purpose of this review is to discuss recent advances in research focused on antimicrobial peptides from frogs and the challenges in conducting research in this area in southern Africa. An extensive literature review of relevant articles published between 1980 and the present was conducted using PubMed, ScienceDirect, Sabinet, Elsevier and GoogleScholar. There has been little research done on anurans from southern Africa which are endemic to the region, and there is therefore a need to focus on this group for the purposes of bioprospecting for potentially new antimicrobial peptide compounds.

Introduction

Antibiotics have been termed the single most significant discovery in medicine. The discovery of penicillin by Alexander Fleming in 1929 ushered in the modern antibiotic age. The real potential for penicillin was, however, only recognised with the advent of the Second World War during which the antibiotic was extensively used in the treatment of septic wounds for soldiers.[1] The post-war era marked what has now been termed 'The Golden Era' of antibiotic research and development.[2,3] This era saw an explosion in the number of antibiotic drugs available for clinical use. However, even at that early stage, antibiotic resistance had already begun to emerge. Antibiotic resistance arises when resistant strains in a population are selected and become dominant over susceptible bacteria.[4]

The gains made in public health care from the use of antibiotics have been in part lost because of the emergence of antibiotic-resistant organisms and the increased incidence of newly described pathogenic fungi and bacteria.[5] Antibiotic resistance results in increased human morbidity, mortality, and attendant costs in health care and has thus been acknowledged as a major global public health problem.[6]

Consequently, there have been renewed efforts in the search for new antimicrobial agents. Antimicrobial peptides have shown promise as lead compounds for new antibiotics. Here we review the information available on the bioprospecting of novel antimicrobial agents from anuran dermal secretions. We mainly discuss the status quo of relevant research in southern Africa – a region which possesses great floral and faunal biodiversity and hence the potential for novel bioactive compounds.

Materials and methods

An extensive literature search was conducted using the following keywords: frog or anuran secretions, frog skin properties, frog antimicrobial activity, frog antifungal activity, antimicrobial peptides, African frog secretions, antibiotic resistance, frog species pharmacological importance and frog secretion techniques. The search was conducted using PubMed, ScienceDirect, Sabinet, Elsevier and GoogleScholar and was limited to articles published between 1980 and the present. The literature obtained was then closely examined to determine the extraction and peptide isolation methods, chemical elucidation and biological activity testing. Whilst there may have been work done prior to 1980, its relevance to this review was deemed limited for various reasons (e.g. the isolation and elucidation techniques are outdated).

Discussion

The use of animal parts in traditional medicine

Animals and animal parts have been used for medicinal purposes by humans since ancient times.[7] Popular remedies often were obtained from animal body parts or animal products, such as skin, horn, corporal secretions and excrement, or from animal housing (e.g. nests and cocoons).[8] Anurans (frogs and toads) feature prominently in materia medica. The Chinese have traditionally administered frog skin and secretions of toad parotid glands to regulate internal corporal functions and fertility or as a treatment for dog bites.[9] Extracts of scraped skin secretions of the giant leaf frog (*Phyllomedusa bicolor*) are used in Chinese folk medicine for the treatment of depression, stroke, seizures and cognitive loss in ailments such as Alzheimer's disease.[10] Traditional healers in Nagaland, India use the dorsal skin of frogs to cover the wounds of their patients.[11]

Amongst the Peruvian Matses Indians, the rubbing of dried skin secretions called 'sapo', from *Phyllomedusa bicolor*, into exposed areas of the skin results in gastrointestinal, cardiovascular and central nervous system effects which have shamanic significance.[12] Several potent peptides, including phyllocaerulein, phyllomedusin and dermorphins, have subsequently been isolated from this species.

In Vietnam, the lack of adequate medical supplies to treat napalm burns during the Vietnam War in the 1960s led surgeons to investigate traditional Vietnamese remedies for burns. They found that the use of amphibian skins from the genus *Rana* as temporary grafts for patients with severe skin loss was a successful means of treatment.[13] When testing these grafts in Wistar rats, experimental wounds dressed with frog skin healed much faster than wounds dressed with cotton gauze. Biochemical assessments of wound granulation were carried out every 2 days until complete healing was achieved. These experiments showed that the group of rats treated with frog skin produced higher levels of the amino acid hydroxyproline than did the control group.[11] Hydroxyproline is a component of collagen, which constitutes fibrous tissue including skin and ligaments.

Anatomy of amphibians

Anurans have limbs which bear fingers and toes, external eardrums, eyelids, skin glands, a tongue, voice box and sternum.[14] They possess a three-chambered heart, and most have paired lungs. Frogs and toads are characterised as cold blooded and their ectoderms are warmed by the external environment.[14] There are few physical differences between frogs and toads. Frogs have a smooth, moist skin with few warts and live near or in water, whereas toads have a rough, drier skin with warts, live on land and use water for breeding purposes.[15] Toads have large parotid glands behind their eyes.[14] Frogs have a narrower body and waist; their hind legs are long for hopping and their feet are webbed for swimming. In contrast, toads have broader, flatter bodies, short hind legs and walk rather than hop.

Amphibian skin is a morphologically, biochemically and physiologically complex organ which fulfils a wide range of functions necessary for the organism's survival. The skin of the frog is a thin, flexible integument that aids in respiration and water absorption.[16] The skin is highly vascular which facilitates dermal respiration, but at the same time it excludes pathogens.[16] The integument consists of two major layers: epidermis and dermis. The epidermis is made up of germinative layers which in turn are made up of basal cells. These cells produce a non-keratinised layer, which is frequently shed during summer months.[17] The dermis contains connective tissue and the layer beneath the germinative layer contains the mucous and pigment cells (chromatophores).[16] These cells enable frogs to alter their colour for protective purposes and thermoregulation.[14]

Defence against invading microbes is a problem faced by all multicellular organisms. The skin provides a potential avenue of entry for bacteria, fungi and other invaders.[18] One key component of the host-resistance apparatus is innate immunity,[19] which for anurans includes glands in the skin which may produce substances that are toxic to other animals.[14] These glands are either scattered throughout the skin or concentrated in specific areas.[14] The compounds secreted by the glands play various roles, either in the regulation of physiological functions of the skin or in defence against predators and/or pathogens.[20,21] The skin glands produce a range of noxious substances that may induce mammalian morbidity and mortality. The cytoplasm of the skin gland cells is rich in granules and the lumen is reduced into a small empty cavity. Contraction of myocytes surrounding the glands causes a synchronous discharge of their contents with a holocrine mechanism.[21] These secretions contain peptides which have the ability to inhibit the growth of pathogenic microorganisms[22] and have been called antimicrobial peptides.

Pharmacological investigations of frog secretions

Amphibians exist in microorganism-rich environments, and as a result they produce potent antimicrobial peptides as a defence. The antimicrobial peptides are secreted by non-lymphoid cells on the mucosal surfaces of the respiratory and gastrointestinal tracts, and by the granular glands of the skin.[20] Given the respiratory and antimicrobial functions of the amphibian skin, it is likely that some of the molecules found in their granular gland secretions may be of use in the treatment of skin and respiratory infections.[23] What follows is a discussion focused on the work done on frogs, the most widely studied of the anurans.

Studies have shown that bactericidal and fungicidal peptides synthesised in the skins of certain frogs represent a promising source of potential therapeutic agents.[22] For example, a compound effective against *Staphylococcus aureus* (which often causes abscesses and boils) and against viruses that are rarely affected by antibiotics was discovered from a frog species of the genus *Rana*.[18] The skin secretions of the African clawed frog, *Xenopus laevis*, have been shown to contain

high concentrations of a diverse array of biologically active components that include thyrotropic hormones and the myotropic peptides caerulein, xenopsin and levitide.[24] Their helical, amphiphilic structures have an affinity for microbial membranes causing dissipation of ion gradients.[25,26] These peptides are water soluble and non-haemolytic and have been shown to inhibit *Candida albicans*.[25] The peptides identified from *X. laevis* appear to represent a previously unrecognised class of vertebrate antimicrobial peptides.

Extensive studies have been conducted on antimicrobial peptides of frogs belonging to the genus *Rana*.[27,28,29,30] This genus comprises more than 250 species distributed worldwide, except for the polar regions, southern South America and most of Australia.[31] Frogs of this genus have proved to be a rich source of peptides with antibacterial and antifungal activity.[32] About 160 antimicrobial peptides have been identified from more than 20 ranid amphibians.[20,28,33,34] Peptides isolated from *Rana ornativentris*,[35] *Rana japonica*,[36] *Rana tagoi, Rana pirica*,[28] *Rana okinavana*[37] and *Odorrana grahami*[38] have shown broad-spectrum antibacterial and antifungal activities. For example, the dermaseptins produced by the South American arboreal frog *Phyllomedusa sauvagii* are lytic, linear, cationic, lysine-rich peptides.[39] Another South American tree frog, *Phyllomedusa bicolor*, produces skin-PYY (SPYY) which is an antifungal compound closely related to neuropeptide Y (NPY) and gastrointestinal tract peptide (PYY).[40] SPYY permeates phospholipid membranes and inhibits the growth of *Cryptococcal neoformans, Candida albicans* and *Aspergillus fumigatus*.[40] A study conducted on the skin secretions of the pickerel frog, *Rana palustris,* led to the isolation of 22 peptides with different inhibitory activities on bacteria and fungi.[41] More recently, the temporins isolated from the European red frog *Rana temporaria* and the North African *Rana saharica* have been the focal point of many studies.[42,43,44] These antimicrobial peptides have shown good activity against Gram-positive bacteria (with mean inhibitory concentrations of between 2 μM and 5 μM), protozoa (*Leishmania donovani*) and fungi (*C. albicans*).

There has been increasing interest in frogs from Africa, as evidenced by recent studies by Marenah et al.[45] on *Rana saharica* (syn. *Pelophylax saharicus*) and Wang et al.[46] on African hyperoliid frogs. However, apart from studies on *Xenopus laevis*, which although is a native of South Africa is now found in most of Africa and has been introduced elsewhere, there is still a paucity of studies on southern African anurans. This dearth exists despite the fact that the region possesses large biological diversity with high endemicity.

The class Amphibia, which comprises more than 5000 species, is represented in South Africa by the orders Anura and Gymnophiona.[14] The southern part of the Western Cape Province of South Africa is a unique biogeographic region with a high amphibian density of 21–30 species per grid cell (676 km^2).[47]

Antimicrobial peptides

The innate immunity of vertebrates to microbial invasion is mediated by a network of host-defence mechanisms, which involve, in part, a non-specific chemical defence system that includes broad-spectrum antimicrobial peptides.[48] Antimicrobial peptides are gene-encoded, ribosome-synthesised peptides comprising of ~10–50 amino acids.[49] Most are synthesised as pre-pro-peptides with an N-terminal signal sequence, a pro-segment and a C-terminal cationic peptide.[50] Most anurans secrete peptides within the 1 kDa – 10 kDa range.[51] Antimicrobial peptides are linear, cyclic or open-ended cyclic in structure with one or two disulphide bridges.[52] They are highly amphipathic with hydrophobic and cationically charged surfaces.[50] It has been shown that antimicrobial peptides inhibit the growth of enveloped viruses, bacteria, protozoa, fungi and even cancer cells in *in-vitro* assays.[22,53]

Although debate continues over the specific mode of action of antimicrobial peptides, it is thought that the cationic nature of the peptides leads to cell membrane disruption and subsequent unregulated ion exchange with the environment.[54] This proposed mechanism has been validated by the observation that antimicrobial peptides work rapidly – apparently far too quickly for any process that involves translocation and binding to an intracellular target molecule.[54] Thus the speed of action seems to point to the mechanism of action being cell lysis when the peptide interacts with the membrane (phospho)lipids rather than acting by binding to specific receptors on the cell membrane. Therefore microorganisms develop resistance to antimicrobial peptides at rates that are less than those observed for conventional antibiotics. On the negative side, the toxicity of many of the peptides and their rapid rate of clearance may present challenges in their potential therapeutic application.[28]

Molecular studies of antimicrobial peptides

Manual sequencing of antimicrobial peptides was used in the 1960s, but this process is time-consuming, inefficient and requires a large number of specimens to be sacrificed, which poses major ethical problems in the present day.[55] Peptide separation has been performed through various techniques including capillary electrophoresis, two-dimensional gel electrophoresis, liquid chromatography and surface-enhanced laser desorption and ionisation.[55] Structural elucidation can then be performed by circular dichroism spectroscopy and nuclear magnetic resonance spectroscopy, but matrix-assisted laser desorption and ionisation mass spectrometry (MS) techniques have gained favour more recently. Mass spectrometry deduces molecular structure by determining the mass of peptide and amino acid fragments with high accuracy and thus allowing peptide mass fingerprinting in which the fragments are matched to theoretical digests or fragmentation patterns of protein databases.[55] It has been shown that the majority of skin peptides do not terminate in arginyl residues and usually contain multiple prolyl

residues, blocked N-terminals and amidated C-terminals, all of which make acquisition of appropriate MS/MS spectra and their interpretation very difficult.[55] To complement mass spectrometry studies, novel peptides structurally assigned by Edman degradation can have structures confirmed by molecular cloning of precursors.[56]

Possible applications of antimicrobial peptides

Diverse applications have been proposed for antimicrobial peptides as therapeutic agents.[57] It is thought that it is the complex interaction of cationic, hydrophobic, α–helic and amphipathic characteristics that confers the cytolytic activity to frog skin peptides.[58] Their broad-spectrum activity positions them for consideration as 'chemical condoms' to limit the spread of sexually transmitted infections, including chlamydia, HIV and AIDS,[59] herpes simplex virus[21,60] and those caused by *Neisseria*. Microbial colonisation and growth on the surfaces of synthetic polymeric materials is a problem that complicates the use of medical devices such as intravenous catheters. One solution is the use of magainin peptides, which, when covalently bound to insoluble polymeric beads, retain antimicrobial activity.[21,61] The antifungal properties of peptides have been studied for nearly 40 years.[3] During the past 10–15 years, interest in their antifungal nature has expanded as a result of increased resistance of fungal pathogens to, and toxicity of, currently used antifungal drugs.[3]

Challenges in conducting research on frogs

Numerous challenges are experienced when conducting research on frogs. These challenges can be both ethical and methodological. Before any research is conducted there is a need to obtain ethical clearance from the relevant ethics boards of institutions and conservation organisations. A licence from the nature conservation authorities has to be obtained and must specify the number and species of frogs to be collected and their specific locality. Because such information is scarce this requirement can pose a problem. The time of collection is also important and may cause logistical problems. The greatest number of frogs is collected at night during the rainy season or near dams, but the specimens have to be stored overnight in an environment that will not aggravate the animals, or allow them to harm themselves, before being transported to the laboratory. Once captured, the methods used for collecting the secretions may also have bioethical implications. Three methods are used for the collection of the secretions: electrical stimulation, chemical stimulation and skin harvesting.

Electrical stimulation has been used in previous studies.[62,63] Skin secretions are obtained by mild electrical stimulation – a process that does not appear to harm the amphibians. Secretions are thoroughly washed from the skin surface with distilled water, collected in a beaker and lyophilised. Other studies describe the frogs being repeatedly stimulated with electrodes at 30 V, 15 mA for 3 s,[64] to much higher frequencies of voltage (150 V) and low amperage. Electrical stimulation appears to produce copious amounts of secretion but the method cannot be easily applied because of the specialised equipment required. It cannot be applied in the field and throughput is limited. Electrical stimulation can also be painful,[65] which has ethical implications.

Chemical stimulation has been widely applied either by the physiological stimulation of the parasympathetic nervous system or by exposing the frog to irritant chemicals. In physiological stimulation, norepinephrine is injected bilaterally to induce secretion.[65] The procedure is repeated after 21 days. The drawbacks of this method are that it involves a controlled drug (norepinephrine) and a level of specialised technical training is needed. It is also invasive and the treated frogs may subsequently die. Another chemical stimulation method involves the use of a chemical irritant. The technique has been successfully applied[29] and appears to be the least complex and least invasive method. Several frogs are put into a cylinder containing a piece of absorbent cotton saturated with anhydrous ether. Following exposure to the ether for 1 min to 2 min, the frogs' skins exude copious secretions which are then collected by washing the dorsal region of each animal with a buffer solution.

While electrical and chemical stimulation methods are considered humane and non-destructive, skin harvesting involves sacrificing the frogs and then excising their skins. The secretions are obtained through homogenisation and clean-up by solid phase extraction. This method poses huge ethical problems and conservation authorities are unlikely to approve such studies, especially in urban areas where frog populations are already under threat. The extraction process may also result in reduced yields of the peptides.

In all cases, once the secretions are collected they should be placed immediately on ice to inhibit the activity of endopeptidases. The process of extraction of the compounds may then proceed by centrifugation and lyophilisation of the supernatant. In general, yields are low and so the use of a large number of animals is strongly recommended. The animals can then be released back into their environment after being taken care of for at least 24 h.

Bioprospecting of South African frogs

Frog species from a limited number of families and locations have been studied for antimicrobial activity.[20] In sub-Saharan Africa, amphibians are represented by a large number of frog families, many of which are endemic to the region and remain unexplored for therapeutic agents. South Africa is home to 114 frog species.[47] The Western Cape Province has 51 frog species, of which half are endemic to the south Western Cape (De Villiers A 2008, personal communication, June 15). The Cape Floristic Region of South Africa, designated as a global biodiversity hotspot and world heritage site, possesses a high endemism of frog and toad species.[65] The

high species diversity may reflect a high molecular diversity of frog secretions and a potential for novel peptides to be discovered. Few studies on the antimicrobial properties of southern African frogs have appeared in the literature, and there is thus a need to conduct research on frog species from this part of Africa. However, there are various problems that have to be addressed, such as obtaining ethical clearance and developing improved extraction techniques for obtaining the frog secretions. Testing of the extractions can be done by microtitre plate methods which requires small quantities of the sample and can be used for a large number of samples.[66] The bioassay could be beneficial when testing frog skin secretions for antimicrobial activity, because of the small quantities that are used in the assay.

Failure of antimicrobial peptides in clinical drug development

Despite the positive picture painted by the foregoing discussion, the successful exploitation of antimicrobial peptides into clinical candidates has hitherto met with dismal failure.[67] Of seven antimicrobial peptide-based drugs which were in clinical trials in the past decade, none has obtained FDA approval, either because of poor clinical outcomes or because of toxicity and safety concerns. Antimicrobial peptides are attractive therapeutic agents because they have broad-spectrum activity and a non-specific mechanism of cidal action. However, because they cause membrane disruption, they can cause non-selective systemic and local toxicity. For example, intravaginal administration of magainin derivatives was shown to inhibit pregnancy establishment in monkeys because of its binding to placental trophoblast cells.[68]

Some of the non-pharmacological causes of failure cited have been stability of formulated peptides, the confounding biological activities of peptides and the potentially high manufacturing costs involved.[50,67] Despite the current concerns and setbacks, research and development of antimicrobial peptides is still in its infancy and continues to hold promise for the future.

Conclusions and recommendations

There is increasing resistance of microbial pathogens to antibiotics as a result of misuse and subsequent natural selection of resistant strains. There is therefore a need to develop new pharmacophores as lead compounds for antimicrobial research and development. Amphibian skin is a rich source of biologically active compounds that are assumed to have diverse physiological and defence functions.[20] In addition to the range of pharmacologically active peptides present, some of which have mammalian homologues, amphibian skin secretions contain a broad spectrum of antimicrobial peptides. Peptides from only a few species have been studied and screening of other species is expected to yield new antimicrobial agents.[19] The proteomic work done on frogs in southern Africa to date is limited and further work in this area is recommended.

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Competing interests

We declare that we have no financial or personal relationships which may have inappropriately influenced us in writing this article.

Authors' contributions

T.G. carried out the research as part of his Master's thesis and drafted the manuscript. A.D. devised the concept, and funded and supervised the research. A.J.E. funded and supervised the research. D.R.K. coordinated the laboratory work on which this review reports and revised the manuscript.

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