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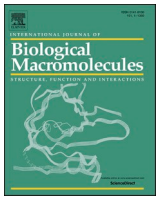

Review

Unlocking the potential of snake venom-based molecules against the malaria, Chagas disease, and leishmaniasis triad

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ABSTRACT

Malaria, leishmaniasis and Chagas disease are vector-borne protozoal infections with a disproportionately high impact on the most fragile societies in the world, and despite malaria-focused research gained momentum in the past two decades, both trypanosomiases and leishmaniases remain neglected tropical diseases. Affordable effective drugs remain the mainstay of tackling this burden, but toxicicty, inneficiency against later stage disease, and drug resistance issues are serious shortcomings. One strategy to overcome these hurdles is to get new therapeutics or inspiration in nature. Indeed, snake venoms have been recognized as valuable sources of biomacromolecules, like peptides and proteins, with antiprotozoal activity. This review highlights major snake venom components active against at least one of the three aforementioned diseases, which include phospholipases A2, metalloproteases, L-amino acid oxidases, lectins, and oligopeptides. The relevance of this repertoire of biomacromolecules and the bottlenecks in their clinical translation are discussed considering approaches that should increase the success rate in this arduous task. Overall, this review underlines how venom-derived biomacromolecules could lead to pioneering antiprotozoal treatments and how the drug landscape for neglected diseases may be revolutionized by a closer look at venoms. Further investigations on poorly studied venoms is needed and could add new therapeutics to the pipeline.

1. Introduction

The malaria, leishmaniasis and trypanosomiasis triad remains a terrible economic, healthcare, and human burden [1,2] causing significant mortality and life-long morbidity [3]. These parasitic diseases fall disproportionately on tropical and subtropical regions of the world, which are particularly affected by inequality, high rates of poverty, unplanned urbanization, lack of adequate health and education systems, and other socioeconomic liabilities [4]. According to latest data from the World Health Organization (WHO) [5,6] the global distribution of these vector-borne protozoan diseases is depicted in Fig. 1. These infectious diseases are rather complex to deal with, given their multifactorial nature and limited therapeutics. Indeed, the catalogue of commercially available drugs as first treatment regimens for these parasitic conditions is considerably far from ideal [7]. Drug-resistant parasite lines, efficacy loss, long treatment courses, and several adverse effects have been

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described for the already reduced chemotherapeutic arsenal frequently used [8]. Collectively, these barriers become an additional impetus to search for new candidates. Moreover, the inclusion of the elimination of these poverty-related diseases within the priority goals of the United Nations 2030 Agenda for Sustainable Development has spurred a revival of interest and investment in recent years, as well as the establishment of public-private partnerships [9].

The selection of new and appropriate chemical leads for parasitic infections has not often been motivated by pharmaceutical and market interests [8]. As a result, drug rescuing, repurposing, and repositioning have been a shorter and more beneficial path to finding compounds to tackle these non-priority conditions [10]. For example, Gomes and co-workers have put their effort into the rescuing of classical antimalarials, like chloroquine, primaquine, and quinacrine, through simple chemical modifications aimed at improving the therapeutic indices of these drugs [11–16]. The potential of some of these modified antimalarial drugs to be repurposed for leishmaniasis was also investigated [17,18]. Also, in recent years, several known drugs have been considered for repurposing to tackle leishmaniasis [19,20] as well as both African and American trypanosomiases [21–24]. Complementary to these approaches, biodiversity-driven drug discovery has been steadily explored by many medicinal chemists, biochemists and parasitologists, as a crucial part of antiparasitic drug development initiatives [25,26]. Among the many natural sources that have been and are being explored

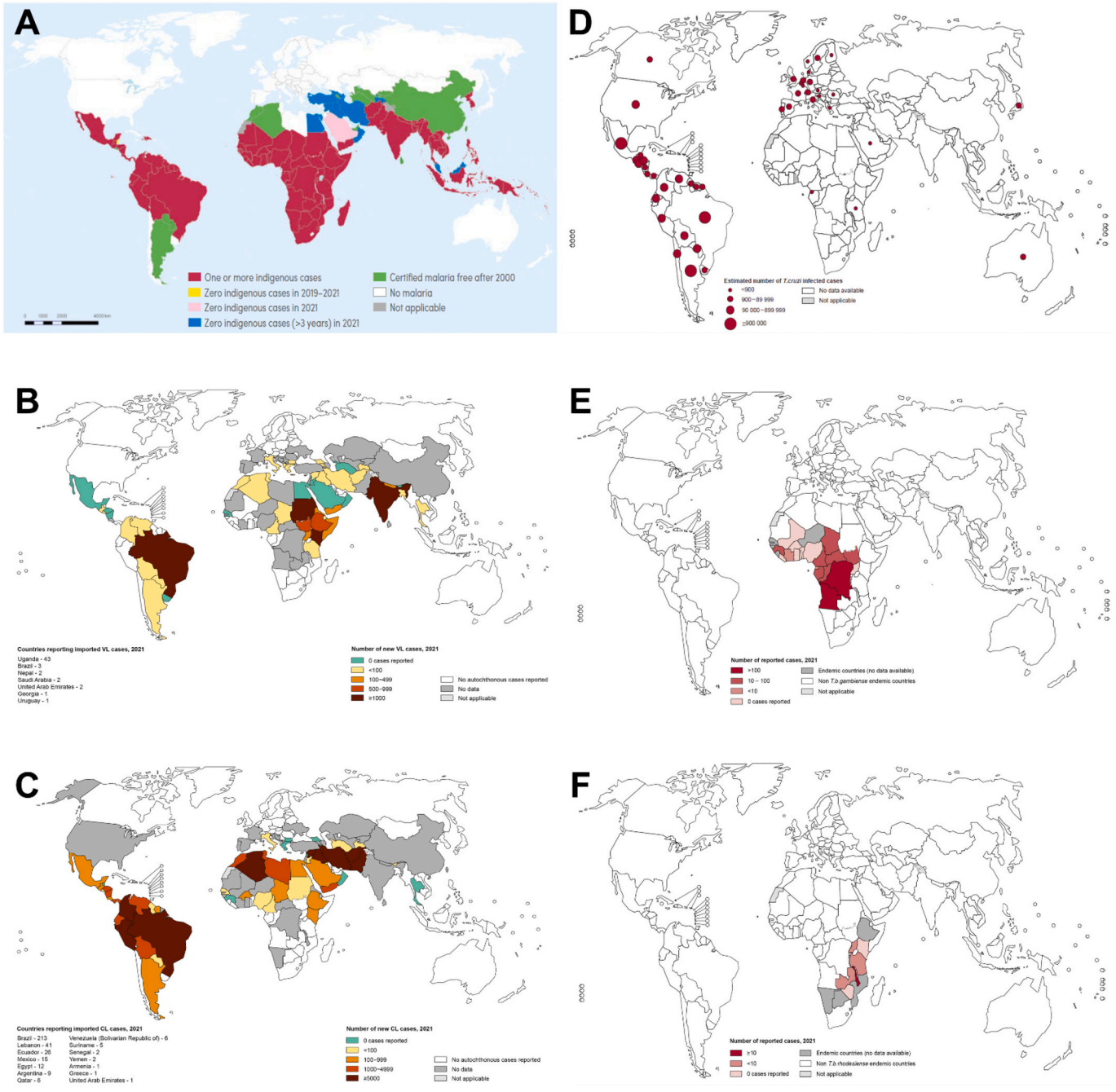

Fig. 1. Worldwide distribution of (A) malaria, (B) visceral leishmaniasis, (C) cutaneous leishmaniaisis, (D) American trypanosomiasis (Chagas disease), (E) Human African trypanosomiasis (sleeping sickness) caused by *Trypanosoma brucei ghambiense*, and (F) by *T. b. rhodesiense*. All maps were sourced from the World Health Organization and refer to 2021, with exception of (D) that refers to 2018 [5,6]. Blue-shaded background in (A) highlights the fact that malaria is the only of the vector-borne protozoal infections herein addressed that is no longer classified as a neglected tropical disease (NTD).

in this context, snake venoms have been playing a leading role in the discovery of clinically relevant anti-protozoal agents [27,28].

The notable history of snake venom-inspired therapeutics has positioned these biochemical secretions as promising drug discovery platforms [29]. Biomedically-focused research on venoms has offered a wide repertoire of biomolecules for programs targeting the identification of lead compounds for different diseases, including those caused by medically important parasites [30]. The Food and Drug Administration (FDA) approval of captopril, a snake venom-derived peptide, for clinical use as an anti-hypertensive agent opened a new era in the pharmacological application of venom-based molecules, which are traditionally stigmatized as mainly lethal and toxic due to the long-term health consequences or even fatalities due to snakebite envenoming [31]. Fortunately, the genomic, proteomic, structural, and functional analyses of snake venoms have shifted the classical paradigm putting in evidence the other side of the coin, marked by benefits to human health [32]. Venom-derived scaffolds have provided a tantalizing prospect of avant-garde therapies to drive the control of malaria [33], leishmaniasis [34] and trypanosomiasis [28,34]. Snake venoms therefore emerge as alternative and diversified platforms to find novel leads for antiparasitic drug discovery. Venomic studies by combined chromatography and mass spectrometry techniques have highlighted the significant inter- and intra-species variability in snake venom composition. This is modulated by factors like age, habitat, available preys, among others, and has important functional and toxicological consequences. Moreover, venoms may show different relative amounts of distinct toxin families, each of which comprising a myriad of protein isoforms differing in only a few amino acid residues, as unveiled by genomics and proteomics. These apparently minor structural differences have impact on physicochemical parameters and, consequently, on biological effects and potential applications. In other words, the range and potency of the biological action can vary substantially within the same protein family of a same venom. [35–37]

An increasing number of investigations have confirmed *in vitro* and *in vivo* antiprotozoal action of different families of snake venom-derived proteins and peptides [38,39]. However, as an underserved area that receives uneven attention, no purified snake venom molecule has been licensed for the treatment of these pathologies. Furthermore, many venom components remain poorly characterized. For example, in Ecuador, >85 % of venomic profiles remain unknown. This percentage increases if the complete primary structure and cytotoxic effect on parasites are considered. Therefore, a significant number of venoms remain in unique and underexplored libraries for future investigations and pharmaceutical advances. This offers fertile ground on which to thrive, especially because snake venoms are excellent research samples [40,41]. Several multidisciplinary questions can arise due to their richness and variability in terms of composition, structure, and function [42]. Refined by evolution, the levels of toxins' expression and diversity of isoforms vary in these mixtures, even within a species [43]. In other words, the venoms milked from individuals belonging to the same species can present different relative abundance of certain families of toxins [44]. Additionally, many distinct isoforms of the same protein family with specific kinetic parameters, primary structure, singular functional properties, and antiparasitic potencies can be found [45]. In line with this, the effects triggered by isolated venom components are also quite diverse [46]. A single toxin can induce more than one effect, while an isoform of to the same family can be specific or even inactive [47]. This variability has also been reported for anti-parasitic effects, meaning that toxin isoforms are a complex puzzle to be deciphered in further structure-function studies to better explore their biomedical potential. Gathered knowledge in this area provides important clues for the rational design and generation of more effective venom-derived anti-protozoal drugs [48].

2. Malaria

2.1. The complexity of the disease and the demand for new treatment options

Malaria puts a heavy toll on health and wellbeing, with mortality and morbidity rates of, respectively, 619 thousand and 247 million people worldwide, in 2021 [6]. This disease is caused by intracellular parasites belonging to the genus *Plasmodium*, of which five species can cause malaria in humans. Of these, *Plasmodium falciparum* poses the major public health problem. *P. falciparum* has a very complex life cycle involving two different hosts, humans and *Anopheles spp.* mosquitoes, and progressing through multiple developmental stages [49]. Thus, malaria infection is initiated by the bite of an infected female mosquito that injects 50–100 sporozoites into the skin of a human host [50,51]. Then, sporozoites enter the blood circulation and reach the liver, where they infect hepatocytes, each of which producing thousands of merozoites that are released into the bloodstream. Once there, merozoites invade red blood cells (RBC) initiating the intraerythrocytic developmental cycle (IDC) that causes disease symptoms [51,52]. During the 48-h IDC, intraerythrocytic *P. falciparum* parasites evolve into a ring stage, then trophozoites and finally schizonts, the latter filled with mature merozoites that are released into the bloodstream upon disruption of the RBC, to repeat the cycle. The transmission of the parasites to the mosquito vector depends on differentiation to sexual stages, the gametocytes [53,54]. Gametocytes that are taken up by a mosquito during an infected blood meal are converted into ookinetes in the mosquito midgut, to which they adhere to next enter the midgut epithelium and form oocysts. These develop into motile sporozoites that migrate to the mosquito's salivary glands, ready to be injected into a new human host when the mosquito takes another blood meal, completing the malaria life cycle [55].

The complexity of the life cycle of *Plasmodium* parasites in a way explains why this disease has not been eradicated even after the many efforts done over the past centuries: thus far, there are no truly effective vaccines against malaria [56]. This means that most antimalarial strategies rely on the use of drugs, several of which were developed many decades ago and pose a few challenges mainly due to parasite drug resistance [6]. Currently, the WHO recommends artemisinin-based combination therapies (ACT) as first line antimalarial therapy which, although useful, underlies increasing parasite resistance to artemisinin and related antimalarials, therefore exhausting available options to tackle chloroquine-resistant malaria [57,58]. Thus, it is urgent to disclose novel efficient and nontoxic compounds capable of interfering with the growth and transmission of malaria parasites, hampering the spread of this millenary threat to human health.

2.2. Antimalarial potential of snake venoms – the past two decades in a glimpse

Snake venoms from sixteen species have been studied regarding their antiplasmodial activity, which has been correlated to a number of different effects mainly on blood-stage parasites (Fig. 2). These snakes belong to seven different genera of the *Elapidae* and *Viperidae* families [43,59]. Snake venoms toxins include enzymes such as phospholipases A_2 (svPLA$_2$s), oxidases, zinc-dependent snake venom metalloproteinases (svMPs) and other proteases, as well as non-enzymatic proteins like disintegrin or neurotoxins, and diverse peptides [59,60]. Antimalarial activity has been investigated on whole venom and/or derived fractions (Table 1), in the latter case mostly on svPLA$_2$s. This is not surprising, as svPLA$_2$s are ubiquitously found in *Elapidae* and *Viperidae* snakes [61,62] and have already provided leads for the development of new therapies for other diseases [63]. svPLA$_2$s are usually divided into Group I (GI-PLA$_2$) and Group II (GII-PLA$_2$), the first one typically found in *Elapidae* snakes, whereas the second group is exclusive of the *Viperidae* family [47,62,64–66]. Though to a lesser extent, svMPs have also been

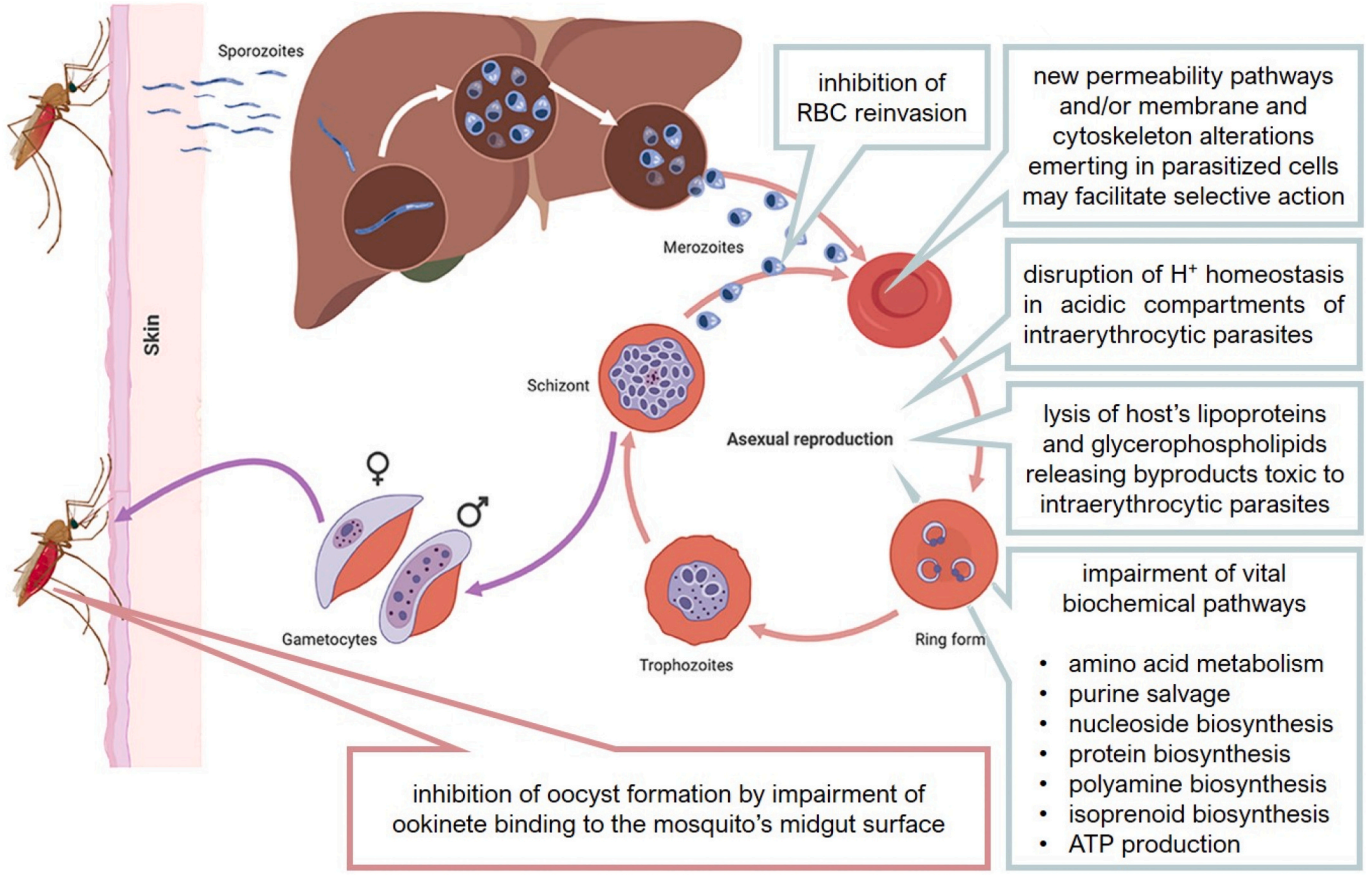

Fig. 2. Reported effects underlying the antiplasmodial action of snake venoms and derived proteins and peptides (adapted from [69] with permission).

investigated for their antimalarial activity. svMPs are a complex family of zinc-dependent endoproteases that, although more abundant in the venoms of *Viperidae* snakes [67], are also found in the *Elapidae* family [68].

2.2.1. *Venoms from Elapidae spp. snakes*

Whole venoms and/or isolated fractions from different *Elapidae* species, namely, *Naja mossambica*, *Naja naja oxiana*, *Notechis scutatus scutatus*, and *Micrurus spixii*, were found to inhibit the *in vitro* growth of *P. falciparum* intraerythrocytic stages, in some cases at nanomolar concentrations, as shown in Table 1 [67,70–73]. Stage-specific action of *N. mossambica* and *N. scutatus scutatus* venoms was reported, as trophozoites 2.5 mg/kg were found to be more sensitive to their action than schizonts; *e.g.*, though the venom from *N. mossambica* was only a moderate inhibitor of erythrocyte reinvasion during the schizont stage, it showed a > 90 % inhibition of reinvasion in the trophozoite stages [70].

The antimalarial properties of the venom from the Iranian *N. naja oxiana* were also investigated in a set of studies by Hajialiani et al.; these authors reported nanomolar inhibition of intraerythrocytic development of *P. falciparum* for a not specified "fraction 4". Interestingly, despite the mode of action of this fraction was not elucidated, the metabolites found in ring stage parasites when under venom challenge suggest that amino acid metabolism and the pathways for protein/isoprenoid biosynthesis are affected. Moreover, the *N. naja oxiana* venom could significantly decrease parasitemia in *P. berghei*-infected mice, which could tolerate 2.5 mg/kg for 10 days without any noticeable adverse effects; however, the curative dose (5 mg/kg) was found to induce chills, diarrhea, and weight loss, which might be correlated with the fact that all but one of the fractions of this venom were hemolytic [71–73]. In contrast, Terra et al. reported a highly selective antimalarial action for the venom of *M. spixii* snakes, which displayed sub-micromolar activity *in vitro* against *P. falciparum* alongside very low toxicity to human hepatoma cells (HepG2), resulting in a selectivity index (SI) above 250 [74].

Recently, Fang et al. reported a cathelicidin-derived peptide from *Bungarus fasciatus* snakes, LZ1, able to display strong antiplasmodial activity *in vitro* (IC_{50} value of 3.045 μM against blood-stage *P. falciparum*) that was translated into a significant antimalarial activity *in vivo* on *P. berghei*-infected mice. Peptide LZ1 was further able to attenuate infection-induced liver inflammation and dysfunction, while apparently owing its antimalarial action to impairment of adenosine triphosphate (ATP) production in parasite-infected erythrocytes by selective inhibition of pyruvate kinase [75].

2.2.2. *Venoms from Viperidae spp. snakes*

Most reports on the antimalarial properties of snake venoms and their components corresponding to the *Viperidae* family, focus on GII-PLA_2. A wide range of *in vitro* activities against *P. falciparum* was found *in vitro*, from picomolar levels to no activity observed up to the micromolar range (Table 1). For example, GII-PLA_2 fractions isolated from *Vipera ammodytes* (ammodytoxin A) and from *Agkistrodon halys* were able to inhibit *P. falciparum* intraerythrocytic stages at nanomolar concentrations and induce selective lysis of parasitized erythrocytes [70,76]. In turn, venoms from *Bothrops atrox* and *B. jararacussu* displayed poor antimalarial activity and moderate toxicity towards mammalian cell lines [77–79].

2.2.2.1. Bothrops spp. snake venoms. Venoms from *Bothrops spp.* snakes, and purified fractions thereof, have been the focus of many studies in search for antimalarial activity. Bothropic venoms consist of a mixture of both non-catalytic proteins, such as Type C lectins, disintegrins and

Table 1
Venoms and/or venom components investigated for antimalarial activity.

Snake species (family)	Venom /venom component	Antimalarial activity (IC_{50})[a]	Specific effects	Toxicity data	Reference
Bothrops asper (*Viperidae*)	Crude venom Fraction V (GII-PLA_2) Fraction V (PLA_2-like)	0.13 μg/mL 1.42 μg/mL 22.89 μg/mL	Not specified	CC_{50} *in vitro* on PBMC: venom, 38.46 μg/mL fraction V, 26.98 μg/mL fractionVI, 67.43 μg/mL *In vivo* toxicity (mice): venom, 2561–3693 μg/kg fraction V > 15,000 μg/kg fraction VI > 15,000 μg/kg	[80]
	BaspB-II (GII-PLA_2) BaspB-IV(GII-PLA_2)	2.460 μM 0.019 μM	Not specified	not specified	[81]
Bothrops atrox (*Viperidae*)	Bax3k fraction Synthetic Bax peptides	not active at 100–1.56 μg/mL	Not specified	Not hemolytic against human erythrocytes at 250–0.49 μg/mL	[79]
Bothrops diporus (*Viperidae*)	BdTX-I BdTX-II BdTX-III	2.44 μg/mL 15.3 ng/mL 0.59 μg/mL	Not specified	CC_{50} (HepG2) > 100 μg/mL CC_{50} (HepG2) > 10 μg/mL CC_{50} (HepG2) > 100 μg/mL	[82]
Bothrops brazili (*Viperidae*)	Venom Fraction BbMP-1 (SVMP)	0.17 μg/mL 3.2 μg/mL	Not specified	MDL_{50} (HepG2), 31.5 μg/mL CC_{50} (HepG2) > 200 μg/mL	[84]
Bothrops jararacussu (*Viperidae*)	Bj3k fraction Bj-derived synthetic peptides	not active at 100–1.56 μg/mL	Not specified	1.1–1.8 % hemolysis at 250 μg/mL for Bj3k and Bj-derived synthetic peptides	[79]
Bothrops marajoensis (*Viperidae*)	Crude venom Bmaj (GII-PLA_2)	0.14 μg/mL 6.41 μg/mL	Not specified	CC_{50} (HepG2), 43.64 μg/mL CC_{50} (HepG2) > 150 μg/mL	[92]
Bothrops moojeni (*Viperidae*)	BmooMPα-I (SVMP)	16.14 μg/mL	Interaction with PfPNP	Non-hemolytic in the range of concentrations tested	[86]
Vipera ammodytes (*Viperidae*)	Ammodytoxin A (GII-PLA_2)	2.8 nM (~39 ng/mL)	Inhibition of erythrocyte reinvasion by 80 % in schizonts and > 90 % in trophozoites	Not specified	[70]
Agkistrodon halys (*Viperidae*)	GII-PLA_2	82.3 pM (1152 pg/mL)	inhibition of erythrocyte reinvasion by >80 % in schizonts and > 90 % in trophozoites	Not specified	
Crotalus adamanteus (*Viperidae*)	GII-PLA_2	Not determined	inhibition of ookinete binding to midgut and oocyst formation	Reported as having low toxicity to MCF-7 cells	[33,126]
Crotalus durissus cumanensis (*Viperidae*)	Crude venom Crotoxin B (GII-PLA_2)	0.17 μg/mL 0.6 μg/mL	Not specified	CC_{50} (PBMC), 38.59 μg/mL CC_{50} (PBMC), 18.23 μg/mL	[102]
Crotalus durissus terrificus (*Viperidae*)	Crotamine	1.87 μM	Not specified	Toxicity to human tumor lines in the μg/mL range, reported separately [127]	[96,97]
Naja mossambica (*Elapidae*)	GI-PLA_2	2.3 pM (3.2 pg/mL)	Inhibition of erythrocyte reinvasion by 50 % in schizonts and > 90 % in Trophozoites	Toxic to MCF-7 at 4.5 U/mL	[70,126]
Naja naja oxiana (*Elapidae*)	not specified venom fractions	0.026 μg/mL (ED_{50} 2.5 mg/kg on *P. berghei*-infected mice)	Interference with the Krebs cycle and metabolism pathways of nicotinamide and pyruvate	Not toxic to human fibroblasts *in vitro*; *in vivo* toxicity (mice): < 5 mg/kg	[71–73]
Notechis scutatus scutatus (*Elapidae*)	Notexin (GI-PLA_2)	2.6 nM (36 pg/mL)	Inhibition of erythrocyte reinvasion by 750 % in schizonts and > 80 % in trophozoites	Not specified	[70]
Micrurus spixii (*Elapidae*)	Crude venom	≤ 0.78 μg/mL	Not specified	MDL_{50} (HepG2) > 200 μg/mL	[74]
Bungarus fasciatus (*Elapidae*)	Peptide LZ1 (cathelicidin-derived)	3.045 μM (lowered parasitemia growth rate and prolonged survival in *P. berghei*-infected mice)	Regulates cytokine production and improves liver function during infection Impairs ATP production by inhibiting pyruvate kinase	Negligible hemolytic action	[75]

[a] Except otherwise indicated; ATP, adenosine triphosphate; CC_{50}, concentration leading to 50 % inhibition of cell proliferation; ED_{50}, 50 % effective dose; HepG2, human hepatoma cell line; IC_{50}, concentration leading to 50 % inhibition of parasite growth *in vitro* (against *Plasmodium falciparum*, unless otherwise stated); K562-R/S, drug-resistant/sensitive human chronic myeloid leukemia cell lines; MCF-7, human breast cancer cell line; MDL_{50}, minimal lethal dose for 50 % of the cells; PfPNP, *P. falciparum* purine nucleoside phosphorylase; PLA, phospholipase A; SK-BR-3, human breast cancer cell line; SVMP, snake venom metalloproteinase; PBMCs, human peripheral blood mononuclear cells.

cysteine-rich secreted proteins (CRISP), and enzymes like GII-PLA_2 and MMP, [79] the last two being the most studied and promising for their antimalarial activity.

Fractions from the *B. asper* venom containing catalytically active GII-PLA_2s displayed higher antimalarial activity than other fractions that, despite being analogous to PLA_2s, were devoid of enzymatic activity (Table 1) [80,81]. While this seems to suggest that enzymatic activity is important for the inhibition of the intraerythrocytic growth of *P. falciparum*, non-enzymatic venom fractions from *B. diporus* have shown antimalarial activity *in vitro* in the nanomolar range (fractions BdTX-III; IC_{50} = 590 ng/mL) with no significant hemolytic effects and a SI higher than those of other fractions (*e.g.*, BdTX-I and BdTX-II; Table 1) [82]. Still, one must consider that the lack of selectivity *in vitro* not always correlates with *in vivo* toxicity; in fact, despite fractions V and VI

from the venom of *B. asper* had been found toxic *in vitro* against peripheral blood mononuclear cells (PBMC), with SI of 19 and 3, respectively, they had low acute toxicity on mice [80].

As previously mentioned, svMPs are amid the most abundant constituents of venom proteins [67,68,83] and among the most studied venom proteins regarding diverse biological properties, including antimalarial. Two botropic venom fractions identified as MMPs, BbMP-1 from *B. brazili* and BmooMPα-I from *B. moojeni*, showed moderate activity (Table 1) against *P. falciparum in vitro* and low toxicity to human cell lines [84–86]. Both fractions showed fibrinogen and fibrinogenolytic activity (α-fibrinogenase) and were weakly hemorrhagic [84,85], a characteristic shared with other bothropic venoms [87–91]. Interestingly, the whole venom from *B. marajoensis* was much less toxic to HepG2 cells (SI > 312) than its Bmaj (GII-PLA_2) fraction (SI = 23) [92]. The same trend was observed when comparing the whole venom of *B. brazili* with its fraction BbMP-1, but the SI of this fraction was 3 times higher than that of BmooMPα-I [84,85]. This latter fraction was further studied *in silico* by Martins et al. [86], to investigate whether its antimalarial action was due to inhibition of the *P. falciparum* purine nucleoside phosphorylase (PfPNP) enzyme. PfPNP is related to the purine salvage pathway and polyamine biosynthesis, necessary to produce nucleosides and nucleic acids, and therefore essential for parasite survival [93]. Molecular docking simulations of the interactions between BmooMPα-I and PfPNP led to identification of one peptide (Pep1BM) as having potential binding affinity to the catalytic site of PfPNP, and to be capable of inhibiting its catalytic activity [86].

2.2.2.2. Crotalus spp. snake venoms. Among the best-known bioactive snake toxins are crotamine and crotoxin, present in the venoms of *Crotalus spp.* snakes. Crotamine is a small myotoxic polypeptide and crotoxin is a neurotoxic svPLA$_2$ [94], but both possess PLA_2 activity and possess diverse pharmacological properties [94,95], including antimalarial action *in vitro* [96,97].

Crotoxin is a major component of several *Crotalus* snakes that was originally isolated from *Crotalus durissus terrificus* [98]. It is a PLA_2 generally found as a heterodimeric complex with two subunits: CA, also known as crotapotin, and CB, or crotoxin B [98,99]. The subunit CA is not toxic, but the subunit CB generally has PLA_2 activity and is toxic to mammals [100], *Leishmania* [101], and *P. falciparum* [33,102]. Quintana and co-workers reported that both the CB subunit and the whole venom from *C. durissus cumanensis* had sub-micromolar activity against intraerythrocytic stages of *P. falciparum*. They further found that, despite having no significant toxicity *in vitro* against PBMC and a remarkable SI of 227, the whole venom was highly neurotoxic to mice; in contrast, CB presented moderate cytotoxicity against PBMC with an SI of 30 but showed no neurotoxic effects on mice (Table 1) [102].

Besides crotoxin, the venom of *C. durissus terrificus* also contains crotamine [103,104] and the latter has also been investigated regarding its antimalarial potential [96,97]. Crotamine is a small cationic polypeptide that, like crotoxin, was also firstly isolated from *C. durissus terrificus* [105]. Antimicrobial and/or antitumoral activities of crotamine were shown to be mostly dependent on the ability to target acidic cellular compartments (as lysosome vesicles) and to form complexes with nucleic acids [106–108]. Crotamine is moderately active (IC_{50} = 1.87 μM; Table 1) against the development of *P. falciparum* and specifically targets Plasmodium-infected RBCs where its internalization efficacy is stage-specific (higher in trophozoites) and energy-dependent (glycolysis) [96,97]. Given its cell-penetrating peptide (CPP) properties and ability to form self-assembled nanomaterials with a variety of other molecules, it been proposed for development of nanocarriers for selective delivery of drugs or nucleic acids into actively proliferating cells, such as in tumors, worms or *Plasmodia* [107–109].

2.2.3. The endless search for multi-stage selective antimalarial action

Owing to the complexity of the life cycle of *Plasmodium* and to its ability to rapidly acquire resistance to antimalarial drugs, new drugs should have multi-stage activity to reduce the chance that viable resistant strains are developed. In line with this, studies on the antimalarial properties of snake venoms and their components against exoerythrocytic parasite forms, *e.g.*, liver and mosquito parasite stages, should be actively pursued. Yet, to the best of our knowledge, only one study from 2001, by Zieler and colleagues, addresses this issue; these authors studied the effects of venoms, and venom fractions, from *C. adamanteus*, *C. durissus terrificus*, *N. mossambica mossambica* and *N. naja* against the mosquito stages of *P. falciparum*. They could demonstrate that the PLA_2 fractions in these venoms inhibited the binding of ookinetes to the midgut surface and, consequently, oocyst formation. The PLA_2 fractions from the *Viperidae* snakes showed stronger inhibition, particularly *C. durissus terrificus*, than those from the snakes of the *Elapidae* family [33]. This agrees with reports that PLA_2 accumulated in the epithelial cells of the mosquito's midgut before a blood meal are released into the lumen upon an infective blood meal, disrupting malaria parasite development in the insect vector [110].

In addition to multi-stage activity, selectivity is highly desired when developing new antimalarial (and other therapeutic) agents, derived or not from snake venoms. Selective action against malaria-infected erythrocytes was observed for venoms and venom fractions from snake species belonging to either the *Elapidae* (*e.g.*, notexin from *N. scutatus scutatus*) or the *Viperidae* (*e.g.*, crotamine from *Crotalus durissus terrificus*) families [70,96]. It has been hypothesized that this selectivity could be due to the structural and functional modifications of the erythrocyte cytoskeleton and membrane, such as cytoskeleton remodeling, higher fluidity of the membrane, membrane localization of new proteins, new permeability pathways, and altered lipid packing, which occur upon infection by *Plasmodium spp.* [111–113]. It is known that svPLA$_2$s promote erythrocyte membrane perturbation (such as lipid peroxidation, osmotic fragility, or membrane phospholipid hydrolysis [114,115], which in turn can increase the permeability of an already substantially altered membrane, due to the infection [80]. Although this mechanism seems plausible, the toxic lipid byproducts resulting from the hydrolysis of human lipoproteins by PLAs were also proposed as a mechanism of PLA$_2$s' antimalarial activity *in vitro*, rather than disruption of erythrocytic cell membrane [70,116,117]; this proposal is supported by the results of *in vitro* inhibition assays using semi-defined culture medium with and without human lipoproteins and the fact that the level of human PLA$_2$s is increased in the plasma of malaria patients [118–120]. Another hypothesis is that some PLA$_2$s have selective antimalarial action due to their ability to hydrolyze glycerophospholipids to produce free fatty acids [92], which are known to inhibit *Plasmodium spp.* growth *in vitro* and *in vivo* [116,117,121,122]. Notwithstanding, wider and deeper studies are needed to have a clearer insight into how and why some snake venoms and venom components have selective antimalarial action.

2.3. Tackling current limitations

It is now established that snake venoms and venom components exist which can: (i) inhibit the *in vitro* proliferation of *P. falciparum* inside the erythrocytes; (ii) hamper erythrocyte reinvasion by the parasites; (iii) promote selective lysis of *P. falciparum*-infected erythrocytes; and (iv) impede development of mosquito stages, namely ookinetes and oocysts. Yet, these findings have not been thus far translated into a precise definition of the targets and mechanisms of action of antimalarial snake toxins and derived components enabling a faster advancement towards promising drug candidates. Desirably, an antimalarial lead should have an IC_{50} in the low nanomolar level and a SI above 100 [7,123]. Based on these criteria and on data compiled in Table 1, few of the tested snake venoms, or fractions and molecules thereof, seem promising. However, distinct experimental methods were used in the different reports, therefore it is unreasonable to make direct comparisons of antimalarial activity data obtained from those studies. This is another critical issue

that needs to be addressed in the future; for instance, it was found that a mere alteration on the composition of culture media, *e.g.*, replacement of human plasma (containing human PLA_2s) by AlbumaxII, could drastically decrease antimalarial activity from nM range to negligible [79]. Therefore, more than engaging into an endless search for the next potent antimalarial venom or venom component, clear universal guidelines should be first established regarding the antimalarial activity screening of this type of substances. Moreover, antimalarial activity studies should not be exclusively focused on the intraerythrocytic stage of parasite development. Despite this is the illness-causing stage, the preceding asymptomatic liver stage infection is the path through which thousands of merozoites ready to invade erythrocytes are produced per each sporozoite injected by the mosquito vector [124]. Eliminating liver forms means impeding progression to the symptomatic blood stage of infection, which has a double benefit: alleviating the patient from the prostrating symptoms of malaria, and avoiding blood-stage parasites to evolve and, eventually, differentiate into gametocytes. These are the only forms infective to the mosquito, which means that eliminating gametocytes blocks transmission and spread of the disease. For this reason, activity against both gametocytes and mosquito stages should be routinely investigated when searching for new antimalarial leads [125]. Yet, as already mentioned in the previous section, studies addressing the activity of snake venoms against a malaria developmental stage other than the intraerythrocytic one are scarce. Overall, there is still a long path to follow, but available data are sound enough to make the journey worthwhile.

3. Chagas disease

3.1. The huge yet neglected burden of Trypanosoma cruzi

Chagas disease (CD) or American trypanosomiasis is one of the two types of parasitic diseases caused by protozoans of the genus *Trypanosoma* that affects humans, the other being the human African trypanosomiasis or sleeping sickness. The incidence of the latter, which is caused by either *Trypanosoma brucei gambiense* or *T. brucei rhodesiense*, has been decreasing significantly for the past two decades to <1000 reported cases in 2021 [128,129]. This is mainly due to a continued eradication effort in Africa alongside a portfolio of available medications and treatment regimens tailored for the specific subtype and stage of the disease. In clear contrast, CD affects 6 to 7 million people worldwide with 12,000 deaths per year, mainly in South America, and 70 million people being at risk [130,131]. This is a huge burden that has been largely neglected, but as more people move to places like North America, Europe, and Japan, CD is growing into a worldwide public health problem [132].

CD is caused by *T. cruzi* that, in its life cycle, alternates between the human host and the insect vector, the triatomine bug (*Triatoma spp.*) when humans come into contact with faeces and/or urine of infected triatomines. Transmission can also occur through blood transfusion or organ transplantation, congenital, and by ingestion of contaminated food or liquids [129–131,133].

T. cruzi parasites are mostly transferred from hematophagous triatomine bugs at the biting site to humans as metacyclic trypomastigote forms (non-dividing). Either the wound or a nearby mucosa is the entry point. Once inside cells, infectious trypomastigotes invade and develop into intracellular amastigotes. These amastigotes then multiply by binary fission to differentiate into trypomastigotes, which are then discharged into the circulation to infect further cells or to be consumed by another vector. In the midgut of the vector, the blood trypomastigotes ingested change into epimastigotes, proliferate, and subsequently differentiate into infectious metacyclic trypomastigotes, ready to be transferred into another host.

CD control relies solely on chemotherapy, as there are no human vaccines available. Targets for pharmacological intervention in the clinically important life-cycle stages of the parasite include the infective trypomastigotes and intracellular replicative amastigotes. Current treatment for CD is limited to nifurtimox and benznidazole, the only available drugs for decades. These drugs are far from the WHO standard, due to their high toxicity and limited efficacy, mainly for late-stage chronic disease, but also to the growing concern of parasites' resistance [135].

3.2. Antichagasic potential of snake venoms – a three decades' quest

The venoms and their purified components that have been most explored as antichagasic agents belong to the viperid snakes from the genus *Bothrops*, present in Central and South America [28,136,137]. The first study exploring the potential of snake venoms against *T. cruzi* parasites was performed by Fernandez-Gomez et al., in 1994; they showed that crude venom from *Cerastes cerastes* and *Naja haje* present a strong inhibitory effect (> 90 % at 100 μg/mL) that was lost upon heating, suggesting that the active factors were thermolabile [138]. Since then, many other snake venoms from different species, or purified proteins and peptides thereof, were evaluated for their trypanocidal action, mostly associated to stage-specific apoptosis- or necrosis-like effects (Fig. 3), as compiled in Table 2. Many of these studies also included an evaluation of anti-leishmanial activity, which is separately presented in Section 4.

3.2.1. Phospholipases under the spotlight again

As already mentioned, svPLA$_2$s have potent effects against many pathogens namely in *Plasmodium spp.* But also in *T. cruzi*, which agrees with the fact that these enzymes, both their Asp49 and Lys49 isoforms, are among the most promising and best researched antimicrobial components of snake venom [41,139]. For instance, in their 2017 investigation of anti-protozoal components in the venom from *B. marajoensis*, Grabner et al. isolated BmajPLA$_2$-II, a basic Lys49 svPLA$_2$ active against *T. cruzi* epimastigotes; this protein reached 61 % growth inhibition (at 100 μg/mL) while showing no significant toxicity to HepG2 human hepatic cells (CC_{50} > 150 μg/mL). Despite having stronger antiprotozoal action than BmajPLA$_2$-II, the crude venom was more toxic to host cells, eventually due to additive or synergistic interactions with other venom components [92]. Another basic Lys49 svPLA$_2$ homologue, the BmatTX-IV fraction of the venom from a Paraguayan specimen of *B. mattogrossensis*, was also found active against *T. cruzi* epimastigotes (IC_{50} = 13.8 μg/mL), and less cytotoxic effect in murine fibroblasts with an IC_{50} of 81.2 μg/mL [140].

Acidic Asp49 svPLA$_2$s have also been investigated for their antichagasic potential. For instance, PLA$_2$ isoforms were isolated from the venom of *B. asper*, resulting in four acidic PLA$_2$s which presented activity against *T. cruzi* epimastigotes [81]. Other acidic PLA$_2$s isolated from the venom of *B. brazili* have also presented trypanocidal effect; examples are Braziliase-I and Braziliase II, which presented 31.5 % and 33.2 % trypanocidal activity (at 100 μg/mL), respectively, against *T. cruzi* epimastigotes [141]. Nevertheless, as observed in other similar studies, the crude venom was much more active than the purified protein fractions (91.2 % trypanocidal activity at 100 μg/mL). These Braziliases showed no myotoxicity in murine mice plasma serum. Multiple sequence alignments showed high similarity of Braziliase-I and II with other acidic PLA$_2$s such as BmooPLA$_2$ from *B. moojeni* and PLA$_2$ from *B. diporus*.

3.2.2. Venoms and venom components as apoptosis/necrosis inducers in T. cruzi

There is evidence that snake venoms are responsible for the induction of programmed cell death in *T. cruzi*. In 2005, Deolindo et al. used the benzenidazol-resistant Y strain of *T. cruzi* to show that apoptosis was triggered on epimastigotes after exposure to the venom of *B. jararaca*, as a result of cell stress, with alteration of mitochondrial membrane permeability, caspase activation, phosphatidylserine exposure, nuclear and cytoplasmic condensation, and DNA fragmentation [142]. Later,

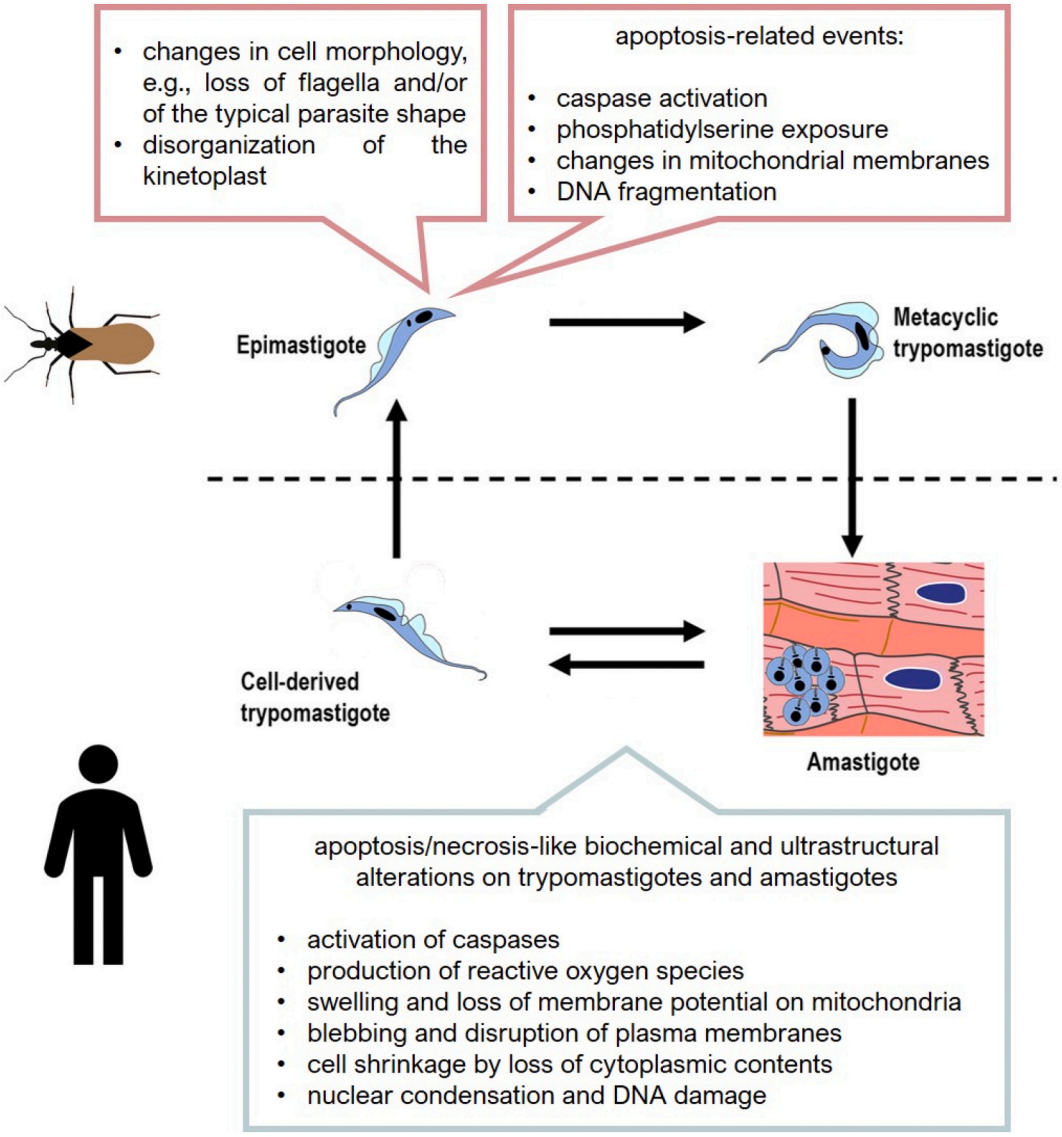

Fig. 3. Reported effects underlying the antichagasic action of snake venoms and derived proteins and peptides (adapted from [134] with permission).

this group was able to demonstrate that the antiprotozoal activity observed for different fractions of the venom from *B. jararaca* (fractions FI and FII) correlated with their respective L-amino acid oxidase (LAAO) activity, implying that H_2O_2 produced *via* the action of this enzyme was likely responsible for parasite killing action [143]. In line with this, the acidic enzymes and BatroxLAAO, isolated from the venoms of *B. jararacussu* and *B. atrox*, respectively, exerted a strong trypanocidal effect with IC_{50} of 4.85 μg/mL (BjussuLAAO-II) [144] and EC_{50} of 62.8 mg/mL (BatroxLAAO) [145], in the latter case against the benzenidazol-resistant Y strain. These findings agree with the fact that LAOO are flavoenzymes that catalyse the oxidative deamination of L-amino acids producing H_2O_2, which is a known inducer of programmed cell death (PCD) in metazoans [146–148]. Alongside their proven antibacterial and antiparasitic properties, LAAO show cytotoxic and apoptosis-inducing effects in different cell lines through activation of caspases, loss of mitochondrial membrane potential, and DNA damage [149,150]. Similar ultrastructural changes, such as swelling of mitochondria, blebbing and disruption of the plasma membrane and loss of cytoplasm components, were observed on all forms of *T. cruzi* when treated with the crude venom from the *C. viridis viridis* rattlesnake [151]. Yet, these effects were ascribed to a venom component other than a LAAO, as the same group later isolated crovirin, a CRISP from the *C. viridis viridis* venom with low toxicity to host cells, but significant activity against key infective stages of *T. cruzi*, namely, trypomastigotes ($IC_{50} = 1.10$ μg/mL; SI = 18.2) and amastigotes ($IC_{50} = 1.64$ μg/mL; SI = 12.2) [34]. Actually, apoptosis/necrosis-like biochemical and ultrastructural alterations, such as the production of ROS, alteration of mitochondrial transmembrane potential and damage of cellular membrane have been also reported for venom-derived antimicrobial peptides like, *e.g.*, Batroxicidin (BatxC), a cathelicidin from *B. atrox*; BatxC proved to have strong and selective activity against *T. cruzi* trypomastigotes and amastigotes, which are the clinically relevant forms of the parasite [152]. Another similar example of antichagasic chatelicidin-related peptides is that of crotalicidin from the venom of *C. durissus terrificus*, which was able to inhibit all developmental forms of the *T. cruzi* benznidazole-resistant Y strain, showing high selectivity against trypomastigotes (SI > 200); as in the previous examples, crotalicidin induced necrosis in *T. cruzi*, causing several morphological alterations, including loss of membrane integrity and cell shrinkage [153]. The stage-selective antichagasic action displayed by snake venom components like BatxC or crotalicidin is likely related to stage-dependent changes on the parasite's membrane, including its surface protein composition [154].

Table 2
Venoms and/or venom components investigated for antichagasic activity.

Snake species (family)	Venom /venom component	Antichagasic activity (IC_{50})[a]	Specific effects	Toxicity data	Reference
Bothrops jararaca (Viperidae)	Crude venom (fresh and boiled)	0.1–0.3 μg/mL (strain Y epimastigotes)	Ultraestructural alterations in epimastigotes and amastigotes (mitochondrial swelling, disorganization of the kinetoplast, and membrane fragmentation)	Not specified	[155]
	Crude venom	10 μg/mL (strain Y epimastigotes)	Ultraestructural alterations in epimastigotes and trypomastigotes (mitochondrial swelling, disorganization of the kinetoplast, fragmentation and disappearance of the mitochondrial membrane; cytoplasmic condensation, loss of mitochondrion membrane potential)	Not specified	[142]
	Fractions FI, FII (LAAO activity)	LD_{50} 2.4 μg/mL (FI) LD_{50} 16.6 μg/mL (FII)	production of toxic H_2O_2	Not specified	[143]
Bothrops atrox (Viperidae)	BatroxLAAO	EC_{50} 62.8 mg/mL (strain Y trypomastigotes)	Not specified	Not specified	[145]
	Batroxicidin (BatxC)	11.3 μM (strain Y epimastigotes) 0.44 μM (strain Y trypomastigotes)	generation of ROS; decrease in mitochondria transmembrane potential; loss of cell membrane integrity observed by scanning electron microscopy	CC_{50} (LLC-MK2) > 100 μM	[152]
Bothrops marajoensis (Viperidae)	Crude venom	61 % inhibition at 100 μg/mL (strain CL Brener, clone B5 epimastigotes)	Not specified	Not specified	[92]
	BmajPLA$_2$-II (Lys49 svPLA$_2$)	42 % and 61 % inhibition at, respectively, 6.25 and 100 μg/mL (strain CL Brener, clone B5 epimastigotes)			
Bothrops brazili (Viperidae)	Crude venom	75.6 % inibition at 100 μg/mL (strain CL Brener clone B5)	Not specified	Not specified	[141]
	Braziliase-I (Asp49 svPLA$_2$)	31.5 % inibition at 100 μg/mL (strain CL Brener, clone B5)			
	Braziliase-II (Asp49 svPLA$_2$)	33.2 % inibition at 100 μg/mL (strain CL Brener, clone B5)			
Bothrops jararacussu (Viperidae)	BjussuLAAO-II	4.85 μg/mL (strain CL Brener, clone B5 amastigotes)	Not specified	Not specified	[144]
Bothrops lutzi (Viperidae)	Crude venom (BltTV)	50.1 μg/mL (strain Y epimastigotes)	Not specified	Not specified	[156]
Bothrops leucurus (Viperidae)	Crude venom	1.14 μg/mL (strain Y epimastigotes)	loss of characteristic morphology, namely, lacking flagella and a spherical format	Not specified	[157]
Bothrops mattogrossensis (Viperidae)	BmatTX-IV	13.8 μg/mL (strain CL Brener, clone B5 epimastigotes)	Not specified	Not specified	[140]
Bothrops asper (Viperidae)	Crude venom	10 and 1 μM (strain Jennifer epimastigotes and amastigotes, respectively)	Not specified	Not specified	[158]
		25 and 3 μM (strain CL Brener epimastigotes and amastigotes, respectively)			
Bothrops nummifer (Viperidae)		11 and 6 μM (strain Jennifer epimastigotes and amastigotes, respectively)			
		57 and 7 μM (strain CL Brener epimastigotes and amastigotes, respectively)			
Bothrops picadoi (Viperidae)		27 and 3 μM (strain Jennifer epimastigotes and amastigotes, respectively)			
		60 and 47 μM (strain CL Brener epimastigotes and amastigotes, respectively)			
Bothriechis schlegelli (Viperidae)		21 and 1 μM (strain Jennifer epimastigotes and amastigotes, respectively)			
		22 and 1.4 μM (strain CL Brener epimastigotes and amastigotes, respectively)			
Crotalus durissus (Viperidae)		20 and 4 μM (strain Jennifer epimastigotes and amastigotes, respectively)			
		39 and 6 μM (strain CL Brener epimastigotes and amastigotes, respectively)			

(*continued on next page*)

Table 2 (*continued*)

Snake species (family)	Venom /venom component	Antichagasic activity (IC_{50})[a]	Specific effects	Toxicity data	Reference
Crotalus viridis viridis (Viperidae)	Crude venom	76–93 % inhibition (strain CL Brener epimastigotes, trypomastigotes, and amastigotes)	Ultrastructural alterations		[151]
	Crovirin (CRISP)	1.10 and 1.64 μg/mL (strain CL Brener trypomastigotes and amastigotes, respectively)			[34]
Crotalus durissus terrificus (Viperidae)	Crotalicidin	reported active against strain Y epimastigotes, amastigotes and trypomastigotes, with SI > 200 for the latter			[110]
Calloselasma rhodostoma (Viperidae)	CR-LAAO	47 % inhibition at 32 μg/mL (strain CL Brener clone B5 trypomastigotes)	Production of toxic H_2O_2	CC_{50} (PBMC), 2.43 μg/mL CC_{50} (HepG2), 10.78 μg/mL CC_{50} (HL-60), 1.7 μg/mL	[149]
Cerastes cerastes (Viperidae)	Crude venom (heating abolished activity)	> 90 % inhibition at 100 μg/mL (epimastigotes, after 72 h incubation) ~ 60 % inhibition at 5 μg/mL (amastigotes and trypomastigotes, after 2 h incubation)	Not specified	not specified	[138]
Vipera lebetina (Viperidae)		50 % inhibition at 100 μg/mL (epimastigotes)			
Naja haje (Elapidae)		inhibition >90 % at 100 μg/mL (epimastigotes)			

[a] Except otherwise indicated; CRISP, cysteine-rich secretory proteins; HL-60, human promyelocytic leukemia cells; LAAO, L-amino acid oxidase; LLC-MK2, Rhesus monkey kidney epithelial cells; SI, selectivity index. Other abbreviations as defined in the footnote for Table 1.

3.3. From and to Latin America

The current treatments for CD, benznidazole or nifurtimox, are 100 % effective if taken at the onset of the acute phase of infection. Then, how come there are about 12,000 deaths, 30,000 new cases, and 8000 newborns infected annually in the Americas, where an estimated total of 6 million people are infected [130,131]? One main reason is the late emergence of symptoms that in most cases only appear when the infection has reached a stage on which the available drugs are much less effective, or not effective at all. Also, neither of these drugs can be administered to pregnant women, while passage from an infected mother to her child during pregnancy or childbirth is a major route of transmission. Moreover, none of the treatments can be used on people with liver or kidney insufficiency, and nifurtimox cannot be taken by people with a history of psychiatric or neurological disorders. Thus, CD usually evolves into a chronic infection in which parasites hide mainly in the digestive and cardiac muscle, in time leading to cardiac, digestive, and neurological ailments that ultimately may cause sudden death due to heart failure. Moreover, salubrious and healthcare conditions are often very deficient in many of the endemic areas for CD, which cross more than twenty continental Latin American countries. Therefore, new treatments must be urgently identified that are active also at later stages of infection, when symptoms appear, and safe for pregnants, newborns, and people afflicted with conditions that hamper the use of currently available drugs. One wise and sustainable option to attain this goal is to take advantage of the vast biodiversity in this region of the globe. Searching antichagasic molecules in the venoms of snake species from Latin America, as in the examples cited above, is a very important yet still incipient move in that direction. Massive prospection of anti-chagasic substances in the secretions of a much wider set of endogenous species, from amphibians (skin secretions) to snakes (venoms), without endangering these animals, should be promoted.

4. Leishmaniasis

4.1. A bird's-eye view on epidemiology and etiological agents in leishmaniasis

Leishmaniasis is another major NTD that is endemic to 98 countries and affects nearly 1 million people worldwide; it is caused by several species of protozoa belonging to the *Leishmania* genus, which are transmitted *via Phlebotominae* sandfly vectors (*Phlebotomus spp.* and *Lutzomyia spp.* in the Old and New Worlds, respectively) [159]. Clinical manifestations include: (i) involvement of liver, spleen, and bone marrow in the case of visceral leishmaniasis (VL), which is the most severe form of the disease also known as *kala-azar*; (ii) from self-healing to disfiguring skin lesions caused by cutaneous leishmaniasis (CL), which is the most common form of the infection; or (iii) in addition to skin, also mucosal lesions (mostly nasal and/or buccal mucosa), in the case of mucocutaneous leishmaniasis (MCL), which is the most disabling type [159–162]. Asymptomatic leishmaniasis infections also occur depending on the geographic location that may contribute to the transmission of the disease, although there are still missing both a clear-cut definition of this type of leishmanial infection and methods for its diagnosis [163]. Parasite species, vertebrate host, and other factors determine whether the infection becomes symptomatic and whether VL, CL or MCL arises. Thus, while VL predominates in the Indian subcontinent, East Africa, and Brazil, nearly 95 % of CL infections occur in Latin America, Central Asia, the Middle East, and the Mediterranean Basin; in turn, MCL seems mostly confined to four countries, namely, Brazil, Bolivia, Ethiopia, and Peru, where about 90 % of the MCL cases occur [160].

At least 20 different species of *Leishmania* parasites can cause the disease in humans, and their prevalence varies with the region of the globe. Thus, the so-called "Old World" leishmaniasis is mainly caused by species that prevail in Africa, Asia, Middle East, India, and the Mediterranean Basin, typically, *L. infantum*, *L. major*, *L. donovani*, *L. tropica*, and *L. aethiopica*. In turn, "New World" leishmaniasis is mostly due to the L. *amazonensis*, *L. braziliensis*, *L. mexicana*, and *L. panamensis* species, as well as by L. *infantum*, all of which occur in America [160]. *Leishmania* parasites have a dimorphic life cycle, with an additional level of

complexity over *Plasmodium* and *Trypanosoma*, as they can be transmitted to pets that may act as major reservoirs for human infection in some cases [164,165].

Briefly, when an infected female phlebotomine uptakes blood meal from a vertebrate host, flagellated parasites (promastigotes) that were lodged in the sand fly's proboscis are inoculated into the dermis of the vertebrate host. Next, promastigotes are phagocytized by mononuclear phagocytes – especially macrophages – where they transform into non-flagellated intracellular forms called amastigotes that are capable to replicating by binary fission and are then released to infect other mononuclear phagocytic cells in diverse tissues. When infected macrophages are ingested by another sandfly, amastigotes transform into promastigotes, with parasite population expansion in the gut, and migrate to the sand fly's proboscis as infective forms able to initiate a new cycle of parasite transmission.

4.2. Constraints of antileishmanial therapies and the rising interest on snake venoms

The main obstacles related to leishmaniasis chemotherapy rely mostly on the use of drugs that are toxic and require parenteral administration, such as pentavalent antimonials and amphotericin B. Less harmful drug options are miltefosine (oral drug administered to patients with VL specially in the Indian subcontinent and another limited locations for specific disease forms) and liposomal amphotericin B, but still with limitations that curb their widespread use, for instance, the high cost and cold chain requirements of the latter [166,167]. Therefore, there is a continuous search for new alternatives to treat leishmaniasis, through numerous approaches mostly based in *in vitro* and *in vivo* screenings using different *Leishmania* species and varied methodologies and host cell models, which may difficult the data interpretation and reproducibility [167,168]. In this context, snake venoms have also been explored as interesting sources of biological compounds with leishmanicidal activity. A total of 46 reports could be found from 1994 onwards, in which venoms or venom components from different snake species have been tested against diverse *Leishmania* parasite species and parasite developmental stages, as summarized in Table 3. Interestingly, nearly half of such studies were carried out in the past five years, implying that the interest on these potential natural sources of antileishmanial compounds is on the rise. Yet, only a limited number of studies have addressed possible mechanisms of antileishmanial action of snakes' venoms and/or their components. The most frequently reported effects are permeabilization of parasite's membranes and changes in mitochondrial membrane potential, mainly on promastigotes, as well as increased production of reactive oxygens species (ROS) and proinflammatory cytokines by infected host macrophages – Fig. 4.

4.2.1. The Viperidae snake family as the prototypical focal point

Reports on the search of antiparasitic, including antimalarial and antichagasic, activity in snake venoms and their components or component analogues and derivatives have mainly addressed species of the V*iperidae* family, and investigations on antileishmanial activity make no exception to this rule. Indeed, nearly 85 % of the reports revised in this section concern snake species of that family, ~64 % and ~ 20 % of which belong to the *Bothrops* and *Crotalus* genera, respectively. Other snake species covered belong to either the *Elapidae* (7 studies) or the *Colubridae* (5 species in a single study) families.

4.2.1.1. Studies on bothropic snakes. The antileishmanial activity of crude venom and its components from *B. jararacussu* was the focus of many studies, namely, by Caldeira et al. [79], Barros et al. [170,171], Barbosa et al. [172], and Carone et al. [144]; these studies respectively reported that: (i) the venom Bj3k fraction and Bj-derived synthetic peptides did not display antileishmanial activity, similarly to what was observed against malaria parasites in the same work (see Section 2, Table 1) [79]; (ii) the whole venom as well as its LAAO-II enzyme component significantly reduced the counts of L. *amazonensis* and L. *braziliensis* promastigotes *in vitro* [172]; (iii) Asp49 svPLA$_2$ were active *in vitro* against L. *amazonensis* promastigotes either in solution or as liposomal formulation, whereas only the latter was confirmed both to be active against amastigote forms [170] and to decrease parasite burden *in vivo* on infected BALB/c mice [171]; (iv) the activity of the LAAO-II enzyme component against promastigotes of L. *amazonensis* could be confirmed, though the IC$_{50}$ value 4.56 μg/mL determined in this study [144] was 80-fold higher than that determined by Caldeira et al. [79], reflecting how the use of different experimental methods influences quantitative data obtained.

The *B. moojeni* species was the second most used in the search for venom-derived substances with antileishmanial activity. In earlier works, Tempone et al. and Stábeli et al. respectively tested the antileishmanial activity of the whole venom and its LAOO fraction [173], and of its myotoxin-II (MjTX-II) fraction [174] against *L. amazonensis*, *L. braziliensis*, L. *major* and *L. donovani* promastigotes; IC$_{50}$ values determined by Tempone et al. reflect a higher activity for the LAOO fraction as compared to the whole venom [173]. Recently, Barbosa et al. confirmed that another L-amino acid oxygenase fraction (LAOO-II) of the *B. moojeni* venom was active *in vitro* against *L. amazonensis* and L. *braziliensis* [172].

The venoms and venom components from *B. jararaca*, *B. marajoensis*, *B. pauloensis*, *B. leucurus*, *B. atrox*, *B. mattogrossensis*, *B. brazili*, *B. asper*, *B. pirajai*, and *B. goodmani* were also subject of several studies targeting identification of antileishmanial substances. Gonçalves et al. and Ciscotto et al. studied the antipromastigote action, respectively, of the venom of *B. jararaca* on L. *major* [155] and of its LAAO fraction on L. *amazonensis* [175]; modest results were obtained, with the venom reported to inhibit multiplication of L. *major* promastigotes at 50 μg/mL. Torres et al. [176] and Grabner et al. [92] also tested the crude venom and its components, or derivatives/analogues thereof, from *B. marajoensis* snakes; the LAAO fraction was found more active than the crude venom against L. *amazonensis* and L. *infantum* promastigotes [176], whereas a basic Lys49 svPLA$_2$ homologue (BmajPLA$_2$-II) was significantly active against L. *infantum* promastigotes at 100 μg/mL [92]. Rodrigues et al. [177], Nunes et al. [178], and Castanheira et al. [179] turned their attention into *B. pauloensis* venom and its components; this allowed identification of antileishmanial activity in the LAAO fraction [177] and in the Lys49 svPLA$_2$ fraction (BnSP-7) [178], the latter being found to disturb proliferation, ultrastructure and infectivity of L. *amazonensis* parasites [178]; in turn, a type-C lectin of the same venom was inactive [179]. Curiously, a galactose-binding lectin (BLL) from the venom of *B. leucurus* showed sub- to low micromolar activity against promastigotes and amastigotes of L. *amazonensis* and *L. braziliensis* [180], whereas the LAAO fraction from the same venom showed sub-micromolar activity against promastigotes of L. *infantum* and *L. braziliensis* [181]. Other components from bothropic snake venoms tested for their antileishmanial action concern the LAAO fractions from the venoms of *B. atrox* [145] and *B. pirajai* [182], svPLA$_2$ fractions from the venoms of *B. asper* [82], *B. brazili* [85,141], and *B. mattogrossensis* [140], as well as synthetic peptides derived from, or inspired in, venom components from *B. atrox* [79,183], and from *B. marajoensis*, *B. moojeni*, and *B. godmani* [184]; although active substances could not be identified in all of these studies, significant activities were observed especially in the case of the *B. atrox* LAAO fraction, showing EC$_{50}$ values of 23.34, 4.3 and 4.5 μg/mL respectively against promastigotes of L. *braziliensis*, L. *donovani* and L. *major* [145], and the *B. pirajai* LAAO fraction, with EC$_{50}$ values in the 1–1.5 μg/mL range against promastigotes of the same three species plus L. *amazonensis* [182]. Regarding synthetic peptides, none of the three (pCergo, pBmTxJ, and pBmje) derived from the acidic Asp49 svPLA$_2$ enzymes from, respectively, *B. marajoensis*, *B. moojeni*, and *B. godmani* venoms showed potent antipromastigote activity, although pCergo was able to

Table 3
Venoms and/or venom components investigated for antileishmanial activity.

Snake species (family)	Venom /venom component	Antileishmanial activity (IC_{50})[a]	Specific effects	Toxicity data	Reference
Bothrops jararacussu (Viperidae)	Asp49 $svPLA_2$	185 μg/mL (*L. amazonensis* promastigotes)	Not specified	SI (over MPM), 0.82	[170,171]
	Asp49 $svPLA_2$ (liposomal formulation)	14.36 and 12.5 μg/mL (*L. amazonensis* promastigotes and amastigotes, respectively)	Decreased parasite burden on infected BALB/c mice	SI (over MPM), 10.65	
	BjussuLAAO-II	4.56 μg/mL (*L. amazonensis* promastigotes)	Not specified	Viability of MCF10A cells reported as unaffected; IC_{50} (MCF-7) 1.80 μg/mL	[144]
		active at 0.195 and 0.391 μg/mL against promastigotes of L. *amazonensis* and L. *braziliensis*, respectively	Parasite's mitochondrial membrane potential altered	Not specified	[172]
	Venom fraction Bj3k and derived Bj peptides	reported as inactive against L. *amazonensis* promastigotes	–	–	[79]
Boothrops moojeni (Viperidae)	Crude venom	EC_{50} 7.56 μg/mL (*L. amazonensis* promastigotes)	Not specified	Not specified	[173]
	LAAO	EC_{50} 1.44, 1.08, and 1.19 μg/mL (promastigotes of L. *amazonensis*, *L. chagasi*, and L. *panamensis*, respectively)			
	BmooLAAO	active at 6.25 μg/mL against promastigotes of *L. amazonensis* and *L. braziliensis*	Parasite's mitochondrial membrane potential altered	Not specified	[172]
	Myotoxin II (MjTX-II)	reported as active against L. *amazonensis*, *L. braziliensis*, *L. major*, and L. *donovani*	Not specified	Not specified	[174]
	Peptide pBmTxJ	EC_{50} 264.24 and 142.88 μM (promastigotes of L. *braziliensis* and L. *amazonensis*, respectively)	Not specified	CC_{50} (BMDM), 492 μg/mL (SI 3.44 and 1.96 for L. *amazonensis* and L. *braziliensis*, respectively)	[184]
		reported significant dose-dependent decrease in amastigote survival			
Bothrops jararaca (Viperidae)	Crude venom	reported to inhibit replication of L. *major* promastigotes at 50 μg/mL	Not specified	Not specified	[155]
	LAAO-active fraction	reported active on L. *amazonensis* promastigotes (50 % viability reduction)	Not specified	Not specified	[175]
Bothrops marajoensis (Viperidae)	Crude venom	reported active against L. *infantum* promastigotes in the 6.25–100 μg/mL range	Not specified	CC_{50} (HepG2), 43.64 and > 150 μg/mL for crude venom and $BmajPLA_2$-II, respectively	[92]
	$BmajPLA_2$-II (Lys49 $svPLA_2$)				
	Crude venom	86.56 and 79.02 μg/mL (promastigotes of L. *amazonensis* and *L. infantum*, respectively)	Not specified	Not specified	[176]
	LAAO-active fraction	2.55 and 2.86 μg/mL (promastigotes of *L. amazonensis* and *L. infantum*, respectively)	Not specified	Not specified	
	Peptide Bmje	125.31 and 184.50 μM (promastigotes of *L. braziliensis* and *L. amazonensis*, respectively)	Not specified	CC_{50} (BMDM), 550.83 μM (SI 2.98 and 4.39 for *L. amazonensis* and *L. braziliensis*, respectively)	[184]
Bothrops pauloensis (Viperidae)	BpLAAO	EC_{50} 1.48, 1.59, 1.03, and 1.29 μg/mL (promastigotes of *L. amazonensis*, *L. donovani*, *L. braziliensis*, and *L. major*, respectively)	Not specified	Not specified	[177]
	BnSP-7 (a Lys49 $svPLA_2$)	58.7 and 28.1 μg/mL (*L. amazonensis* promastigotes and amastigotes, respectively)	Not specified	CC_{50} (MPM), 5.6 μg/mL	[178]
	BpLEC (type C lectin)	reported as inactive against *L. amazonensis* promastigotes	promastigote agglutination observed after 24 h incubation with 25, 5 and 1 μg of BpLEC	Not specified	[179]
Bothrops leucurus (Viperidae)	BLL (galactose-binding lectin)	1.5 and 0.88 μM (*L. amazonensis* promastigotes and amastigotes, respectively)	Observed decrease in promastigote mitochondrial transmembrane potential	CC_{50} (MPM), 37.57 μM (SI 25 and 42.6 for L. *amazonensis* promastigotes and amastigotes respectively; SI 28.9 and 43.6 for L. *braziliensis* promastigotes and amastigotes, respectively)	[180]
		1.3 and 0.86 μM (*L. braziliensis* promastigotes and amastigotes, respectively)			
	LAAO	EC_{50} 0.07 and 0.08 μM (promastigotes of *L. infantum* and L. *braziliensis*, respectively)	Not specified	Not specified	[181]

(*continued on next page*)

Table 3 (*continued*)

Snake species (family)	Venom /venom component	Antileishmanial activity (IC_{50})[a]	Specific effects	Toxicity data	Reference
Bothrops atrox (Viperidae)	BatroxLAA	EC_{50} 23.34, 4.3 and 4.5 μg/mL (promastigotes of L. *braziliensis*, *L. donovani*, and L. *major*, respectively)	Not specified	~35.32 % and 42.8 % inhibition of proliferation of PBMC and HL-60 cells, respectively, at 50 μg/mL	[145]
	Batroxicidin (BatxC) BatxC(C-2.15Phe) BatxC(C-2.14Phe) des-Phe1	EC_{50} 4.90 μM EC_{50} 8.86 μM EC_{50} 6.74 μM (*L. amazonensis* promastigotes)	Damage of parasite's cell membrane observed	Not specified	[183]
	Fraction Bax3k and derived synthetic peptides	reported inactive against L. *amazonensis* promastigotes	–	–	[79]
Bothrops brazili (Viperidae)	Crude venom	75.6 % inibition at 100 μg/mL (*L. infantum* promastigotes)	Not specified	Not specified	[141]
	Braziliase-I (Asp49 $svPLA_2$)	26.2 % inibition at 100 μg/mL (*L. infantum* promastigotes)			
	Braziliase-II (Asp49 $svPLA_2$)	19.2 % inibition at 100 μg/mL (*L. infantum* promastigotes)			
	Myotoxic $svPLA_2$ fractions	~40–60 and > 100 μg/mL (promastigotes of *L. amazonensis* and *L. braziliensis*, respectively)	Not specified	Not specified	[85]
Bothrops mattogrossensis (Viperidae)	BmatTX-I, II and III ($svPLA_2$s)	reported as poorly active against *L. amazonensis* promastigotes	Myotoxic effects and release of proinflammatory cytokines on host (phospholipase-type action)	30–50 % and 10–20 % cell death at 100 μg/mL for Jurkat and SK-BR-3 cells, respectively	[207]
	BmatTX-IV ($svPLA_2$)	11.9 μg/mL (*L. infantum* promastigotes)	Not specified	CC_{50} (NCTC929), 81.2 μg/mL	[140]
Bothrops asper (Viperidae)	Crude venom	8.6 μg/mL (*L. infantum* promastigotes)	Not specified	Not specified	[81]
	Acidic $svPLA_2$	> 100 μg/mL (*L. infantum* promastigotes)	Not specified	Not specified	
Bothrops pirajai (Viperidae)	BpirLAAO-I	EC_{50} 1.46, 1.06, 1.26, and 1.20 μg/mL (promastigotes of *L. amazonensis*, *L. braziliensis*, *L. donovani*, and *L. major*, respectively)	production of toxic H_2O_2 (antiparasitic activity reduced by catalase)	Not specified	[182]
Bothrops godmani (Viperidae)	Peptide pCergo	EC_{50} 93.69 and 110.40 μM (promastigotes of *L. braziliensis* and *L. amazonensis*, respectively)	Not specified	CC_{50} (BMDM), 448 μM (SI 4.05 and 4.78 for *L. amazonensis* and *L. braziliensis* promastigotes, respectively)	[184]
Crotalus durissus terrificus (Viperidae)	LAAO	parasite viability decreased at 55 mEAU	Production of toxic H_2O_2	Not specified, but LAAO referred to as highly toxic to L929 cells	[185]
	$svPLA_2$	29.9 μg/mL (*L. amazonensis* amastigotes)	Not specified	CC_{50} (BMDM) > 50 μg/mL	[191]
	Crotamine	reported to decrease parasite burden in BALB/c mice when combined with pentavalent antimonials	Combination of glucantime with crotamine increased NO production by macrophages	Not specified	[188]
	Crotamine encapsulated in PGLA microparticles	reported as poorly active against L. *amazonensis* promastigotes and amastigotes	–	Reported as non-toxic for MPM	[189]
	Crotoxin	22.86 μg/mL (*L. amazonensis* promastigotes)	Not specified	Reported as non-toxic for MPM after 48 h incubation at at 1.2, 2.4 and 4.8 μg/mL, but host cells showed increased mitochondrial activity	[190]
	Crotamine	25.65 μg/mL (*L. amazonensis* amastigotes)	Not specified	CC_{50} (BMDM) > 50 μg/mL	[191]
	Crotoxin	28.15 μg/mL (*L. amazonensis* amastigotes)			
	Gyroxin	31.35 μg/mL (*L. amazonensis* amastigotes)			
	Convulxin	52.7 μg/mL (*L. amazonensis* amastigotes)			
Crotalus durissus cascavela (Viperidae)	LAAO	2.39 μg/mL (*L. amazonensis* promastigotes)	Production of toxic H_2O_2	Not specified	[186]
Crotalus durissus collilineatus (Viperidae)	$svPLA_2$	reported inactive against *L. amazonensis in vitro* and *in vivo*	–	Reported as non-toxic to MPM	[187]
Crotalus viridis viridis (Viperidae)	Crovirin (CRISP)	> 4.8 μg/mL at 24, 48 and 72 h (*L. amazonensis* promastigotes)	–	No significant toxicity to MPM observed	[34]
		2.38 (24 h), 1.05 (48 h), and 1.21 (72 h) μg/mL (*L. amazonensis* amastigotes)			

(*continued on next page*)

Table 3 (*continued*)

Snake species (family)	Venom /venom component	Antileishmanial activity (IC_{50})[a]	Specific effects	Toxicity data	Reference
Cerastes cerastes (Viperidae)	Crude venom	reported as having stronger inhibition effect than chlorimipramin at 50 μM (*L. infantum* promastigotes)	Impairment of nucleic acid biosynthesis	Not specified	[138]
	Disintegrin	reported as inactive against L. *infantum* promastigotes	–	Not specified	[192]
Lachesis muta (Viperidae)	Crude venom	decreased parasite burden and footpad swelling in L. *amazonensis*-infected BALB/C mice	Increased NO levels in macrophages	not specified	[193]
	LmLAAO	2.22 μg/mL (*L. braziliensis* promastigotes)	Not specified	CC_{50} (AGS), 22.7 μg/mL CC_{50} (MCF-7), 1.4 μg/mL	[194]
Agkistrodon contortrix laticinctus (Viperidae)	peptide pAcl	EC_{50} 50.98, 57.23 and 220.32 μM (promastigotes of L. *amazonensis* 2269, *L. amazonensis* PH8, and L. *infantum*, respectively)	Permeabilization of the parasite's plasma membrane	CC_{50} (BMM), 232.88 and 273.70 μM (pAcl and pAclR7, respectively)	[195]
	Peptide pAclR7	EC_{50} 27.19, 36.83 and 70.71 μM (promastigotes of L. *amazonensis* 2269, *L. amazonensis* PH8, and L. *infantum*, respectively)			
Calloselasma rhodostoma (Viperidae)	LAAO	16.66 and 24.47 μg/mL (promastigotes of *L. infantum* and L. *braziliensis*, respectively)	Changes in production of H_2O_2	$CC5_{50}$, 10.78 and 1.7 μg/mL (HepG2 and HL-60 cells, respectively); little effect on PBMC	[149]
Naja naja oxiana (Elapidae)	Crude venom	0.36 and 14.12 μg/mL (*L. major* promastigotes and amastigotes, respectively)	Not specified	Not specified	[197]
	NNOV-FK	46.59 μg/mL (*L. tropica* promastigotes)	Modulation of iNOS and cytokines expression in L. *tropica*-infected macrophages	CC_{50} (J774), 0.51 μg/mL (SI = 2.8)	[198]
	NNOVF9	32.32 μg/mL (*L. infantum* promastigotes)	Not specified	Not specified	[199]
	NNOVF11	12.76 and 0.03 μg/mL (*L. infantum* promastigotes and amastigotes, respectively)	Immunomodulatory effects combined with induction of ROS, stimulation of apoptotic-like mechanisms, and impairment of arginine metabolism	CC_{50} (macrophages), 0.51 μg/mL (SI = 17)	
	NNOVF13	98.26 μg/mL (*L. infantum* promastigotes)	Not specified	Not specified	
Naja haje (Elapidae)	Crude venom	reported as having stronger inhibition effect than chlorimipramin at 50 μM (*L. infantum* promastigotes)	Not specified	Not specified	[138]
Micrurus spixii (Elapidae)	Neurotoxic $svPLA_2$	1.24 μg/mL (*L. amazonensis* promastigotes)	Not specified	MLD_{50} (HepG2) > 200 μg/mL (SI ≥ 256.4)	[74]
Micrurus lemniscatus (Elapidae)	ML-LAAO	0.14 and 0.039 μg/mL (promastigotes of *L. amazonensis* and *L. infantum chagasi*, respectively)	Possible changes in H_2O_2 production	Not specified	[200]
Bungarus caeruleus (Elapidae)	Crude venom	14.5 and 11.2 μg/mL (*L. donovani* promastigotes and amastigotes, respectively). Decreased parasite burden in L. *donovani*-infected BALB/c mice.	Immunomodulatory effects including increased production of TNF-α, IFN-γ, ROS, and NO in infected mice	Observed activation of MPM upon incubation with crude venom	[201]
Philodryas patagoniensis (Colubridae)	Crude venom	51.5 % inhibition at 1.7 mg/mL (*L. major* promastigotes)	Not specified	Not specified	[202]
Philodryas olfersii olfersii (Colubridae) *Philodryas baroni (Colubridae)* *Hypsiglena torquata texana (Colubridae)*		no significant activity observed	–	Not specified	
Trimorphodon biscutatus lambda (Colubridae)		108.6 μg/mL (*L. major* promastigotes)	Not specified	Not specified	

[a] Except otherwise indicated; AGS, human gastric adenocarcinoma cells; BMDM, murine bone marrow-derived macrophages; EAU, enzyme activity units; IFN-γ, interferon gamma; iNOS, inducible nitric oxide synthase; J774, human reticulum cell sarcoma cells; Jurkat, immortalized line of human T lymphocytes; MCF10A, spontaneously immortalized, non-malignant fibrocystic breast cell line; MLD_{50}, minimum lethal dose for 50 % of the cells; MPM, murine peritoneal macrophages; NCTC929 (same as L929), strain L mouse subcutaneous connective tissue fibroblasts; ROS, radical oxygen species; TNF-α, tumor necrosis factor alpha. Other abbreviations as defined in the footnotes for Tables 1 and 2.

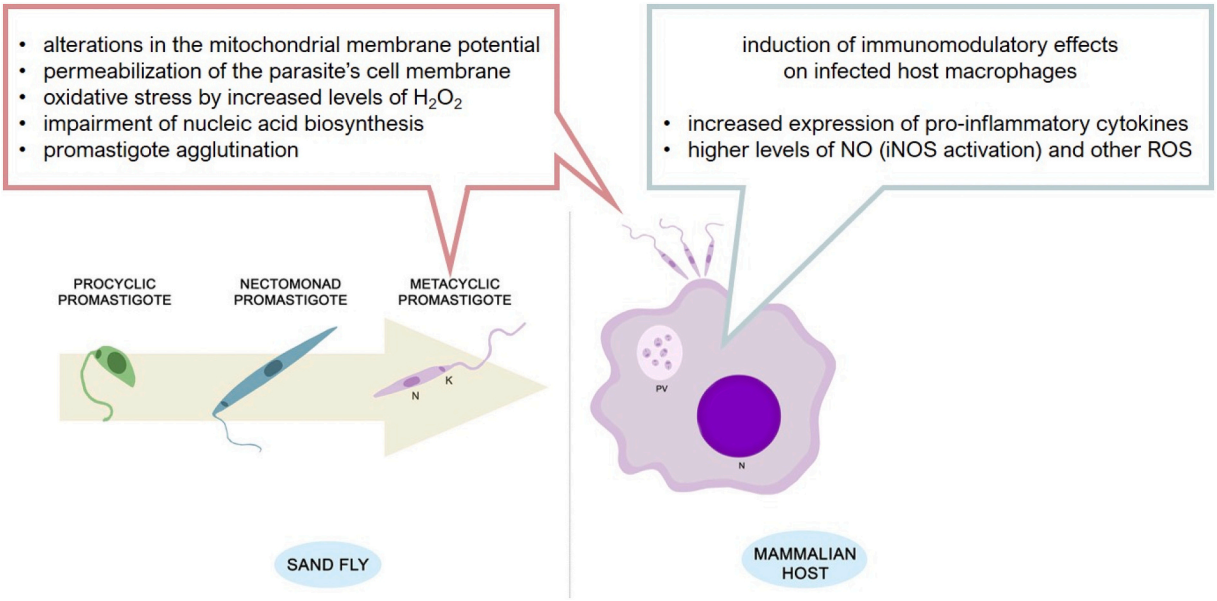

Fig. 4. Reported effects underlying the antileishmanial action of snake venoms and derived proteins and peptides; N, nucleus; K, kinetoplast; PV, parasitophorous vacuole
(adapted from [169] with permission).

reduce the intracellular amastigotes burden *in vitro* [184]. In turn, batroxicidin and its synthetic analogues developed by Dematei et al. showed low micromolar activity against L. *amazonensis* promastigotes [183].

4.2.1.2. Studies on other Viperidae snakes. Non-bothropic snake species of the *Viperidae* family, especially but not exclusively those from the *Crotalus* genus, have also been explored as potential sources of antileishmanial substances. Different sub-species of the *Crotalus durissus* species have been the most used in this context. Hence, LAAO fractions from the venoms of *C. durissus terrificus* and *C. durissus cascavela* were found active against L. *amazonensis* promastigotes by, respectively, decreasing parasite viability at 55 mEAU (EAU, enzyme activity units) [185] and inhibiting by 50 % parasite growth at 2.39 μg/mL [186]. In turn, svPLA$_2$ fraction from *C. durissus collilineatus* was devoid of antileishmanial action [187]. Antileishmanial activity was also investigated in the most emblematic peptides from the venom of *C. durissus terrificus*, *i.e.*, crotamine and crotoxin, alongside other peptides originated from the same venom; thus, Silva et al. tested crotamine against L. *amazonensis* amastigotes *in vitro* and *in vivo*, and observed a decreased parasite burden when the peptide was combined with a standard pentavalent antimonial [188]; curiously, Macedo et al. found that encapsulation of this peptide in PGLA microparticles led to poor antileishmanial activity [189]; Farias et al. also determined a significant activity of crotoxin *in vitro* against promastigotes and amastigotes of L. *amazonensis* [190]; more recently, Katz et al. determined IC$_{50}$ values of 25.65, 8.15, 31.35 and 52.7 μg/mL for crotamine, crotoxin, gyroxin and convulxin peptides against L. *amazonensis* intracellular amastigotes [191]. Interestingly, crovirin – a CRISP from *C. viridis viridis* mentioned in the previous section due to its anti-*T. cruzi* activity *in vitro* (see Section 3, Table 2), was also found active against promastigotes and amastigotes of *L. amazonensis* [34].

The venoms, venom components, and derived peptides from other non-bothropic vipers that have been scrutinized for leishmanicidal action include the crude venom [138] and the disintegrin fraction [192] from *Cerastes cerastes*, the crude venom [193] and the LAAO fraction [194] from *Lachesis muta*, the LAAO fraction from *Calloselasma rhodostoma* [149], and synthetic peptides derived from the basic svPLA$_2$ fraction of the venom from *Agkistrodon contortix laticinctus* [195]; these studies delivered interesting outputs, whereby the venoms of *L. muta* and *C. cerastes* were respectively found to decrease parasite burden and leishmaniasis-related footpad swelling *in vivo* [193], and to have a stronger inhibitory effect on the growth of L. *infantum* promastigotes than chlorimipramine [138], an anti-depressant drug that induces apoptosis in *Leishmania* parasites by impairing their redox metabolism [196]. Also, the LAAO fraction of *L. muta*, LmAAO, showed significant activity (IC$_{50}$ 2.22 mg/mL) against promastigotes of *L. braziliensis in vitro* [194]. Relevant *in vitro* activities were also found for p-Acl and p-AclR7, short (13 amino acid residues) synthetic peptides inspired in the *C*-terminus of the Lys49 svPLA$_2$ from the venom of *A. contortix laticinctus*; p-AclR7 is an analogue of the native sequence p-Acl where all original lysine (Lys) residues were replaced by arginines (Arg) that showed increased potency against promastigotes of L. *infantum* and of L. *amazonensis* strains 2269 and PH8, compared to p-Acl [195].

4.2.2. Beyond vipers: studies on Elapidae and Colubridae snakes

As in the studies targeting antimalarial and antichagasic snake-venom derived substances addressed in previous sections, the *Elapidae* family is the runner up in the search for venom-derived molecules with antileishmanial properties. Still, the total number of reports focused on this family is markedly lower than those concerning *Viperidae* snakes. On the other hand, except for the pioneering work by Fernandez-Gomez in 1994 where the crude venom of *Naja haje* was reported as more active than chlorimipramine against L. *infantum* promastigotes *in vitro* [138], most other reports involving non-*Viperidae* snakes date from the past two years, which highlights that antileishmanial drug discovery focused on snake venoms has only recently started to expand beyond vipers.

Within the *Elapidae* family, *Naja* has been the most explored genus. The crude venom from the *N. naja oxiana* subspecies was reported active against both promastigotes (IC$_{50}$ 0.36 μg/mL) and amastigotes (IC$_{50}$ 14.12 μg/mL) of L. *major* [197], whereas several of its protein fractions, *e.g.*, NNOV-FK and NNOVF11 were respectively found active *in vitro* against L. *tropica* [198] and L. *infantum* [199] amastigotes; actually NNOVF11 was more active *in vitro* than meglumine antimoniate and its leishmanicidal action seems to emerge from a combination of immunomodulatory, oxidative, and pro-apoptotic effects [199]. The genus *Micrurus* was also explored, though apparently in two studies only: Terra et al. have found significant activity (IC$_{50}$ 1.24 μg/mL) of neurotoxic

svPLA$_2$s from *M. spixii* against promastigotes of L. *amazonensis* [74], whereas Soares et al. discovered that the LAAO enzyme fraction of *M. lemniscatus* was significantly active *in vitro* against promastigotes of L. *amazonensis* (IC_{50} 0.14 μg/mL) and *L. infantum chagasi* (IC_{50} 0.039 μg/mL) with its activity may explained by the phospholipase-induced changes in hydrogen peroxide production [200]. The only additional study that, as far as we know, was reported with a snake species from the *Elapidae* family concerns the evaluation of the crude venom from the *Bungarus caeruleus* snake species both *in vitro* and *in vivo*: Bhattacharya et al. found significant *in vitro* activity against promastigotes (IC_{50} 14.5 μg/mL) and amastigotes (IC_{50} 11.2 μg/mL) of L. *donovani*, and additionally observed decreased parasite burden in leishmania-infected BALB/c mice. Interestingly, immunomodulatory effects seem to be recognized as a possible mechanism of action in this case due to increased production of TNF-α, IFN-γ, ROS, and NO in infected mice [201].

Besides *Viperidae* and *Elapidae*, the *Colubridae* snake family has been the only one covered in a single antileishmanial drug discovery initiative by Peichoto et al. These authors investigated the activity of the venoms of *Philodryas baroni*, *Philodryas olfersii olfersii*, *Philodryas patagonensis*, *Hypsiglena torquate texana*, and *Trimorphodon biscutatus lambda*, against promastigotes of L. *major*; this study allowed to find that the venom of *P. patagonensis* was able to inhibit parasite proliferation by over 50 % at 1.7 μg/mL, whereas that from *T. biscutatus lambda* displayed an IC_{50} of 108.6 μg/mL [202].

4.3. Promises and pitfalls in the search for snake-derived antileishmanial compounds

The reports highlighted in this section openly demonstrate that the venoms of diverse snake species enclose great potential to deliver new substances with antileishmanial action. Still, a few shortcomings impair a clear interpretation of knowledge available as well as of the path to follow ahead; first, as already mentioned at the beginning of this section, a uniformized standard methodology for *in vitro* and *in vivo* assessment of antileishmanial action is lacking, so comparison of data from different studies is questionable [167,168]. Moreover, most studies focused on *in vitro* activity and cannot be regarded as predictive of *in vivo* efficacy; especially when considering that at least half of the research articles collected from the literature in this review did not use macrophages as host cells in or even a mammalian cell type to evaluate any cytotoxicity parameter. Several studies reported cytotoxic concentrations for tumoral and non-tumoral cell lines, e,g,: MCF7, HL-60, AGS, HepG2, Jurkat, SK-BR-3, NCTC929 cells (Table 3). Again, this absence of a defined protocol may difficult the interpretation and comparison of data delaying the advance in the accurate description of the leishmanicidal activity of new compounds, as recently discussed by Brioschi et al. [168].

In fact, while there is common sense pointing out that translation of *in vitro* into *in vivo* efficacy is often hampered in drug discovery pipelines, the opposite may also occur as the context within a living organism is much more complex than what can be mimicked in *in vitro* models. In this case, the best *in vivo* performer does not necessarily correspond to the most potent one *in vitro* [203]. Furthermore, in the majority of reports found in the literature, activity assays were not conducted on the intracellular amastigote forms; these are actually the most concerning ones, given their ability to take advantage of host cell mechanisms to establish and "perpetuate" the infection [204] and being the clinical relevant stage of the parasite. Last, but not least, most of the studies targeting the identification of snake venom components with antileishmanial activity have focused on *Leishmania* species mainly responsible for CL or, less frequently, MCL, whereas only four out of the 46 studies cited herein have included L. *donovani*, the major causative agent of VL that is fatal in 95 % of the cases if timely treatment lacks or fails [205] and which is further aggravated by the rising number of VL + HIV co-infections [206]. Altogether, while existing data brings hope regarding the discovery of snake-derived antileishmanial substances, it also clearly demonstrates that there is still a long way to go, preferably through converging methodological approaches leading to meaningful data.

5. Concluding remarks – from nature's gifts to drug-like candidates

The enormous therapeutic potential of snake venoms and their components are widely recognized since long ago and go way beyond the components and therapeutic applications herein highlighted. For instance, natriuretic peptides from the venoms of vipers and mambas have been explored over the past three decades for their potential therapeutic potential to address heart failure [208]. The diversity as well as the chemical basis for the therapeutic action of snake venom-derived peptides have been very nicely and thoroughly reviewed in [209]. Many drugs exist in the market, especially for cardio-vascular indications, which have snake venoms in their origin [210]. But going from the natural scaffold to a drug-like molecule is rarely an easy task, as most bioactive snake venom components are proteins and peptides that have several issues, especially from a pharmacokinetics viewpoint. Indeed, despite therapeutic use of natural peptides and proteins dates to 1920 with the introduction of insulin for the treatment of diabetes, peptide-based therapeutics have been traditionally regarded as unappealing, given their typically low membrane permeability and stability *in vivo* [211]. Still, the high specificity and diversity of proteins and peptides makes them unmatched bioactive agents; therefore, we have been witnessing a paradigm shift over the past two decades, especially regarding peptides, due to their smaller size, easier chemical synthesis, better cost-effectiveness, and higher membrane permeability, as compared to proteins; as such, the development of peptide-based drugs is now a priority topic in pharmaceutical research [212]. Consequently, several strategies have been advanced to take advantage of the best that peptide therapeutics have to offer, while bypassing their shortcomings; using non-canonical amino acids (*e.g.*, D-amino acids, *N*-methylated amino acids, among others) or peptide bond bioisosteres, downsizing or cyclizing the native peptide, modifying the peptide's *N*- and/or *C*-terminus, attaching an anchoring (*e.g.*, albumin-binding) or targeting (*e.g.*, nuclear localization signal sequences) moiety, and encapsulating in diverse nano-delivery systems, are among the most popular approaches [213]. This means that Medicinal and Pharmaceutical Chemists have already in hand a plethora of tools adequate to convert promising snake venom components into drug-like candidates; yet, not all of them are of universal application or interest as, for instance, expensive and/or thermolabile nanoencapsulation formulations are not convenient to address diseases that are endemic to tropical and sub-tropical low-income regions of the world.

In view of the above, moving forward from the current standpoint in antiprotozoal drug discovery based on snake venoms means finding simple and cost-effective ways to enhance the drug likeness of the most promising snake venom components; therefore, future research in this area should privilege: (i) bioactive peptide sequences as short as possible as in, *e.g.*, [39,184,195], preferably having a modified *N*- and/or *C*-terminus, as illustrated by the emblematic example of captopril, a rationally-modified dipeptide based on the bradykinin potentiating factor of *B. jararaca* and worldwide used for decades as an antihypertensive drug [214]; (ii) backbone cyclization leading to increased stability, like in, *e.g.*, [215] or the marketed antiplatelet drug eptifibatide, a cyclic heptapeptide mimic of barbourin from the American Southeastern pygmy rattlesnake (*Sistrurus miliarus barbouri*) [216]; and (iii) use of non-canonical amino acids and/or conjugation to other moieties, as in the case of the antiplatelet peptidomimetic vipegitide, derived from a type-C lectin (vixapatin) found in the venom *of Vipera xanthina palestinae*; vipegitide incorporates α-aminoisobutyric (Aib) residues and requires further *N*-terminal modification (*e.g.*, PEGylation) for enhanced stability in human serum [217]. In other words, the

knowledge is there to convert antiprotozal snake venom components into drug-like candidates, and new approaches are emerging everyday. For instance, recent reports on the enhanced enzymatic stability and bioactivity associated to *N*-terminal modification of small peptides with unusual moieties like, *e.g.*, antimicrobial ionic liquids [218–220] demonstrate that the frontiers of knowledge in drug design are continuously being pushed forward.

In spite of the above, there is still a long way to go until snake venom-derived/inspired molecules are translated into potent and safe antiprotozoal drugs. The major obstacle is the existing gap in the knowledge regarding mechanisms of selective antiprotozoal action of snake venoms and their components. As highlighted in Figs. 2-4, a number of effects on distinct developmental stages of malaria, trypanosoma, and leishmania parasites has been observed upon treatment with snake venoms and/or derivatives, but their specific molecular targets remain largely unknown. This may appear counter-intuitive as the elucidation of therapeutic targets became much easier in the 'omics' era, but the high complexity and variability of snake venoms's composition adds an extra level of difficulty. This is further aggravated by the fact that a number of snake venoms and derivatives were found to trigger immunomodulatory effects on parasitized mammalian host cells, which means that therapeutic action can be parasite- and/or host-related. That not only increases the number of possible targets to be scrutinized for each specific venom or venom component, as also amplifies concerns on off-target effects, *i.e.*, toxicity to the host. Still, a selective antiprotozoal action has been observed in many of the reports herein addressed. Moreover, despite the neurotoxic, myotoxic, and necrotic effects, among others, caused in humans by the bite of some venomous snakes, most snake toxins are not as toxic as generally assumed [40].

In conclusion, a detailed and comprehensive understanding of the biomolecules and biochemical pathways targeted by snake toxins in both parasites and their host cells, is of utmost importance for the future design and fine tuning of potent and selective antimalarial, antichagasic and antileishmanial drugs derived from snake venoms. The reports herein reviewed show that the interest, knowledge, and will to advance towards this goal is steadily growing. Moreover, the snake venom-derived/inspired compounds that are currently in use for other therapeutic and cosmeceutic indications [36,40,209] demonstrate that such a goal is fully achieavable. Indeed, all conditions are gathered to progress in this field, provided policy- and decision-makers support initiatives that aim at using the Nature's gift of biodiversity to create drugs to save from neglect the millions of people afflicted with protozoal infections.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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