

Exploring the global animal biodiversity in the search for new drugs -Amphibians

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Abstract

Many breakthrough drugs have been developed from plants with medicinal properties on the basis of ethnopharmacological information. In the search for structurally novel and mechanistically unique lead compounds, drug discovery and development programs are now also paying attention to the myriad of bioactive molecules provided by the animal biodiversity. A well-known example emerging from these efforts is the breakthrough angiotensin-converting enzyme inhibitor captopril used for the treatment of hypertension and congestive heart failure. Captopril has been developed as an analogue of bradykinin in the venom of the Brazilian pit viper *Bothrops jararaca* (Viperidae) following the observation that the latter agent lowered blood pressure by vasodilation. In the mean time, dozens of clinically useful and potentially therapeutically applicable compounds from animal origin have been identified. The skin gland secretions of amphibians have been refined during millions of years of evolution to highly efficient defensive chemicals. These compounds have remarkable pharmacological activities and may also represent a treasure chest of potentially novel drugs against pathophysiological conditions. This paper addresses the importance of bioactive compounds in the skin secretions of frogs, toads, salamanders, newts, and caecilians, and elaborates on the therapeutic potential of some of them as anti-alzheimer, cardiotonic, antidiabetic, anti-HIV, analgesic, antimicrobial, and antiparasitic compounds.

Introduction

Natural products represent historically important sources of bioactive compounds with medical applicability [1]. Particularly the plant biodiversity has been explored, and this has yielded numerous breakthrough and sometimes life-saving medications [1]. Well-known examples are the cardiotonic digoxin from foxgloves in the genus *Digitalis* (Plantaginaceae) [2]; the oral hypoglycemic biguanide metformin that originates from the French lilac *Galega officinalis* (Fabaceae) [3]; the phyto steroid sapogenin diosgenin from yam species in the genus *Dioscorea* (Dioscoreaceae) that serves as precursor for, among others, oral contraceptives and cortisone [4]; the antineoplastic tubulin-interfering agent vincristine from the Madagascar periwinkle *Catharanthus roseus* (Apocynaceae) [5]; and the cholinesterase inhibitor galantamine from snowdrops in the genus *Galanthus* (Amaryllidaceae) that is indicated for the treatment of mild to moderate dementia and Alzheimer's disease [6].

Other sources of successful drugs have been certain microorganisms which have produced antibiotics with a high therapeutic index. For instance, fungi in the genus *Penicillium* (Trichocomaceae) [7], and strains of the bacteria *Streptomyces griseus* (Streptomycetaceae) [8] and *Micromonospora purpurea* (Micromonosporaceae) [9], produced powerful antibacterial agents such as the β -lactam antibiotics ampicillin and cloxacillin, and the aminoglycosides streptomycin and gentamicin, respectively. And the highly efficacious antifungal compounds amphotericin B (for treating systemic fungal infections) and griseofulvin (for treating dermatophytic fungi) have been produced on the basis of the products from the bacterium *Streptomyces nodosus* (Streptomycetaceae) [10] and the fungus *Penicillium griseofulvum* (Trichocomaceae) [11], respectively.

The animal biodiversity, from the smallest invertebrates to the largest vertebrates, has also represented a highly useful source of novel

therapeutics. For instance, the antileukemic antimetabolite cytarabine was first encountered in the Caribbean demosponge *Tectitethya crypta* (Tethyidae) [12]; trabectedin, an orphan drug for treating soft-tissue sarcomas and ovarian cancer, has first been identified in the mangrove tunicate *Ecteinascidia turbinata* (Perophoridae) [13]; and the powerful analgesic ziconotide was molded on the basis of the extremely potent conotoxins produced by predatory cone snails in the genus *Conus* (Conidae) [14]. Furthermore, the venom from the South American lancehead viper *Bothrops jararaca* (Viperidae) led to the development of angiotensin-converting enzyme-inhibiting agents such as captopril, enalapril, and lisinopril which proved ground-breaking in the treatment of hypertension [15]. And a number of compounds from insects and other arthropods displayed encouraging pharmacological activities for developing novel therapeutics (reviewed in references [16,17]).

From an evolutionary perspective, amphibians were the first to abandon aquatic life and come ashore in the Late Devonian, roughly some 370 million years ago [18]. They successfully adapted to the challenges of land life and refined their skills for surviving. Thus, they developed strong appendages, well-attached large and sturdy pectoral and pelvic girdles, and a vertebral column that resists bending; changes in eyes, ears, and nose to properly function in air; modifications in the skin to prevent desiccation and maintain water balance; adaptations to breath on lands; and effective defensive mechanisms to improve their chances for survival. The latter structures involve an astonishingly

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diverse chemical armamentarium of skin toxins with a wide range of pharmacological activities. In this paper, some of these compounds have extensively been addressed from the point of view of their potential usefulness as candidates for new drug design programs.

Background

Characteristics of amphibia

The first amphibians had developed from primitive four-limbed animals called Tetrapodomorpha who lived in the Late Devonian, 365 to 360 million years ago [18]. The latter, in their turn, had developed from ancient bony fishes called Sarcopterygii which presumably had fleshy, lobed, paired fins with finger-like protrusions and articulations [18]. These proto-limbs allowed them to move on the sea floor and crawl on land to search for food, and evolved into legs [18]. Some of the primitive amphibians also developed primitive lungs which enabled them to breathe when the shallow Devonian swamps dried up [18]. These suppositions are supported by the body plan of *Ichthyostega*, an early genus of tetrapodomorph ancestors of the amphibians that lived some 370 million years ago [19]. *Ichthyostega* had many similarities to prehistoric sarcopterygian fishes, but also important adaptations to terrestrial life including nostrils, more efficient lungs, four strong legs, and a bony skeleton to support their weight [19].

Eventually, the tetrapods abandoned aquatic life and became the ancestors of, among others, the amphibians [18]. The amphibians became the dominant group of land animals in the Early Carboniferous, 360 to 345 million years ago [18], but still needed to return to water to lay their vulnerable shell-less eggs [18]. The Carboniferous rainforest collapse - a minor mass extinction event that occurred about 305 million years ago [20] - followed by the Permian-Triassic extinction event roughly 100 million years later [21], led to the decimation of the amphibians and the dominance of the reptiles [18,20,21]. Ultimately, only a fraction of the previously abundant amphibians survived [18].

The modern Amphibia comprise a class of ectothermic, tetrapod vertebrates which in general still characteristically start their life cycle as aquatic free-swimming larvae with gills, and metamorphose to partly terrestrial and partly aquatic adults with lungs and limbs but without a tail. The name of this animal class is derived from the ancient Greek words '*amphi*' and '*bios*' meaning 'both' and 'life', referring to their aquatic and terrestrial forms of life. Amphibians have colonized a wide diversity of ecosystems including moist terrestrial, fossorial, and arboreal habitats as well as freshwater aquatic environments. They are in general not found in marine habitats with the exception of, among others, the crab-eating frog *Fejervarya cancrivora* (Dicroglossidae) that inhabits mangrove swamps and marshes in south-eastern Asia [22], and Anderson's salamander *Ambystoma andersoni* (Ambystomatidae) that lives in the brackish water of Lake Zacapu in the Central Mexican Plateau [23].

Most adult amphibians not only breathe through their lungs but also through their skin. As the skin is permeable to water, the animals are able to respire in water without rising to the surface, and to hibernate at the bottom of ponds. Some terrestrial frogs and salamanders even lack lungs and exclusively breathe through their skin. To make cutaneous respiration possible, the skin must always remain moist to allow the oxygen to diffuse at a sufficiently high rate. Examples of amphibians that primarily or solely rely on cutaneous respiration are aquatic species such as the Titicaca water frog *Telmatobius culeus* (Telmatobiidae) and the hellbender *Cryptobranchus alleganiensis* (Cryptobranchidae), as well as small terrestrial lungless salamanders in the family Plethodontidae.

Because of their permeable skin, amphibians are sensitive indicators of the status of the ecosystem they live in [24]. Amphibians also have mucus glands in their skin, the secretions of which help keep the skin moist and make the animals difficult to grip and hold firmly [25]. Most species have, in addition, clusters of granular skin glands or parotoid glands which secrete distasteful or poisonous substances to deter predators [25]. In many cases, the poisonousness is accompanied by bright aposematic colors to warn or detract predators [26].

The circulatory system of juvenile amphibians (aquatic tadpoles) resembles that of fish, comprising a two-chambered heart that pumps the blood through the gills where it is oxygenated, after which it supplies the rest of the body and reenters the heart in a single loop. In most adult amphibians, the gills have disappeared and a double circulatory system has developed that includes a three-chambered heart consisting of two atria and one ventricle. Deoxygenated blood from the body enters the right atrium and passes to the ventricle that pumps it to the pulmocutaneous circuit where gas exchange occurs in the lungs and through the skin. The oxygenated blood reenters the heart via the left atrium, again passes to the ventricle, which then distributes it through the rest of the body.

The blood is filtered by two kidneys and the urine is stored in a urinary bladder. Larvae and most aquatic adult amphibians excrete nitrogenous waste as ammonia in large quantities of dilute urine. Terrestrial species have a greater need to conserve water and excrete their metabolic waste in the form of urea. Species with limited access to water such as some tree frogs excrete most of their waste as uric acid. The nervous system of amphibians is comparable to that of other vertebrates, consisting of a central brain, a spinal cord, and nerves throughout the body. However, it is more similar to that of fish than to that of reptiles, birds, and mammals: the brain consists of three components, the forebrain, the midbrain, and the hindbrain, which are mainly involved in olfaction, vision, and motor skills, respectively.

As most amphibians locate prey by sight, binocular vision is their most important sense. Frogs and toads are also able to hear both low frequency sounds (usually from predators) and high-frequency sounds (mostly from mates), while salamanders can only detect low vibrations via the ground. Many amphibians have chemoreceptors in the mouth, on the tongue, and in the skin which help them recognize potential mates, locate food, and detect noxious and poisonous chemicals. Frogs and toads have a larynx and vocal chords which enable them to make species-specific sounds to mark their territory, give distress calls, and attract females.

Most amphibian larvae are herbivores and live on algae and different plant matter, while most adults are carnivores. In general, they swallow their prey whole, which is then passed via a short esophagus into the stomach where the enzyme chitinase helps digest the chitinous cuticle of arthropod prey. Other digestive enzymes are produced by the pancreas, the gall bladder, and the relatively large liver.

Depending on the order, fertilization occurs externally or internally. In all cases, the eggs lack coverings and must be laid in a moist environment to develop into tadpoles. Parental care is only seen in about 10% of amphibians, and in these cases it usually is the male that protects the nest and broods the eggs. The tadpoles of most species undergo a dramatic metamorphosis which may involve, among others, losing their tail, fins, and gills and developing lungs and limbs [27]. Metamorphosis is incomplete in some cases which retain, for instance, their gills and/or tails as adults [27]. Metamorphosis in all anurans is regulated by a balance between blood concentrations of thyroxine

which stimulates metamorphosis, and those of prolactin which counteracts thyroxine's effect [27]. After metamorphosis, the redundant organs are removed by apoptosis [27]. There is considerable variation in developmental responses to specific environmental circumstances, and this results in many modifications of the process of metamorphosis, even within the same species [28].

Classification of amphibia

The Amphibia are classified into three subclasses, namely the extant Lissamphibia which include all modern amphibians, as well as the extinct Lepospondyli and Temnospondyli which lived during the Paleozoic and Early Mesozoic Eras, roughly 541 to 252 million years ago (Figure 1) [29]. The lissamphibians are subdivided into the orders Anura or Salientia, Urodela or Caudata, and Gymnophiona or Apoda (Figure 1). The Anura or Salientia consist of frogs, toads, and their relatives, and represent about 88% of the total number of about 8,212 currently known amphibian species [29]. Most anurans inhabit moist habitats but there are some species that live in deserts. Fertilization occurs externally, and the eggs and larvae are typically aquatic while the adults are in general terrestrial. The tadpoles have tails which disappear after metamorphosis. The tail-less adults develop muscular hind limbs and webbed feet.

The Urodela or Caudata comprise the salamanders, newts, and their relatives, and encompass 9% of the amphibian species described today [29] which are mostly encountered in the northern hemisphere. Both names of this amphibian order can freely be translated as 'to bear a tail', since most of its members keep their tail after metamorphosis. Some species also retain the external gills characteristic for their larval stages during adulthood. Urodela reproduce by internal fertilization without actual copulation: the male deposits a packet of sperm that is picked up and stored by the female, and the eggs are fertilized as they leave its body through the cloaca.

The Gymnophiona or Apoda encompass the caecilians and their relatives, and make out the remaining 3% of the currently described amphibian species (Figure 1) [29]. These amphibians are 'naked and limbless like a snake', almost blind (their vernacular name 'caecilians' is derived from the Latin word '*caecus*', meaning 'sightless' or 'blind'), and are in general found in tropical regions where they mostly live in underground burrows. Unlike anurans and urodeles, members of the Gymnophiona reproduce by internal fertilization following internal insemination. Some species have live births and some lay eggs.

The smallest amphibian in the world (as well as the smallest vertebrate in the world) is the narrow-mouthed frog *Paedophryne amauensis* (Microhylidae) [30]. This animal attains an average body size of only 7.7 mm and was discovered near Amau village in southern Papua New Guinean [30]. The largest extant amphibian

is the fully aquatic Chinese giant salamander *Andrias davidianus* (Cryptobranchidae) that can reach a weight of 50 kg and a length of 180 cm, and can be encountered in the Yangtze river basin of central China [31]. However, with an average length of 9 m and a weight of over 2,000 kg, the extinct *Prionosuchus* was the largest amphibian that ever existed [32]. *Prionosuchus* was a genus of crocodile-like tetrapode primitive amphibians that lived during the early Perm, some 299 to 272 million years ago [32]. Fossilized fragments of this animal have been discovered in the Pedra do Fogo Formation in the Parnaíba Basin of north-eastern Brazil [32].

Defenses of amphibia

Many amphibians are relatively small and slow-moving creatures with tender bodies and thin skins, and do not have claws, canine teeth, defensive armor, and/or spines. Furthermore, they usually occur in high population densities, are good sources of protein, and contain little indigestible material. Thus, amphibians generally represent easy and attractive prey for predators. For this reason, they have developed a broad arsenal of defensive mechanisms to prevent or minimize predation and increase their chances of survival in all stages of their life cycle. Some of these mechanisms are comprehensively dealt with in the sections about the different lissamphibian orders hereunder.

Among the most distinctive defense mechanisms of all amphibians are the above-mentioned mucous and granular glands in their skin [25,33,34], the secretions of which keep the animals moist and slippery and difficult to grip and often represent powerful defensive chemicals [25,33,34]. The mucous glands are widely distributed throughout the integument and continuously secrete their contents onto the skin surface [25,33,34]. The mucous secretion acts as a physical barrier against pathogens, restricts loss of water, provides lubrication to the skin, reduces friction under water, and minimizes mechanical damage on land [25,33,34]. In some species - such as the African clawed frog *Xenopus laevis* (Pipidae) - it is also distasteful and toxic, providing the animals an opportunity to escape when ingested by predatory snakes [35].

The granular or parotoid glands are in general found in clusters on the skin, mainly behind the ears in toads, along the back in frogs, behind the eyes in salamanders, and on the upper surface in caecilians [25]. These glands synthesize and secrete a large variety of bioactive substances which serve a variety of protective and defensive purposes [25,36,37]. Some have powerful antimicrobial properties which protect the animals against a variety of microbial pathogens, others give them an unpleasant taste or smell, and still others act as irritants, hallucinogens, convulsants, nerve poisons, or vasoconstrictors [38,39]. At this moment, hundreds of bioactive peptides, bioactive amines, alkaloids, and steroids have been identified in amphibian granular skin secretions [25,36,37]. In most cases, the toxic ingredients in the secretions are derived from the poisonous plants and small insects the animals consume or live amongst [25,40], but the Australian corroboree frogs *Pseudophryne corroboree* and *Pseudophryne pengilleyi* [41] and certain members of the Salamandridae family (true salamanders and newts) [42] synthesize the toxic compounds themselves.

As also indicated earlier, many toxic amphibians combine their toxicity with aposematic coloring to warn potential predators to keep distance [43]. Well-known examples are the poison dart frogs in the genus *Phylllobates* (Dendrobatidae) from Central and South America [44], and the fire salamander *Salamandra salamandra* (Salamandridae) from southern and central Europe [45]. These amphibians combine the production of the highly toxic alkaloids curare

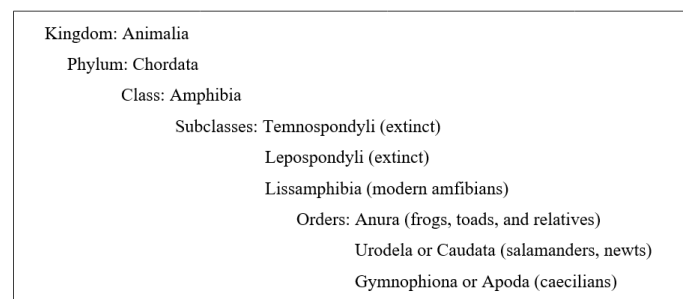


Figure 1. Taxonomy of the amphibia in the animal kingdom

and samandarin, respectively, in their skin secretions with vividly bright skin coloration. Some species such as rocket frogs in the genus *Colostethus* (Dendrobatidae) and Zaparo's poison frog *Allobates zaparo* (Aromobatidae) have cashed in on the success of their aposematic relatives by applying Batesian mimicry, *i.e.*, using equally extremely bright (pseudoaposematic) coloration while having only minimal or no toxicity [44,46].

Some of the defensive chemicals of amphibia elicit remarkable pharmacological effects and may be exploited as lead compounds for developing novel diagnostic compounds and therapeutic drugs [47]. A few of these compounds from Anura, Urodela, and Gymnophiona are comprehensively addressed hereunder.

Anura

Generalities about anura

The Anura or Salientia include 7,243 species of living frogs and toads and as mentioned above, represent the largest order of the Amphibia, comprising nearly 90% of this class of animals [29]. Frogs and toads are sometimes distinguished from each other with respect to the structure of their skin and their habitat: frogs would have in general smooth and moist, slimy skin and would spend most of their life in or near water, while toads would have dry and warty skin and would spend their life much more on land. However, taxonomically, this distinction is incorrect. All anurans - including so-called toads - are frogs, and only members of the family Bufonidae are considered 'true toads'. Thus, the Panamanian golden frog *Atelopus zeteki* has a smooth skin and is a 'true toad' in the toad family Bufonidae, whereas the European fire-bellied toad *Bombina orientalis* has a slightly warty skin but is a 'frog' in the family Bombinatoridae.

A unifying characteristic of all anurans is the absence of a tail, which is captured in the name 'Anura' of the order that is derived from the ancient Greek words '*a(n)*' and '*ourá*' meaning 'without' and 'tail', respectively. In addition, all anurans have a relatively short trunk; broad, flat heads; and relatively long hind limbs which fold underneath them, enabling them to assume a squatting position and to leap and jump (the synonym 'Salientia' for this order is derived from the Latin verb 'salere' meaning 'to jump'). With the exception of polar areas, frogs and toads have colonized all ecological niches throughout the world, particularly damp habitats and trees in tropical areas. As mentioned earlier, the smallest anuran in the world is the narrow-mouthed frog *P. amauensis* from Papua New Guinea that measures about 7.7-millimeter [30]. The largest is the goliath bullfrog *Conraua goliath* (Conrauidae) from western Africa that can grow up to 32 centimeters in length and weigh up to 3.25 kilograms [48].

The oldest members of the frog lineage probably were extinct proto-frog-like amphibians of the genus *Triadobatrachus* that had lived during the Early Triassic about 250 million years ago [49]. Millions of years later, in the Early Jurassic (about 190 million years ago), modern frogs arose, the earliest example of which was *Prosalirus* (Prosaliridae) [50]. These early anurans gave rise to the current 7,243 species which can be grouped in 459 genera, 54 families, and 3 suborders, the Archaeobatrachia, Mesobatrachia, and Neobatrachia [29].

The suborder Archaeobatrachia includes 28 relatively small species of primitive anurans which are placed in 4 families and 6 genera [29]. Well-known examples are the fire-bellied toads belonging to the genus *Bombina* in the family Bombinatoridae [29]. They are considered 'primitive' because they have characteristics that were common in extinct frogs and toads but are no longer present in the more modern

suborders, most notably free vertebrae that are not attached to the ribs instead of fused vertebrae [29]. The Archaeobatrachia are mainly found in Eurasia, New Zealand, the Philippines, and Borneo [29]. The suborder Mesobatrachia consists of 244 species in 16 genera and 6 families of evolutionary more advanced frogs and constitutes the second-largest of the Anura suborders. There are five fossorial families and one obligatorily aquatic family, the Pipidae (tongueless frogs) from Africa and South America such as the Surinam toad *Pipa pipa* [29]. The suborder Neobatrachia has evolved later than the Mesobatrachia and comprises the remaining frogs including the most commonly known species throughout the world such as those in the families Hylidae (true tree frogs), Microhylidae (narrow-mouthed frogs), Bufonidae (true toads), and Ranidae (true frogs) [29]. With roughly 5,000 species in over 350 genera and more than 30 families, it is by far the largest anuran subgroup, comprising over 96% of all existing frogs and toads [29].

Fertilization in Anura mainly occurs externally, taking place by amplexus, involving the male grasping the female with its front legs and covering the eggs with sperm as they are released from the female's body [51]. After fertilization, the life cycle of most anurans starts in water when the eggs hatch into limbless larvae with gills. Within a few days they develop into tadpoles with limbs, lungs, and a relatively long, spiral-shaped gut to digest the vegetarian diet they live off. Eventually, the tadpoles undergo metamorphosis to terrestrial air-breathing animals, involving, among others, the development of the lungs and the disappearance of the gills, apoptosis of the tail, modifications for hearing and (stereoscopic) vision as well as for locomotion on land, changes in the skin to prevent dehydration and facilitate skin breathing, as well as adaptations for a carnivorous lifestyle [52]. Remarkably, all these changes can occur within twenty-four hours [52].

Anurans have developed various strategies to defend themselves against attack or predation in all stages of their life cycle. For instance, the eggs from the red-eyed tree frog *Agalychnis callidryas* (Phyllomedusidae) escape predation by immediately hatch in response to vibrations caused by a potential predator and drop from the undersides of the leaves they have been laid on into the ponds beneath [53]. The (non-toxic) leaf litter frog *Ischnocnema henselii* (Brachycephalidae) feigns death as a way to avoid predation [54], a behavior called thanatosis after Thanatos, the god of death in ancient Greece. When threatened, the red tree frog *Leptopelis rufus* (Arthroleptidae) combines thanatosis with the release of a strong ammonia-like smell through its open mouth, adding to the illusion of a dead frog [54].

Furthermore, the Pacific tree frog *Pseudacris regilla* (Hylidae) uses camouflage, changing its skin color depending on the time of year and the general background color [55]. The European common toad *Bufo bufo* (Bufonidae) adopts a characteristic aggressive stance with its body inflated, its hindquarters raised, and its head lowered when confronted with an assailant [54]. When grasped, the smoky jungle frog *Leptodactylus pentadactylus* (Leptodactylidae) emits a high-pitched scream to startle the assailant [54]. And when facing danger, the American bullfrog *Lithobates catesbeianus* (Ranidae) crouches down with eyes closed and head tipped forward, placing the parotoid glands in the most effective position for defense while the glands on its back begin to ooze noxious secretions [54].

Biochemical and pharmacological studies have shown the existence of hundreds of defensive chemicals in the skin secretions of anurans, including alkaloids, peptides, proteins, steroids, and biogenic amines [56]. These compounds mainly come from the ants and other arthropods they consume [57-61], and may have irritating, hallucinogenic, convulsant, nerve-poisoning, or vasoconstrictive properties [56]. These

meaningful pharmacological properties suggest that ingredients of anuran skin secretions could be useful against human diseases.

Bioactive compounds from anura

Anurans have been the subject of numerous folktales, fables, myths, and legends, ranging from their 'usefulness against ugliness and wickedness' according to the Chinese 'Book of Songs' from 3,000 years ago [62] to the many variations of the Brothers Grimm fairy tale 'The Frog Prince' in which the protagonist is transformed into a handsome prince after having been kissed by the beautiful princess [63]. Likewise, the pharmacological properties of anurans - either perceived or genuine - have been known for a long time. Secretions from the parotoid glands from frogs and toads, but also preparations from whole animals or from their bones or muscle tissues, have been used for millennia for treating, among others, infections, bites, heart disorders, hemorrhages, allergies, inflammation, pain, and even cancer and AIDS [64].

For instance, in China and Japan, the dried secretions from the skin glands of the Asiatic toad *Bufo gargarizans* (Bufonidae) or the Asian common toad *Duttaphrynus melanostictus* (Bufonidae) called *venenum bufonis*, toad cake, or Chansu (Senso) in China and Kyutshin in Japan, are since long used to treat cancer [65,66]. These substances have also extensively been used for hundreds of years, either singly or in combination with other compounds, as analgesics, local anesthetics, diuretics, and antimicrobial substances [65,66]. Notably, an injectable form of Chansu called Huachansu has officially been approved for use in traditional Chinese medicine as an antineoplastic agent, particularly for treating liver and pancreatic cancer [67,68].

Ongoing research efforts are showing that many of these traditional applications may have a scientific rationale and, more importantly, may help in the identification of lead compounds for producing novel therapeutics. For instance, various members of the harlequin toads in the genus *Atelopus* (Bufonidae) produce bufadienolide and tetrodototoxin [69], as well as zetekitoxins [70] and tetrodototoxin analogues called chiriquitoxins [71] in their parotoid glands. Bufadienolides and zetekitoxins are powerful cardiotoxins that are able to reduce cardiac rhythm [70], while tetrodototoxin and chiriquitoxins are neurotoxins which can cause muscle paralysis and respiratory arrest [71]. For these reasons, these compounds may serve as leads for developing novel heart medications and analgesics.

Furthermore, preparations from anuran venoms including toad cake showed encouraging *in vitro* antitumor activity that might be associated with the inhibition of cell proliferation, the promotion of angiogenesis of tumor endothelial cells, the induction of apoptosis, the promotion of differentiation, and the stimulation of tumor-directed immune responses [72-74]. Comparable results were obtained with various isolated bufadienolides [72-74]. Thus, ingredients of anuran skin secretions may also turn out useful against cancer.

Numerous studies have demonstrated the antimicrobial properties of peptides from anuran skin secretions. A few examples are bombinins in European species of fire-bellied toads in the genus *Bombina* [75]; magainins in the African clawed frog *X. laevis* [76]; dermaseptin in the waxy monkey tree frog *Phyllomedusa sauvagii* (Phyllomedusidae) [77]; bombinin-like peptides in two species of *Bombina* [78,79]; brevinins in the Nagoya Daruma pond frog *Rana brevipoda porsa* (Ranidae) [80]; and rugosins in the Japanese wrinkled frog *Rana rugosa* (Ranidae) [81]. It is conceivable that some of these compounds will replace currently available antibiotics which are becoming less efficacious due to the development of microbial resistance.

Together, these data indicate the potential of anuran glandular skin poisons for developing novel heart medications, analgesics, anticancer agents, antibiotics, and a host of other therapeutics [82]. Hereunder, ingredients from anuran glandular skin secretions with potential applicability as anti-alzheimer, cardiostonic, antidiabetic, and anti-HIV compounds is in detail addressed.

Anti-alzheimer's compounds

Alzheimer's disease is a progressive neurodegenerative disorder characterized by a gradual decline in cognitive, behavioral, and social skills that disrupts the ability to function independently, and culminates in the loss of bodily functions and, ultimately, death [83]. This condition is the most common cause of dementia in many parts of the world, affecting about 50 million individuals in 2019 and accounting for more than two-thirds of cases of senile dementia [84]. Its incidence in 2019 was about 10 million and doubles every five years after the age of 65 years due to continued aging of the world population [84], and the prevalence is anticipated to reach over 115 million by 2050 [85].

The etiology of Alzheimer's disease is complex and still incompletely understood, but is believed to involve an interplay of genetic, lifestyle, and environmental factors that increasingly affects the brain with older age [83-85]. These factors contribute to various degrees to the hallmarks of the disease, namely abnormalities in the cerebral cholinergic system, and the deposition of amyloid plaques and neurofibrillary tangles in certain areas in the brain [86-88]. The cholinergic system plays an important role in the regulation of learning and memory processes, and progressive loss and malfunctioning of choline-containing neurons in the brain are believed to substantially contribute to the cognitive decline in patients with Alzheimer's disease [86]. Amyloid plaques are dense, insoluble buildups of β -amyloid peptide and cellular material outside and around neurons that interfere with neuron-to-neuron communication at synapses [87,88]. Neurofibrillary tangles are aggregates of the microtubule-associated protein tau which have become hyperphosphorylated and accumulate inside neurons, blocking the transport of nutrients and other essential molecules inside neurons [87,88]. Other changes in Alzheimer's disease include chronic inflammation, atrophy of the brain, and loss of normal brain function as a result of the immune response of microglia on the presence of β -amyloid and tau proteins as well as other debris, cell loss, and the increasing inability of the brain to metabolize glucose [87-89]. It is gradually becoming clear how all these abnormalities work together in causing Alzheimer's disease [89].

So far, there are no treatments to halt or reverse the progression of Alzheimer's disease, although some medications may temporarily provide symptomatic improvement [90]. Since a deficit in the cerebral cholinergic system is a consistent and early finding in Alzheimer's disease, many drug discovery and development efforts have focused on inhibiting acetylcholinesterase and/or butyrylcholinesterase activities, thus preventing acetylcholine turnover, restoring synaptic levels of this neurotransmitter, enhancing cholinergic transmission, and decreasing the formation of amyloid plaques and tau tangles [91]. As a result, cholinesterase inhibitors such as galantamine, rivastigmine, and donepezil are currently the main medications for the treatment of Alzheimer's disease [90,91]. However, these medications only temporarily relieve symptoms while having substantial side effects, and often lose their effect as the disease progresses [90,91].

For these reasons, the search for novel anti-alzheimer medications with an improved therapeutic index is ongoing. Among these compounds are ingredients of the skin extracts from certain anuran species. For

instance, the skin extracts from several South American hylidan species, namely Cordoba's tree-frog *Hypsiboas cordobae* (Figure 2), the lesser swimming frog *Pseudis minuta*, the Montevideo tree frog *Boana pulchella*, the swimming frog *Pseudis platensis*, and the dwarf treefrog *Dendropsophus nanus* displayed encouraging acetylcholinesterase- and butyrylcholinesterase-inhibiting activities *in vitro* [92,93]. Essentially similar results were found with the skin extracts from the butter frog *Leptodactylus latrans*, Cei's white-lipped frog *Leptodactylus chaquensis*, the mustached frog *Leptodactylus mystacinus*, Hensel's swamp frog *Pseudopaludicola falcipes*, and the Helvetia dwarf frog *Physalaemus santafecinus* in the Leptodactylidae family; and that from the two-colored oval frog *Elachistocleis bicolor* belonging to the Microhylidae [92]. All the skin extracts also exhibited *in vitro* monoamine oxidase B inhibitory activity and displayed *in vitro* antioxidant activity [92], suggesting that they may target additional pathways that may be of relevance to the pathophysiology of Alzheimer's disease [85,89].

The identity of the chemicals in the skin extracts that were responsible for these observations is not clear. However, in the case of that of *B. pulchella*, the *in vitro* (butyryl)cholinesterase-inhibiting activity might be attributed to low molecular weight-compounds [94] as well as the protein Hp-1935 isolated from this extract [95,96]. Hp is an abbreviation of *Hypsiboas pulchellus*, a synonym for *B. pulchella*, and Hp-1935 had shown notable antimicrobial activity in earlier studies with *Mycobacterium tuberculosis* [95] as well as *Escherichia coli* and *Staphylococcus aureus* [97]. The results from molecular dynamics simulations suggested that Hp-1935 is among the most potent natural peptides against acetylcholinesterase [96]. In the mean time, a number of analogues of Hp-1971 have been synthesized that displayed potent inhibition of acetylcholinesterase and butyrylcholinesterase activity [98]. These developments hold the promise to eventually apply anuran-derived products in the clinic against Alzheimer's disease.

Cardiotonic compounds

Heart failure is a complex clinical syndrome characterized by the reduced ability of the heart to pump and/or fill with blood [99]. In other words, in these patients, the cardiac output is not sufficient to meet the metabolic demands of the body [99]. Heart failure has been characterized as an emerging worldwide threat, affecting 64.34 million individuals in 2017 and accounting in that year for USD 346.17 billion expenditure and 9.91 million years lost due to disability [100]. This condition poses the largest disease burden in individuals older than 60 years, and prevalence as well as years lost due to disability have increased by 3.9% and 4.5%, respectively, in very elderly people during the last 28 years [100].



Figure 2. The Cordoba tree frog *Hypsiboas cordobae* (Hylidae) (<https://images.app-goo.gl/Xo8eS6haXdsZ9im26>)

The cardiac glycoside digoxin from foxglove plant species belonging to the plant genus *Digitalis* (Plantaginaceae) has been used for centuries for treating heart failure owing to its cardiotonic effects [101]. Digoxin inhibits the Na^+/K^+ -ATPase transport system in the plasma membrane of cardiomyocytes, which leads to higher intracellular Na^+ concentrations, a reduced efficacy of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, and higher intracellular Ca^{2+} concentrations, leading to increased contractility, reduced velocity of electric conduction, and a decreased heart rate [101]. The end results is improved cardiac output and ejection fraction as well as reduced filling pressures and pulmonary capillary wedge pressure while heart rate is hardly affected [101]. In addition, digoxin reduces the velocity of electric conduction of the vagal nerve which, via a reflexive reduction of sympathetic transmission, also contributes to reduction of the heart rate [101].

Despite the availability of newer and more efficacious treatments for heart failure such as angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, angiotensin-receptor neprilysin inhibitors, If channel blocker, β -blockers, aldosterone antagonists, diuretics, anticoagulants, and cholesterol-lowering drugs (statins) [102], digoxin is still widely used in this condition, particularly in treatment-resistant patients [101]. However, the major drawbacks of digoxin is its narrow therapeutic index [101]. For these reasons, the search for novel clinically useful cardiac glycosides is ongoing.

Among these are the above-mentioned anuran skin gland preparations called venenum bufonis, toad cake, Chansu (Senso), and Kyushin [65,66]. These substances elicited notable cardiotonic effects in an *in vivo* rabbit model of heart failure as well as in anesthetized open-chest guinea pigs or dogs, accomplishing an increase in mean aortic pressure, left ventricular pressure and maximal rate of rise of left ventricular pressure, and a decrease in left ventricular end-diastolic pressure and systemic vascular resistance without significantly changing mean blood pressure and heart rate [103-107]. These effects were comparable to those caused by digoxin [103], and the beneficial effects on the heart might be aided by stimulation of β -adrenoceptors [108] and diuresis [109]. Importantly, Senso and Kyushin caused cardiac arrhythmia - a common side effect of digoxin [101] - at much higher doses than digoxin, suggesting that they had a broader safety margin than digoxin [106]. Interestingly, Kyushin inhibited experimentally induced arrhythmia in guinea pigs, presumably through an inhibitory effect on the conduction system as well as by potentiating the decrease in heart rate by the parasympathetic nervous system [110].

The main ingredients of Chansu and Kyushin are cardiac glycosides including bufadienolide glycosides such as bufalin, resibufogenin, and cinobufagin [110,111]. These compounds elicited cardiotonic effects comparably to digitoxin and ouabain in a guinea-pig working heart preparation and in experimentally induced heart failure in a perfused guinea-pig heart [111]. Furthermore, commercially available bufolin inhibited Na^+/K^+ -ATPase activity in normotensive rats comparably to ouabain and ouabagenin [112], and both bufolin and cinobufagin inhibited myocardial Na^+/K^+ -ATPase activity in the plasma membrane of cardiomyocytes and increased the myocardial contractile force in open-chest guinea pigs without affecting the heart rate [113].

In addition to bufadienolides, bufadienolide-like compounds marinoic acid and marinosin have been identified as inhibitors of Na^+/K^+ -ATPase activity and thus potential cardiotonic compounds in the skin of the cane toad *Rhinella marina* (Bufonidae) (Figure 3) [114,115]. These compounds might be related to the bufadienolides because of some structural similarities and their inhibitory effect on Na^+/K^+ -ATPase activity, and competed with ^3H -ouabain for binding to the

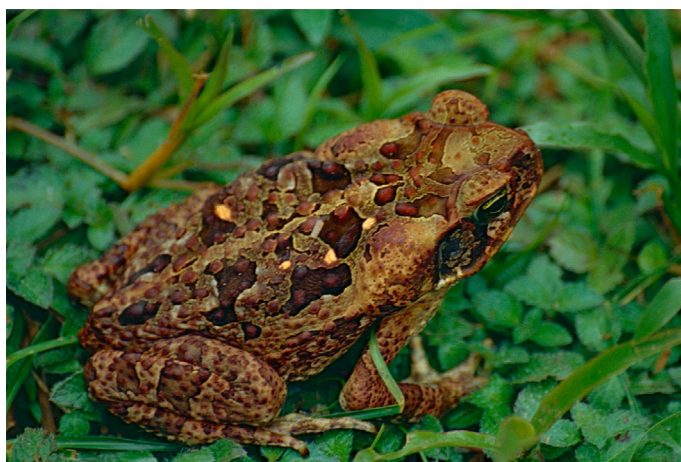


Figure 3. The cane toad *Rhinella marina* (Bufonidae) (<https://images.app.goo.gl/korNjV649ZnLMhrb9>)

digitalis receptor site on Na^+/K^+ -ATPase [114,115]. These observations, together with those from the previous alinea, warrant further research on bufadienolides and bufadienolide-related compounds in anuran skin gland preparations as improved glycoside cardiotonics.

Antidiabetic compounds

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia (fasting plasma glucose > 7.0 mmol/L, or plasma glucose > 11.1 mmol/L two hours after a meal) as a result of insufficient insulin secretion, insufficient insulin action, or both [116]. The defective insulin secretion is the result of inappropriate functioning of the β cells of the pancreas, while the inadequate insulin action is generally associated with resistance of the target tissues to insulin [116]. The resulting abnormalities can lead to distinctive complications of heart, blood vessels, eyes, kidneys, and nerves which can eventually be fatal [116].

Diabetes mellitus is among the most widespread non-communicable diseases in many parts of the world, with a global morbidity and mortality in 2019 of 463 million and 4.2 million individuals, respectively [117]. These numbers are rising rapidly: in 2011, the global prevalence in adults was 6.4% or 350 million, but it is anticipated to reach 7.3% or 700 million by 2045 [117]. Health expenditure for diabetes mellitus amounted in 2019 to USD 760 billion, i.e., approximately 10% of total spending on adults [117]. Still, the proportion of individuals with type 2 diabetes mellitus is increasing in most countries [117], with 79% of adult patients living in low- and middle-income countries in 2019 [117].

Treatment of diabetes mellitus involves both the adoption of lifestyle changes, particularly an adjusted diet and regular exercise, and the intake of drugs [118]. Patients with type 1 diabetes mellitus are treated with one of the nay dosage forms of insulin [118]. Those with type 2 diabetes mellitus may be prescribed oral antihyperglycemic drugs, injectable glucagon-like peptide-1 receptor agonists, insulin, or a combination of these agents [118]. In all cases, regular monitoring of blood glucose levels is essential to prevent complications [118]. The therapy to be delivered is determined by the type of diabetes mellitus involved, i.e., type 1 diabetes mellitus (caused by autoimmune β -cell destruction and ending with absolute insulin deficiency), type 2 diabetes mellitus (caused by a progressive loss of β -cell insulin secretion along with insulin resistance), gestational diabetes mellitus (occurring in the

second or third trimester of pregnancy) and specific types of diabetes due to other causes [119].

Despite the large armamentarium of antidiabetic medications, each of these compounds have their specific drawbacks [118]. For instance, metformin is the first-line medication for type 2 diabetes mellitus, but is infamous for gastrointestinal upset, including diarrhea, cramps, nausea, vomiting, and increased flatulence [120]. For these reasons, the search for improved antidiabetic medications from 'unusual' sources is ongoing. These efforts have led to the development and approval of glucagon-like peptide-1 receptor agonist such as exenatide, synthetic versions of exendin-4, a hormone found in the saliva of the Gila monster *Heloderma suspectum* (Helodermatidae) [121]. These medications are also called incretin mimetics because they simulate the actions of endogenous incretin hormones that the body produces to stimulate insulin release and decrease excessive glucagon release in response to a meal [121]. They are subcutaneously administered before the first and last meal of the day, and are used along with diet and exercise to control blood sugar in adults with type 2 diabetes [121].

More recently, the skin secretions of several species of frogs have also been found to synthesize potent incretin mimetic insulinotropic peptides with therapeutic potential [122-125]. Well-studied examples are pseudin-2 from the paradoxical frog or shrinking frog *Pseudis paradoxa* (Hylidae) (Figure 4); tigerinin-1R from the Chinese edible frog *Hoplobatrachus rugulosus* (Dicroglossidae); magainin-AM2 from the volcano clawed frog *Xenopus amietii* (Pipidae); hymenochirin-1B from the Congo dwarf clawed frog *Hymenochirus boettgeri* (Pipidae); caerulein precursor fragment (CPF) peptides from the African clawed frog *X. laevis* and the Cameroon clawed frog *Xenopus epitropicalis* (Pipidae); esculentin-2CHa from the Chiricahua leopard frog *Lithobates chiricahuensis* (Ranidae) [122-125]. These compounds stimulated the release of insulin from cultured BRIN-BD11 cells and isolated mouse islets [125-130], and reduced body weight, improved glucose tolerance following a high-fat diet, enhanced insulin release and insulin sensitivity, and decreased glucagon and plasma glucagon-like peptide 1 in high fat-fed laboratory mice with impaired glucose tolerance and insulin resistance [126,128,131-133].

Importantly, none of these compounds were cytotoxic *in vitro* [125-128,130] or *in vivo* [133]. Other encouraging properties for their use as antidiabetic agents are the resistance to degradation by plasma enzymes *in vitro* for up to 8 h (in the case of magainin-AM2 [128]), the capacity to lower circulating triglyceride levels by (in the case of CPF-



Figure 4. The paradoxical frog or shrinking frog *Pseudis paradoxa* (Hylidae) (<https://images.app.goo.gl/VXrm2yBbQDhtr4Tu9>)

SE1 [132]), the promotion of β -cell proliferation and survival *in vivo* (in the case of esculetin-2CHa [133]), and the positive results with the magainin analogue pexiganan in the treatment of infected foot ulcers in patients with diabetes [134]. Nevertheless, more work needs to be carried out before the antidiabetic therapies based on frog secretions can be tested on human patients.

Anti-HIV compounds

Human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS) represent a syndrome that is caused by infection with the human immunodeficiency virus (HIV) [135]. HIV/AIDS is characterized by a progressively weakening immune system and a greatly increased risk of contracting (opportunistic) infections with a more serious course than in healthy individuals [135], and developing tumors which are rare in people who have normal immune function such as Kaposi's sarcoma [135,136]. The late symptoms of HIV infection are referred to as acquired immunodeficiency syndrome (AIDS) [135]. There were worldwide about 38 million cases of HIV infection in 2019, and in that year 690,000 patients died from their disease [137]. The total worldwide death toll of HIV/AIDS between the first identified case in the 1980s and 2019 amounted to roughly 32.7 million [137]. Not surprisingly, HIV/AIDS is considered a pandemic, although its spread has been largely controlled by preventive measures and adequate medications [138].

The causative agents of HIV/AIDS are the lentiviruses HIV-1 and HIV-2 [135], which are probably common in other primates including chimpanzees, and have first spread to humans in west-central Africa in the early-to-mid 20th century [139]. HIV-1 is more virulent and more infective than HIV-2, and is the cause of the majority of HIV infections globally [140]. AIDS was first recognized by the United States Centers for Disease Control and Prevention in 1981, and its cause - HIV infection - was identified in the early part of the decade [141]. HIV primarily infects CD4⁺ T cells [142], which play key roles in the adaptive immune system, among others, by releasing cytokines which help activate cytotoxic CD8⁺ T-cells to fight off viruses and other foreign invaders [143].

So far, there is no cure or vaccine for HIV/AIDS. Patients receive highly active antiretroviral treatment (HAART) which can slow the course of the disease and may lead to a near-normal life expectancy [144], as well as preventive and active treatment of opportunistic infections [145]. HAART consists of combinations of at least three medications belonging to at least two classes of antiretroviral agents [146]. The individual drugs that make up HAART interfere with distinct steps in the HIV infection cycle and include nucleoside reverse-transcriptase inhibitors and nucleotide reverse-transcriptase inhibitors, non-nucleoside reverse-transcriptase inhibitors, protease inhibitors, entry inhibitors or fusion inhibitors, and integrase inhibitors [146]. Currently, the World Health Organization recommends the combination of the nucleoside reverse-transcriptase inhibitor lamivudine, the nucleotide reverse-transcriptase inhibitor tenofovir, and the integrase inhibitor dolutegravir as first-line treatment, with the non-nucleoside reverse-transcriptase inhibitor efavirenz instead of dolutegravir as an alternative [147]. Combinations that include protease inhibitors are used if the above regimen loses effectiveness [147].

Amphibian skin secretions have been shown to contain peptides with strong antiviral properties in *in vitro* systems. A few examples are brevenin-1 from the Nagoya Daruma pond frog *R. brevipoda porsa* (Figure 5) that displayed activity against herpes simplex viruses types 1 and 2 [148]; esculetin-2P and ranateurin-2P from the northern

leopard frog *Lithobates pipiens* (Ranidae) that inactivated frog virus 3 and channel catfish herpes virus [149]; and dermaseptins from several hylid frogs - particularly those in the genera *Agalychnis* and *Phyllomedusa* - that exerted potent activity against human papilloma virus and herpes simplex virus [150], herpes simplex virus 1 and 2 including an acyclovir resistant strain [151,152], and rabies virus in mice [153].

Likewise, several ingredients from anuran skin secretions were found to elicit anti-HIV activity. This held true for, for instance, dermaseptins-S4 and S9 from certain species in the genus *Phyllomedusa* [154,155] which reduced cell-free and cell-associated HIV-1 transmission, and impaired HIV-1 attachment to P4-CCR5 indicator cells, human primary T lymphocytes, and monocyte-derived dendritic cells [154]. Notably, certain analogues of these peptides displayed less cytotoxicity to normal cells without affecting the anti-HIV activity [154,155]. Furthermore, a heme-containing protein called BAS-AH isolated from skin secretions of the Asiatic toad *B. gargarizans* inhibited HIV-1 infection and replication at concentrations that showed little effect on the viability of host cells, possibly by interfering with the activity of HIV-1 reverse transcriptase [156]. The bombinin-like antimicrobial peptide maximin 3 from skin secretions of the Chinese red belly toad *Bombina maxima* (Bombinatoridae) also elicited significant anti-HIV activity, albeit through (a) so far unclear mechanism(s) of action [157].

In addition, a relatively large number of protease inhibitors have been isolated from the skin granular glands of various anuran species (reviewed in [158]). Given the impact of protease inhibitors on AIDS morbidity and mortality following their introduction [159], and their importance as second-line options in HAART combinations [147], anuran protease inhibitors may be of interest for developing novel anti-HIV medications. This is particularly relevant when considering the unfavorable bioavailability and toxicity profile of available anti-HIV protease inhibitors [160], and their decreasing therapeutic efficacy due to the development of protease inhibitor-resistant HIV-1 [161].

Urodela

Generalities about urodela

The order Urodela (from the ancient Greek words 'uro' for 'tail' and 'delos' for 'conspicuous') or Caudata (Latin for 'tailed') consists of the salamanders, a group of slender, elongated amphibians with blunt snouts and short limbs projecting sideways, which have retained their laterally flattened fin-like tail as adults. Salamanders are in general



Figure 5. The Nagoya Daruma pond frog *Rana brevipoda porsa* (Ranidae) (<https://images.app.goo.gl/RSWYbWzypbv4JmLd6>)

shorter than 15 centimeters and move close to the ground, bearing some similarity with lizards, but lack claws and have a smooth or warty skin without scutes or scales. These animals are mainly encountered in the non-tropical regions of the Northern hemisphere. Depending on the season, many species reside in terrestrial or freshwater habitats. As mentioned above, Anderson's salamander *A. andersoni* is one of the few species that lives in brackish or salt water [23].

One of the earliest salamanders on Earth was the extinct salamandroid *Beiynerpeton jianpingensis*, fossilized remains of which dating from the Late Jurassic (about 155 million years ago) were found near the county town Jianping in the state of Beiyuan in China [162]. Other archaeological finds in England and Kirghizstan (Central Asia) even date the Urodela to at least the Middle Jurassic, 174.1 to 163.5 million years ago [163]. These early species were the predecessors of the extant 754 species in 69 genera and 10 families [29]. The smallest is the terrestrial Veracruz pigmy salamander *Thorius pennatulus* (Plethodontidae) that can be found near Veracruz in Mexico and reaches a length of 15 to 21 millimeters [164]. The largest is the earlier mentioned fully aquatic Chinese giant salamander *A. davidianus* that can become as large as 1.8 meters [31].

The 10 Urodela families are assigned to three suborders, the Cryptobranchioidea, the Salamandroidea, and the Sirenoidea [29]. The suborder Cryptobranchioidea contains the primitive salamanders and includes, among others, the Chinese giant salamander *A. davidianus* and the hellbender *C. alleganiensis* [29]. The members of this group have retained several larval characteristics in their adult state such as gill slits and unidrid eyes, propagate by external fertilization, and breathe through lungs and the many folds in their thin skin [29]. The suborder Salamandroidea encompasses the evolutionary more advanced salamanders in, among others, the families Salamandridae (true salamanders as well as newts) and Plethodontidae (lungless salamanders which comprise about 70% of extant salamanders) [29]. The females of this suborder fertilize the eggs in their cloaca by spermatophores they have picked up after these have been deposited by males [29]. The suborder Sirenoidea includes eel-like aquatic salamanders with reduced forelimbs and no hind limbs which are classified into four species of sirens in a single family, the Sirenidae [29].

The development of salamanders is highly diverse, some species going through an elaborate metamorphosis while others, such as the axolotl *Ambystoma mexicanum* (Ambystomatidae) display pedomorphosis, retaining typical larval features such as external gills and a caudal fin extending from behind the head to the vent [29]. Due to their semiaquatic lifestyle as adults, alternating between aquatic and terrestrial habitats, newts in general do not undergo the dramatic developmental changes seen in other urodelans [29].

Urodelans have also various strategies to their disposal for their defense against threats or attack. For instance, the larvae of mole salamanders in the genus *Ambystoma* (Ambystomatidae) delay hatching in the presence of predatory flatworms, allowing them to grow to more advanced stages which are more difficult to ingest by the worms [165]. And various adult salamanders produce sounds, adopt threatening poses, bite, and/or autotomize their tail in order to startle, intimidate, ward off or distract attackers, allowing them to escape [166-168].

In addition, like anurans, various urodelans produce mucus and toxins from their skin glands as a defense against predation [169]. The fire salamander *S. salamandra* is even able to squirt a fine jet of toxic fluid from the granular glands on its spine at an attacker from a distance of 80 centimeters [170]. The skin poisons are complex

mixture of various ingredients including biogenic amines such as tryptamine and serotonin, proteins and peptides, steroids, most notably cholesterol, and unique steroidal alkaloids called salamander alkaloids or samandarines [169]. Some of these compounds are so poisonous that they can cause potentially lethal convulsions, even in larger animals and humans [171]. The skin toxins have also been used to taxonomically classify some urodelan taxa [172]. As seen in many toxic amphibian species, toxic salamanders such as the North American tiger salamander *Ambystoma tigrinum* (Ambystomatidae) and the fire salamander *S. salamandra* advertise their toxicity with striking aposematic skin coloration [170].

Bioactive compounds from urodela

The toxicity of Urodela, more specifically, salamanders, has been known since ancient times and has been subject of many fascinating myths and legends. The Roman author, naturalist, and natural philosopher Gaius Plinius Secundus, also known as Pliny the Elder (AD 23/24 - 79), has referred to salamanders as "the most wicked of all venomous animals" [173]. According to various tales, their skin secretions would poison the fruits of trees they had climbed in and would make the water of wells they had fallen into undrinkable, and their saliva would make the hair of human beings fall out [173]. Salamanders were also portrayed as products or even the origin of fire and, consequently, would be able to withstand heat and put out fires [173]. Indeed, it is with good reason that salamanders have extensively been used as weapons to attempt murder [173], as typical ingredients of magic potions of witches [173] and as a powerful symbol of alchemy [173]. This is illustrated in Shakespeare's Macbeth, in which the witches throw "the eyes of a newt and the toe of a frog" in their boiling cauldron [173].

On the other hand, salamanders also have long-standing medicinal uses. For example, a salamander crawling three times over a patient's clothing would treat fever or pain, a salamander carried by a patient in a special bag would treat wasting diseases, salamander skin placed on a woman's thigh would prevent pregnancy, and highly diluted salamander skin poison would treat epilepsy [169]. Even today, the Chinese giant salamander *A. davidianus* represents a luxury food item in China for nourishing the vital life force Qi, and is used in traditional Chinese medicine for treating, among others, neurasthenia, anemia, dysentery, and malaria [174]. Unfortunately, this has resulted in the decline of the wild population by more than 80% since the 1950s, making this giant a critically endangered species [175]. Furthermore, salamander brandy that is supposedly indigenous to Slovenia and is prepared from the toxic skin secretions of live salamanders and alcohol, would cause extraordinary hallucinations and extreme sexual arousal [176].

Since some of the secretions from the granular glands have notable pharmacological properties which should be useful against human diseases. A few of them are addressed hereunder.

Analgesic compounds

One of the most deadly non-proteinaceous substances known to man is the quinazoline alkaloid tetrodotoxin, a neurotoxin in, among others, the skin secretions from the rough-skinned newt *Taricha granulosa* (Salamandridae) (Figure 6) from North America and other members of its genus [177]. Tetrodotoxins have been named after the Tetraodontiformes, an order of fish that includes, among others, pufferfish, globefish, blowfish, porcupinefish, and triggerfish in which they had first been identified [178,179]. The toxin also occurs in blue-ringed octopuses in the genus *Hapalochlaena* (Octopodidae), and



Figure 6. The rough-skinned newt *Taricha granulosa* (Salamandridae) (<https://images.app.goo.gl/ZgWiZst7f39YdBhu6>)

moon snails in the gastropode family *Naticidae* [178,179]. However, tetrodotoxin is neither produced by *Taricha* newt species nor by the other animal species but by certain bacteria that they consume, are infected with, or live symbiotically with such as *Actinomyces*, *Aeromonas*, *Alteromonas*, *Bacillus*, *Pseudoalteromonas*, *Pseudomonas*, and *Vibrio* species [178,179]. Despite its toxicity, a few predators such as the North American common garter snake *Thamnophis sirtalis* (Colubridae) are able to successfully prey on *Taricha* species because of their (partial) resistance to tetrodotoxin [180].

Tetrodotoxin selectively binds to the extracellular pore openings of fast voltage-gated sodium channels in nerve cell membranes [181]. This temporarily disables the function of the ion channels, blocking the passage of sodium ions into neurons and inhibiting the generation of action potentials in neurons [181]. As a result, the transmission of signals to produce muscle contractions is perturbed, which leads to loss of sensation and paralysis of voluntary muscles including the diaphragm and intercostal muscles and eventually death due to respiratory failure [181]. So far, there is no antidote for tetrodotoxin intoxication, therapy being limited to gastric lavage and/or respiratory support or mechanical ventilation until the toxin is completely excreted [182].

Because of its ability to block voltage-gated sodium channel and to cause paralysis, tetrodotoxin can potentially be applied as a pain relieving agent. This was already known to the ancient Chinese, who first used the toxic eggs from pufferfish about 2800 BC to treat convulsive diseases [183]. Also, in Japanese traditional medicine, globefish was given in patients with leprosy in order to alleviate their neuralgia [183]. After the isolation and chemical characterization of tetrodotoxin from globefish ovaries in 1909 [183], this compound was used instead of parts of globe fish to suppress neuralgia associated with leprosy, and also to reduce muscle spasms due to tetanus and as an analgesic for patients with rheumatoid arthritis [183]. Tetrodotoxin has also been studied as a potential biological weapon on human subjects by Japan's infamous Unit 731, a covert biological and chemical warfare research and development unit of the Imperial Japanese Army that undertook lethal human experimentation during the Second Sino-Japanese War (1937-1945) of World War II [184].

The results on the potential efficacy of tetrodotoxin against neuropathic pain in cancer patients who do not respond to opioids and other analgesics were promising [179,185]. Although for

unknown reasons not all patients responded, tetrodotoxin, given either intramuscularly or subcutaneously, elicited meaningful analgesic effects which lasted up to two weeks while toxicity was relatively mild [186-188]. Other clinical studies have shown that tetrodotoxin may also help in reducing cue-induced increases in heroin craving and associated anxiety [189] and in alleviating acute heroin withdrawal syndrome [190] while causing relatively low side-effects [190].

Further clinical trials with tetrodotoxin for inadequately controlled pain related to cancer, against chemotherapy-induced peripheral neuropathy, to manage opiate withdrawal symptoms, and as a local anesthetic (see, for instance, [191]) have been suspended, since the results suggested that it did not meet the primary efficacy goal [192]. However, there are a number of good reasons to conduct further research on tetrodotoxin as a therapeutic agent: it does not cause myocardial depression like other local anesthetics [193]; hardly crosses the blood brain barrier, reducing the risk of seizures or central nervous system depression [194]; is not addictive like, for instance, opioids, which alter neurotransmitter levels [195]; and has long-lasting effects after being administered for a relatively short period of time [188].

Antimicrobial compounds

Apart from the quinazoline alkaloid tetrodotoxin, many members of the Salamandridae family (true salamanders and newts) produce steroidal alkaloids and secrete these compounds from their granular skin glands. Well-studied examples are the fire salamander *S. salamandra* (Figure 7) and the alpine salamander *Salamandra atra* in the skin secretions of which these compounds were first discovered [196-198]. The steroidal salamander alkaloids are called samandarines and have also been identified in other salamandrids such as such as the large alpine salamander *S. lanzai*, the bay Lycian salamander *Lyciasalamandra billae*, and the northern crested newt *Triturus cristatus* [199], and appeared to be the major compounds in their granular secretions [169]. In fact, samandarines are a unique class of steroidal alkaloids that are found nowhere else in nature but in the skin secretions of Salamandridae [197]. Furthermore, in contrast to the toxins of many other amphibians which are sequestered from their diet and symbiotic microorganisms [25,40], samandarines are biosynthesized by the salamanders themselves [196-198], probably from cholesterol as the starting material [198].



Figure 7. The fire salamander *Salamandra salamandra* (Salamandridae) (<https://images.app.goo.gl/jcMZ9dqgGpBwXy1k9>)

Samadarines are neurotoxic compounds that mainly affect the spinal cord, and poisoning - through transdermal exposure or oral ingestion - can cause increased excitability of the medulla and hyperactivation of motoric elements, initially leading to overexcitation of the muscles and restlessness, hypertension, rapid breathing, dilated pupils, and increased secretion of mucus and saliva, and later to convulsions, dyspnea, and even death by respiratory paralysis [169,199]. The main samandarines are samandarine, samandarone and O-acetylsamandarine [169,199]; samandarine seems to be the most neurotoxic one, samandarone the least toxic [36,197,198].

Apart from nerve-blocking and local anesthetic activities [169,199], the samandarines investigated today have shown antimicrobial activity. The crude cutaneous gland secretion of the near Eastern fire salamander *Salamandra maculosa* (Salamandridae) inhibited the growth of several gram-positive and -negative bacteria and fungi [200]. Furthermore, samandarine, samandarone, and samandaridine isolated from the skin gland secretions of this salamander species elicited a more or less specific bactericidal and/or fungicidal activity [201]. The toxins caused signs of lysis in the cytoplasm and the plasma membrane of the baker's yeast *Saccharomyces cerevisiae* (Saccharomycetaceae) [201]. Notably, from the individual compounds tested, samandarone was active against the broadest range of microorganisms and displayed the strongest antimicrobial activity [201]. In a more recent study, the partially purified skin secretions from the smooth newt *Lissotriton vulgaris* (Salamandridae) and the Balkan crested newt *Triturus ivanbureschi* (Salamandridae) also showed broad *in vitro* antimicrobial activity, including activity against antibiotic-resistant bacterial strains [202].

Unfortunately, the samandarines evaluated today were not better than most currently available antibiotics, showing activity at concentrations around 10 µM [201]. For this reason, there are currently no therapeutic uses for samandarins. However, it is conceivable that using modern structure-activity relationship techniques, combinatorial chemistry strategies, and high-throughput screening approaches, analogues can be developed with less toxicity and clinically more useful features.

Gymnophiona

Generalities about gymnophiona

The order Gymnophiona or Apoda comprises the caecilians, which are readily distinguished from the Anura and the Urodela by their elongated, cylindrical, legless and nearly tail-less body which make them look somewhat like snakes or worms. This resemblance is captured in the names of this amphibian order, 'gymnos' and 'ophis' meaning 'naked' and 'serpent', respectively, in ancient Greek, and 'a' and 'poda' standing for 'without' and 'legs', respectively. However, the oldest known caecilian, the extinct *Eocaecilia micropodia* dating from the early Jurassic period, roughly 200 million years ago [203], had short legs reminiscent of its tetrapod origin. The extant Gymnophiona consist of about 256 species in 56 genera and 10 families, comprising the smallest order of the amphibians [29]. The tiniest species is the Makumunu Assumbo caecilian *Idiocranium russeli* (Indotyphlidae) from Cameroon that reaches a length of at the most 9 centimeters [204]. The largest is Thompson's caecilian *Caecilia thompsoni* (Caeciliidae) that is endemic to Colombia and can become 1.5 meters long and can weigh 1 kilogram [205].

Gymnophiona have in general rudimentary eyes, and are mostly encountered in the tropical regions of Africa, Asia, as well as Central and South America, where they spend most of their life underground

in damp soil, among rotten wood, and under plant debris [29]. The main exceptions are the aquatic caecilians or rubber eels in the family Typhlonectidae, which inhabit semi-aquatic or aquatic environments in the northern parts of South America [29]. The ten families are distinguished from one another by the presence or absence of a tail, the degree of fusion of cranial bones, the degree of kineticism of the skull, the nature of the annular grooves, and the structure of the phallodeum, a penis-like appendage in males [29].

Gymnophiona are the only order of amphibians that exclusively uses internal insemination: the male inserts its phallodeum into the cloaca of the female for mating sessions that can last two to three hours [206]. About 75% of caecilians - either aquatic or terrestrial - are viviparous, giving birth to already-developed offspring [207,208]. Certain viviparous caecilians feed their offspring by maternal dermophagy, involving the mother producing a nutritious outer layer of its skin or its uterine for her young to feed on by scraping with their distinctive teeth, with multiple cusps [209]. The remaining 25% are oviparous and lay their eggs in burrows in mud that is close to water [208]. The eggs hatch into free-swimming larvae which have large, leaf-like gills that are resorbed at metamorphosis [208]. Oviparous caecilians can also lay their eggs terrestrially, which hatch into miniature versions of the adult form, skipping the free-swimming larval stage [208]. In fact, most caecilians do not undergo an anuran-like metamorphosis [210].

The skin of Gymnophiona has numerous ring-shaped folds or annuli that partially encircle the body, giving the animals a segmented appearance. In some species of caecilian, the skin bears calcite scales which are not seen in other Lissamphibia [211]. So far, only a limited number have been evaluated for their skin content, but currently available data suggest that most caecilians are toxic to predators [212]. Many toxic species have a purple-, green-, blue-, orange-, and/or yellow-colored skin (in contrast to the gray, brown, or black skin of most non-toxic species) which probably serves as an aposematic warning to predators [213].

Bioactive compounds from gymnophiona

Due to the reclusive lifestyle of Gymnophiona, studies on the bioactive compounds in their skin secretions have so far been limited to a few reports with relatively little focus on the pharmacological activities of the (ingredients in the) substances. For instance, the granular secretion of the common yellow-banded caecilian *Ichthyophis glutinosus* (Ichthyophiidae) from Sri Lanka reportedly contained toxic compounds which would repel potential predators [214], but this was not in agreement with a previous study [215] reporting that it is not venomous. The Cayenne caecilian *Typhlonectes compressicauda* (Typhlonectidae) that lives in the Amazon basin and river systems in the Guianas, also produces toxic mucus in slime glands all over its body which it readily secretes when attacked [216]. In a feeding experiment, this secretion was found to be strongly irritating to the human eye and lethal to the predatory wolf fish [216], but no further pharmacological and biochemical studies have been done on this substance.

More comprehensive studies have been carried out with the skin secretion of Boettger's caecilian *Siphonops paulensis* (Siphonopidae). This substance exerted hemolytic activity [217,218] and also caused indirect cardiotoxic activity in *in situ* toad heart preparations by increasing K⁺ concentration of the medium [217,219]. Furthermore, treatment of an artificial lipid bilayer with this substance led to the formation of voltage-dependent channels in the membrane [220]. However, the clinical significance of these findings is not clear at this moment.

Antiparasitic compounds

Interesting and potentially clinically applicable results on pharmacologically active compounds from Gymnophiona were obtained with the ringed caecilian *Siphonops annulatus* (Figure 8) (Siphonopidae). This creature occupies a broad diversity of ecosystems throughout South America [29]. The skin secretion of this animal elicited meaningful antiparasitic activity against cultured promastigotes of *Leishmania* (*Leishmania*) *infantum* (also known as *L. (L.) chagasi*) and trypomastigotes of *Trypanosoma cruzi* [221]. These activities could be localized to a hydrophobic fraction and were better than those caused by the standard drugs miltefosine and benznidazole, respectively [221]. However, the *S. annulatus* skin secretion was not active against amastigotes of *L. (L.) infantum* and displayed excessive cytotoxicity against normal peritoneal murine macrophages [221].

Nevertheless, this study may have merit, since *L. (L.) infantum* is an important causative agent of visceral leishmaniasis, a slow but progressive illness and the most severe form of leishmaniasis that is invariably fatal if left untreated [222]. More than 90% of cases (about 300,000 per year) occur in India, Bangladesh, Nepal, Sudan, and Brazil, and the worldwide mortality has been estimated at 20,000 to 40,000 [222]. *T. cruzi* is responsible for causing Chagas disease (also known as American trypanosomiasis), a tropical disease that is mostly spread by blood-sucking triatomine bugs tend (also called kissing bugs because they tend to bite people's faces [223]. An estimated 8 million people in Mexico, Central America, and South America have Chagas disease, most of whom do not know they are infected. However, if untreated, infection is lifelong and can be life-threatening, particularly because of cardiac complications which can include an enlarged heart, heart failure, altered heart rate or rhythm, and cardiac arrest [223].

The *S. annulatus* skin secretion had been called 'siphonopsina' [224]. Initial studies indicated that they contain serotonin and bufotenine [225]. So far, however, the role of these compounds in the antiparasitic activity of the secretions is not clear. There are also no clear indications about the chemical structure(s) of the actual pharmacologically active ingredient(s).

Conclusion

Secretions from their grandular skin glands comprise important defense of amphibians against attack by all kinds of microorganisms, parasites, and large predators. The pharmacological activities of their



Figure 8. The ringed caecilian *Siphonops annulatus* (Siphonopidae) (<https://images.app.goo.gl/EPAD9U4QNbDoq4ZD9>)

ingredients have been refined during 370 million years of evolution. Thus, these substances may represent extraordinarily rich sources of bioactive compounds for developing novel medications including medications against these diseases. This paper has extensively reviewed a few compounds from anurans, urodelans, and gymnophiones which may lead to the development of novel and improved medications for treating Alzheimer's disease, a failing heart, diabetes mellitus, HIV/AIDS, chronic pain, as well as microbial and parasitic infections. Notably, a relatively large proportion of amphibians (particularly the reclusive Gymnophiona) has not even been explored for pharmacologically active compounds, while new species are being discovered on a regular basis [29]. Thus, there is high probability that further exploration of this class of animals will help identify many lead compounds for developing novel therapeutics. That this approach may pay off, is illustrated by the alkaloid epibatidine from the skin of the phantasmal poison frog *Epipedobates tricolor* (Dendrobatidae), a species of poison dart frog that is endemic to Ecuador (South America) [226]. Epibatidine has led to the development of a novel class of non-opioid painkillers with a potency ranging of 100- to 200-fold that of the analgesic opioid morphine and 30 times that of nicotine while not being addictive [227]. Unfortunately, its very narrow therapeutic index has prohibited its use in humans [227]. This has led to the synthesis of a number of epibatine analogs that retain the superior analgesics effects but do not cause the toxicity (see, for instance, [228]), and hopefully may someday provide a clinically useful compound.

In the mean time, it should be kept in mind that many amphibians including *E. tricolor* obtain their toxins through their diet and do not produce them in captivity [25,40]. However, a relatively recent assessment suggests that at least 3,000 of the roughly 8,000 species of amphibian species throughout the world are threatened with extinction [229]. This poses an urgent need to implement proper *in situ* conservation strategies in order to protect these creatures before it is too late. At the same time, their glandular skin secretions should thoroughly be evaluated for the presence of effective, novel lead structures using advanced high-throughput screening techniques. When considering the experience with other more conventional sources of novel drugs such as plants and microorganisms, these approaches are virtually guaranteed to produce improved therapeutics with novel structures and unique mechanisms of action.

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