

Review

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## Review

# Neglected Zoonotic Diseases: Advances in the Development of Cell-Penetrating and Antimicrobial Peptides against Leishmaniosis and Chagas

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**Abstract:** In 2020, the WHO established a road map for neglected tropical diseases 2021–2030 that aims to control and eradicate 20 diseases, including Leishmaniosis and Chagas disease. In addition, since 2015, the WHO has been developing a Global Action Plan on Antimicrobial Resistance. In this context, the achievement of innovative strategies as an alternative to replace conventional therapies is a first-order socio-sanitary priority, especially regarding endemic zoonoses in poor regions such as those caused by *Trypanosoma cruzi* and *Leishmania* spp infections. In this scenario, it is worth highlighting a group of natural peptide molecules (AMPs and CPPs) that are promising strategies for improving therapeutic efficacy against these neglected zoonoses, avoiding the development of toxicity and resistance of conventional treatments. This review presents the novelties of these peptide molecules and their ability to cross a whole system of cell membranes, as well as to stimulate the host immune defenses or even serve as a vector of molecules. The efforts of the biotechnological sector will make it possible to overcome the limitations of antimicrobial peptides through encapsulation and functionalization methods to obtain approval for these treatments to be used in clinical programs for the eradication of Leishmaniosis and Chagas disease.

**Keywords:** antimicrobial peptides; cell-penetrating peptides; intracellular pathogen; *Trypanosoma*; *Leishmania*

## 1. Introduction

Protozoan parasites of the genera *Leishmania* and *Trypanosoma* comprise a diverse group of unicellular eukaryotic species that are distributed worldwide and are responsible for serious infections in animals and humans. These zoonotic agents promote intracellular infections accompanied by virulence strategies and evasion of host immune defenses, which hinder the accessibility of the corresponding therapies and the occurrence of adverse effects. Consequently,

treatment strategies based on selective drugs directed towards infected target cells represent a suitable approach to reduce limitations of conventional therapies [1–3]. The abuse of antibiotics in the conventional treatment of microbial infections (not only bacterial, but also in protozoan parasites), favors cumulative toxicity and resistance to antimicrobials, which forces the development of new antibiotics or new strategies for the control of these infections. Currently, the multiresistance generated in microbial agents against various treatments reduces the success rate of antibiotics and has triggered morbidity and mortality rates worldwide. In 2015, a Global Action Plan on Antimicrobial Resistance was created to mitigate this situation [4]. In fact, the World Health Organization (WHO) considers resistance to antimicrobials as one of the main public health threats facing humanity. The magnitude of the problem impairs the achievement of some Sustainable Development Goals (SDG) such as an end to poverty (SDG 1), and health and well-being (SDG 3), complicating the survival of more than 10 million people by the year 2050 [5]. This demonstrates that the development of innovative strategies as an alternative to the substitution of antibiotics is a first-order socio-sanitary priority.

Remarkably, a group of natural peptide molecules that can interact with cell membranes and participate in relevant biological processes has attracted the interest of researchers in recent years. These are promising strategies to increase therapeutic efficacy, avoiding the development of toxicity or resistance; or in a controlled way [6]. The members of this group of peptides share similar physicochemical properties, but their mechanisms of action can vary, thus making it possible to differentiate them into two subgroups: antimicrobial peptides (AMPs) and cell penetrating peptides (CPPs) [7,8]. This review presents the novelties of these peptide molecules, their potential to replace traditional antibiotics, and their activity and mechanisms of action against two of the most relevant neglected zoonoses: leishmaniosis and Chagas disease. Likewise, some biotechnological innovations using peptides are described, with the potential for clinical application focused on neglected zoonotic diseases.

## 2. AMPs and CPPs and Neglected Zoonotic Diseases: An Overview

Currently, the WHO recognizes 20 highly prevalent diseases in tropical areas, which together are known as neglected tropical diseases (NTDs). Many of them constitute life-threatening zoonoses, which further aggravates their socio-sanitary repercussions. The most representative examples of neglected zoonoses are vector-borne diseases such as the three main forms of leishmaniasis, caused by different species of the *Leishmania* genus, as well as Chagas disease which is caused by the parasite *T. cruzi* [9]. These zoonoses have been neglected for a long time by governments and pharmaceutical companies that do not foresee economic benefits in the establishment of control methods. Even the COVID-19 pandemic has further delayed the intervention agenda against these diseases. As a result, the control of NTDs is still inadequate, and remains extremely difficult today [10]. Given this scenario, the WHO has presented a new 10-year plan to put an end to the suffering caused by neglected tropical diseases: a road map for neglected tropical diseases 2021–2030 [11].

The conventional treatment of these diseases has important limitations, since many of the drugs are from the early and mid 20th century, and have limited efficacy in advanced stages of the disease, and are nonspecific and/or highly toxic [12]. Therefore, the goal of finding new starting points for developing new drugs to effectively treat and control these diseases is a priority [12,13]. To overcome these deficiencies, AMPs and CPPs could become a promising alternative for pharmaceutical design and treatment of intracellular infectious diseases. Current studies reflect interesting antimicrobial properties of these peptides against intracellular bacteria, virus, and protozoan genera *Leishmania*, *Trypanosoma*, and *Plasmodium*, the etiological agents of the main forms of leishmaniasis, trypanosomiasis, and malaria, respectively [8,14].

AMPs are short peptide sequences of fewer than 50 amino acids that participate in the innate first line of defense against invading pathogens. AMPs have demonstrated antimicrobial activity against a variety of pathogens, such as viruses, bacteria, fungi, and protozoa [15]. Their mechanism of action usually consists of permeabilizing the membrane, although alternative mechanisms have also been observed that affect the biochemical processes of the pathogen, such as destabilizing its

membrane or interfering with its synthesis of proteins or nucleic acids. By following one of these mechanisms, AMPs lead to the death of the microbial cell. Currently, researchers have access to a large database dedicated to AMPs. The Antimicrobial Peptide Database records 3569 AMP peptides from the six kingdoms of living beings, as well as others of a synthetic nature [16]. Currently, several AMPs have been approved for antibacterial treatment (polymyxins and daptomycin) by the Food and Drug Administration (FDA), and many other AMPs are in clinical development [6,17].

CPPs are relatively short peptides (fewer than 35 amino acids) whose main function is to offer the possibility of transporting a variety of versatile cargoes into the cell. That is, the distribution of various types of molecules: proteins, peptides, nucleic acids, liposomes, nanoparticles, or drugs. This group of natural or synthetically generated peptides has the ability to translocate through cell membranes; in many cases, they do so at low concentrations and without significantly damaging the cell membrane [8]. The entry mechanism can be through an indirect endocytosis (energy dependent) or a direct entry (energy independent) that can be associated with membrane toxic activity, or even through both mechanisms occurring simultaneously. The handicap of endocytosis is that the peptide-cargo complex is not retained inside the endosome but can be released to reach its destination. For this, it is essential that the CPP be equipped with molecules capable of lysing the endosome [18]. There is now a new version of an extensive database dedicated to CPPs [19] to analyze and develop CPP prediction methods. To date, no CPP or CPP-conjugate drugs have been approved by the FDA [20]. One aspect to optimize in relation to vaccines and drugs is adequate administration into the cell. In this context, CPPs have penetration capacity, are not toxic, and their peptide sequences can also be modified to prevent their proteolysis and optimize their function [21].

### **3. How Do AMPs and CPPs Specifically Target Protozoan Parasites without Harming the Infected Mammalian Cell? Cell Entry Mechanisms**

A critical obstacle that peptides must overcome to exert their action on intracellular parasites (such as *Leishmania* spp or *T. cruzi*) in any of their stages is the complex system of membranes that must be crossed in order to enter the infected mammalian cell and the parasitophore vacuole, and to finally get to the parasite that resides inside [22]. Furthermore, the interior of the vacuole is a hostile environment with an acidic pH, where the parasite's proteases can inactivate drugs of peptide nature. The success of therapies is directly related to a high toxicity towards the parasite and a low toxicity to the cells of the host organism; this is known as selective toxicity. In this section, we discuss some possible entry mechanisms that allow peptide molecules to pass through the host cell without damaging it before reaching the parasite to exert their antiparasitic activity. Two entry mechanisms into the cell can be differentiated for nutrients, pathogens, or particles, including AMPs and CPPs. These are direct fusion and endocytosis [23].

The first is based on the differences in composition and charge of the cell envelopes (membranes and glycocalyx) of both parasites and host cells. This is a direct penetration mechanism, which involves electrostatic interaction between peptides and membrane charges, and does not require ATP. Direct penetration is a highly limited entry pathway for conventional drugs, preventing them from exerting their action on their target [24]. However, the peptides can use this route of entry, accumulating locally in the parasite and reaching the necessary concentration to be effective [13]. The cell membranes of these pathogens have an outer hemilayer with a higher percentage of anionic phospholipids, providing global negative charge to the surface. Conversely, the membrane of mammalian cells is characterized by being a bilayer mainly made up of zwitterionic phospholipids, and therefore with a net neutral charge. Rivas and colleagues described in detail that the composition of the trypanosomatid membrane is made up of negatively charged components such as phosphatidylcholine, sphingolipids, and sterols [25]. However, the structure of the membrane and its variations associated with the cell stage of the parasite remains to be studied in detail, as well as if this influences the interaction with AMPs and/or CPPs.

The other entry mechanism is through endocytosis or vesicular trafficking. Trypanosomatids have complex glycocalyx made up of multiple lipids and proteins bound to phosphorylated sugars of different nature. Once more, the composition of the glycocalyx changes depending on the cell stage.

Often these molecules confer a negative charge to the surface; however, they seem to constitute a barrier to the entry of peptides rather than an entry gate. Thus, entrance by endocytosis is limited to the flagellar pocket, a specialized region devoid of glycocalyx that accounts for 5% of the cell surface [26–28]. Endocytosis is the cellular route developed by the cell for the trafficking of substances, with up to five different variants. Notably, the usage of the different pathways depends on the combination of cell surface receptors and on the size of the cargo to be internalized [23]. The detailed mechanism of AMPs and/or CPPs internalization is not fully clear, but some hints have been elucidated [13]. Parasite phospholipases (PLA<sub>2</sub>) are involved in regulating the vesicular traffic, by modifying the membrane phospholipids and inducing the membrane deformation to generate the vesicles [29]. Inhibiting the PLA<sub>2</sub> activity with bromoenol lactone blocks the trafficking of proteins such as transferrin or albumin. On the other hand, the hemoglobin acquisition in *Leishmania* and *Trypanosoma* is very interesting [30]. These parasites are auxotrophic for heme groups, i.e., they are not able to produce heme groups, but their uptake is essential for parasite survival. The most probable source is the heme group from hemoglobin. *Leishmania* amastigotes express a high-affinity hemoglobin receptor (HbR), located in the flagellar pocket. Then, different Rab GTPases/SNARE proteins mediate its internalization to the lysosome, where hemoglobin is degraded, and the heme group is released and transported to its final intracellular destination [31,32]. Therefore, it has been proposed that hemoglobin trafficking pathways could be an unexploited approach for new therapeutic strategies. Indeed, 40 amino acids derived from the hemoglobin binding domain of HbR are able to block hemoglobin uptake, thus inhibiting parasite growth [33]. It is conceivable that, like hemoglobin, AMPs and CPPs can be transported through these endocytic pathways regulated by the multiple Rab GTPases/SNARE family. AMP/CPP use the same entry mechanism pathway of some nutrients, such as hemoglobin, and other particles. Deciphering the components involved, surface receptor, Rab GTPases and SNAREs could constitute an interesting approach to identifying new strategies based on the use of AMPs or CPPs, with enhanced target specificity.

#### 4. AMPs and CPPs as Alternative Therapies vs. Conventional Drugs against Leishmaniosis and Chagas Disease

It is important to consider the parasitic intracellular lifestyle when proposing an adequate therapeutic approach. The trypanosomatids *Leishmania* spp. and *T. cruzi* are examples of obligate intracellular parasites. After their uptake by mammalian cells (especially macrophages in the case of *Leishmania* spp. and macrophages, fibroblasts, and epithelial cells in the case of *T. cruzi*), these parasitic protozoan flagellates lodge in their amastigote form within the acidified parasitophorous vacuole [34]. *Leishmania* amastigotes can multiply within host cells and cause their lysis. The released amastigotes are then responsible for systemic infection. In the case of *T. cruzi*, after multiplying, the intracellular amastigotes differentiate into trypomastigotes, lyse the host cell, and are released into the bloodstream to spread and invade other nucleated cells (cardiomyocytes or cells of the gastrointestinal tract) [22]. Treating these infections implies that the drug or bioactive compound should cross the macrophage cell and the parasitophorous vacuole membrane to reach the parasite. Otherwise, the local concentration inside the vacuole could be too low to be effective against the parasite. Moreover, the stability of the drug once inside the vacuole, under acidic pH, should be considered [35].

To date, there are no vaccines available to protect humans against Chagas disease or any of the main forms of leishmaniasis (visceral, cutaneous, and mucocutaneous). In addition, the treatment of choice with conventional drugs, which include antimonials, miltefosine or liposomal amphotericin B (AmBisome®) against some *Leishmania* species (due to the difference between the clinical forms of leishmaniosis, there is no universal treatment), or benznidazole and nifurtimox against *T. cruzi* infection, are not completely effective at eradicating these parasitoses. Furthermore, conventional pharmacological therapies have been limited due to the toxicity and side effects [36–39]. At this point, AMPs and CPPs represent a very promising opportunity to overcome these limitations and develop new therapies [40]. Natural AMPs and CPPs have been isolated from amphibian, snakes, fish, insects, arachnids, and virus [41]. AMPs represent an essential defense mechanism integrated into the

immune system of vertebrates and invertebrates, which can be active against various types of pathogens, including trypanosomatids [42]. These peptides have been classified into four groups on the basis of their structure ( $\beta$ -sheet,  $\alpha$ -helical, extended and loop) and cationic and amphipathic characters [43]. Cationic peptides preferentially bind to the parasite membrane. This preference is presumably because mammalian cells contain zwitterionic phospholipids in their membrane while, conversely, the membranes of *Leishmania* parasites have an anionic nature. However, even though AMPs and CPPs have shown selective binding to the parasite membrane, some of these compounds exert less activity against amastigote forms [44–47]. This is likely due to the different surface charge between *Leishmania* life stages. The promastigote form is strongly negatively charged, mainly due to the content of surface lipophosphoglycans (LPG, which covers more than 60% of the surface); instead, LPG is present at very low or non-detectable levels in the amastigote form [48]. The external covering of the amastigotes is made up primarily of glycoinositolphospholipids (GIPLs) [49], and the affinity of the peptides for the plasma membrane of amastigotes could be decreased [45]. Thus, it should be noted that these studies show promastigote sensitivity to AMP more than amastigotes [50].

Another useful property of AMPs is their ability to stimulate the host immune response, which can help to eliminate the infecting pathogen and promote the clearance of infected cells [13]. This broad-spectrum activity makes them particularly useful for treating infections caused by complex microorganisms like *Leishmania* or *T. cruzi*. Similarly, CPPs have some properties that make them a powerful alternative to conventional drugs, especially for intracellular pathogens [24,51]. Among these properties, we can highlight their biocompatibility, ease of synthesis, and controllable physical chemistry [52]. Interestingly, CPPs can cross cellular membranes without disrupting them, limiting their toxicity to the target cell [52]. This specificity and low associated toxicity suggests that CPPs constitute powerful tools to combat infectious diseases. Furthermore, there is currently interest in exploiting the use of CPPs for their ability to transport a wide variety of cargo molecules into cells, through covalent or non-covalent binding. Therefore, another complementary scenario seems feasible in which conventional drugs can be conjugated with a reference CPP to give a formulation with high absorption and parasiticide activity capable of both effectively defeating this resistance and increasing the spectrum of susceptible trypanosomatids [8].

The synthetic peptides **pseudomona-derived KDEL and REDKL** cause a severe disruption in membrane and loss of cytoplasmic components in *L. tarentolae* promastigotes [53,54]. Likewise, the *L. tropica* promastigotes exposed to analogues of halictine-2 (a novel AMP from the venom of eusocial honeybee) showed pores on their surface with a significant collapse of the membrane [55] and jellein (an AMP derived from the royal jelly of honeybees) caused pore formation, as well as changing the membrane potential in *L. major* promastigotes [56]. This effect is also produced by amphotericin B (AmB). This polyene antifungal is often used intravenously for systemic fungal infections and is currently the first-line medication for treating leishmaniasis (visceral, cutaneous and mucocutaneous forms) in some endemic regions such as India [57]. AmB treatment was implemented in Bihar state after a large-scale resistance to pentavalent antimony therapy developed [58]. AmB has better selectivity for membranes containing ergosterol than for those containing cholesterol, leading to pore generation and membrane fragility. However, AmB induces nephrotoxicity in a huge percentage of patients, probably due to damage to the glomerular membrane [59]. The liposomal formulation of AmB (AmBisome®) reduces this toxicity, but comes with a high cost of treatment.

In addition to these mechanisms of action, AMPs have also been demonstrated to exert their leishmanicidal activity through other routes. Following the membrane integrity disruption upon peptide treatment, mitochondria are among the most important intracellular targets. For instance, the full-length enterocin AS-48 induces mitochondrial damage to *Leishmania* spp. promastigotes [45]. *Pseudomonas* exotoxin-derived peptides also cause depolarization of promastigote mitochondrial membrane [53]. Additionally, a lauric acid brevinin conjugate limited *L. major* promastigote proliferation by changing the mitochondrial potential [60], and the lethal effect of the recombinant plant-derived defensin Vu-Defr on *L. amazonensis* is the result of mitochondria membrane potential loss, among other mechanisms [61]. This mechanism of action involving mitochondrial electrochemical potential has also been detected after the exposure of parasites to reference drugs

such as miltefosine and paromomycin [62,63]. Miltefosine is a compound derived from phosphocholine, firstly used as an anti-neoplastic drug, that comprises the only oral drug for treating leishmaniasis. Paromomycin is an aminoglycoside antibiotic broadly used for treating Gram-negative bacterial infection, which was introduced for the treatment of leishmaniasis in 2006. As a result of the depolarization of the mitochondrial membrane, the release of ROS to cytosolic space is produced, and consequently, changes in the ionic balance result in the induction of apoptosis. Just like miltefosine [64], AMPs are capable of inducing this form of programmed cell death in *Leishmania* parasites. For instance, a synthetic peptide carrying the core of the *Vigna unguiculata* defensin caused *L. amazonensis* promastigotes culture inhibition by activation of an apoptotic-like cell death pathway [65]. Since the loss of mitochondrial membrane potential is a key indicator for the initiation of programmed cell death, synthetic peptides such as the *Pseudomonas* exotoxin-derived and modified halictine, as well as AMPs from the temporin family, have been demonstrated to induce apoptosis in parasites [53–55,66].

The development of combination chemotherapy against leishmaniasis may prevent drug resistance and shorten the duration of treatment, thus reducing the cost of therapy [58]. In recent years, the synergistic effect of leishmanicidal drugs with AMPs has been analyzed. For instance, the combination of synthetic anti-lipopolysaccharide peptides (SALPs), 19-2.5 and 19-4LF with the paromomycin and AmB enhanced their activity against *L. major* amastigotes in vitro [67]. Conversely, this synergistic effect was not observed when modified halictine-2-derived peptide was employed in combination with those leishmanicidal treatments. Nevertheless, potassium antimony (III) tartrate (PAT) in combination with this peptide showed a synergistic antileishmanial effect against intramacrophagic *L. tropica* amastigotes through some unknown mechanism [55]. As described, synergistic effects between leishmanicidal compounds and AMPs are not always detected. This is the case for curcumin, a natural compound derived from dried ground rhizome of the perennial herb *Curcuma longa* Linn (commonly known as turmeric), with anti-inflammatory, anti-cancer, anti-protozoal, anti-viral and anti-bacterial activity. Despite curcumin showing synergistic effects when applied in combination with paromomycin and miltefosine [68,69], such desirable outcomes were not detected when combining curcumin with CM11 hybrid peptide against *L. major* promastigotes or amastigotes [70,71].

Nowadays, there is global alarm concerning the development of multidrug resistance by microorganisms, and this has also been identified in *Leishmania* [72] and Chagas disease infections [73]. AMPs and CPPs have multiple mechanisms of action, making it difficult for pathogens to develop resistance [74]. Furthermore, resistance to AMPs and CPPs is complex, since it involves important changes in the phospholipid composition of cell membranes, which can result in pleiotropic effects on transport and enzymatic systems, seriously threatening the survival of microorganisms [75].

## 5. Antiparasitic and Immunomodulatory Activities of AMPs and CPPs

The antiparasitic activity of AMPs and CPPs, as we will detail later, is carried out using different mechanisms, for example, through rupture of the plasmatic membrane, alteration of calcium homeostasis (excessive accumulation of intracellular  $\text{Ca}^{2+}$  interferes with metabolism, disorganizing kinetoplast DNA and promoting autophagy and cell death) [76]. Even CPPs cross the membrane and tend to accumulate directly within the cytoplasm to carry out their antiparasitic activity by interfering with enzymatic activity and nucleic acid synthesis. These peptides may be involved not only in antiparasitic activity, but also in immunomodulatory functions, leading to proper regulation of the inflammatory response to reduce damage to different target organs and control infection [40,77].

In Table 1, in vitro studies of natural and synthetic AMPs and CPPs against *T. cruzi* and different *Leishmania* species are summarized. Additionally, many of them were obtained by synthetic routes, although in general they respond to primary structures in the same manner as in nature. In fact, a wide natural source for AMPs and CPPs with antimicrobial activity could be appreciated, including mammals (human host defense peptides account for a large proportion), plants, amphibians,

microorganisms, marine and insects. However, it is notable that mainly organisms used as primary sources are not common hosts of *Leishmania* or *T. cruzi* parasites.

Once internalized, the peptides and/or the cargo they carry can exert their antiparasitic activity. Two main mechanisms of action have been proposed: transient destabilization of membranes, and intracellular targets [77]. In the case of cationic peptides, it has been suggested that electrostatic interactions on the basis of differences in the compositions of the envelopes of host cells and parasites constitute the determining factor allowing preferential binding to the latter, using the host cell membrane as a means of passage. Binding to the parasite membrane induces its destabilization, which can lead to lysis, but also to a loss of membrane potential. In contrast, when the peptides are anionic, hydrophobic, and amphipathic, the mechanism is elusive. In other cases, the target may be intracellular, such as an enzyme or nucleic acids, or organelles as mitochondria [78,79]. As most AMPs are cationic, one of the mechanisms of action of such compounds is the selective binding to membrane from the parasites causing its disruption and pore formation [80]. Those pores might be formed by the dimerization of the peptides within the membrane upon these electrostatic interactions [81]. As previously mentioned, the differences in external membrane charge between *Leishmania* life stages is responsible for the dissimilarity in susceptibility to compounds between the promastigote and amastigote forms. The intracellular nature of amastigotes creates additional barriers for the leishmanicidal activity of peptides. In an attempt to increase our knowledge on this topic, delivery systems, combinations and chemical conjugation strategies have been tested. Since lipopeptides produced by *Bacillus* species target the cytoplasmic membrane and form ion-conducting pores in the lipid membrane, these lipopeptides are endowed of cytotoxicity towards human cells which limits their biomedical application. Their encapsulation in chitosan nanoparticles, which have previously exhibited antileishmanial potential through direct intercalation into the parasitophorous vacuole, enhanced the antileishmanial activity [82]. This improvement could be related to the progressive release of lipopeptides from chitosan delivery system [83]. This system had already showed effectiveness against experimental cutaneous leishmaniosis by using AmB as the incorporated drug [84]. In fact, among the strategies used to increase the therapeutic index and reduce the toxic effects of currently available chemotherapy against leishmaniosis, nanocarriers stand out as having shown potential as a site-specific drug delivery system [85].

### 5.1. Synthetic and Bioinformatic Tools

An important advantage of AMPs is their broad potential for synthetic modification. The molecular characterization of AMPs makes it possible to generate synthetic derivatives by modification of the primary sequence in order to improve some sides related to target specificity, cytotoxicity, potency, stability, or the active site [53,54,67,70,71,86]. This flexibility could enable the development of AMPs with optimized therapeutic properties for the treatment of both diseases. Among the most promising scaffolds for drug development, AMPs have been explored as potent antimicrobials because of their versatility and almost unlimited sequence space. These molecules can be easily tuned to achieve a broad spectrum, a specific activity, or cytotoxicity through changes in the amino acid residues that are part of their sequence. These changes give rise to variations in the structural and physicochemical properties that are closely related to their antimicrobial activity [87]. Cytotoxic activity may be enhanced by changing the amino acids. The synthesis of peptides bearing the sequence responsible for biological activity is also useful [61]. Additionally, among different kinds of peptide modifications, fatty acid conjugation to potentiate antimicrobial activity has been a topic of interest. However, the results have not always been as expected [56]. Either way, AMPs are toxins produced by organisms, such as frogs or snakes, and can require complex and expensive purification processes to be used as therapeutic agents. The synthetic production of AMP can also be expensive and time consuming, but this problem is often overcome through solid-phase peptide synthesis [88].

In addition, bioinformatics is a useful tool for active peptide selection. The physicochemical properties, structure and toxicity of peptides can be predicted using bioinformatic tools, in order to detect antimicrobial regions and to determine the charge, hydrophobicity, isoelectric point (pI), and

peptide mass. Cationic peptides bio-inspired by natural toxins have been recognized as an efficient strategy for the treatment of different health problems. Then, selected peptides sequences were synthesized and tested against cancer cells, bacteria and two *Leishmania* species [89]. Another choice is the generation of hybrids peptides. CM11, which consists of N-terminal domain of cecropin A and hydrophobic C-terminal domain of melittin, has demonstrated activity killing *Leishmania major* promastigotes and amastigotes, with no significant cytotoxicity to murine macrophages [47]. The production of recombinant peptides using cloning strategies has also been tested. The insect defensin rDef1.3 from *Triatoma pallidipennis*, a vector of *T. cruzi*, was produced by transformed *Escherichia coli* and purified using immobilized metal affinity chromatography. Then, its microbicidal activity was analyzed against trypanosomatids species, including two *Trypanosoma* species, as well as *L. major* and *L. mexicana*. Recombinant defensin caused atypical morphology and proliferative activity reduction in *Leishmania* parasites [90].

### 5.2. AMPs and CPPs for Combatting Different Forms of Leishmaniasis

The attractive biological activities of AMPs are prompting active research in the therapeutic application of these agents to combat many infectious diseases [91]. The first reports of the effects of AMPs on *Leishmania* were published in 1998, with *Hyalophora* (both) cecropin A [92] and cecropin A (1–8)–melittin (1–18) (CAMEL) hybrid peptides [93] and components of the target microorganisms, such as macromolecules and organelles [41,94]. To date, several groups of AMPs and CPPs, such as cathelicidins, cecropins, defensins, dermaseptins, eumenitins, histatins, magainins, melittins, and temporins, among others, have been proven to have significant action against diverse *Leishmania* species [67,91]. In this sense, relevant reviews have been published in recent decades [8,79], highlighting this group as an exciting alternative for designing new pharmaceutical alternatives against leishmaniasis and making evident the growing list of AMP and CPPs with antileishmanial activity [8]. Below, a compilation of previously published studies regarding the action of these molecules against *Leishmania* is discussed.

The problem faced by traditional drugs in crossing the protozoa membrane and accessing intracellular amastigotes is well known. AMPs are characterized by their high intracellular penetrability. Its antiprotozoal activity could be direct, altering membranes or focused on internal targets, including DNA, RNA and protein synthesis, lysosomal bilayer, altering key enzymatic activities and mitochondria [95]. In addition to their ability to permeabilize membranes, recent observations have shown that some peptides can also move to the cytoplasm of the microorganism cell and interact with intracellular targets, interfering with the cell wall, the synthesis of nucleic acids or proteins, and with enzymatic activity [96]. The high penetrability of AMPs will undoubtedly contribute to a faster mode of action than traditional drugs. This aspect is especially important in Chagas disease, preventing the disease from progressing to a chronic phase. AMPs, also as a result of their multiple mechanisms of action, could act synergistically with both conventional drugs (such as Bz and nifurtimox) and with other AMPs, leading to better treatment outcomes [97].

The phospholipase A2 (PPA2)-derived peptides are enzymes commonly present in the venom of organisms from all kingdoms, belonging to natural origin, and can hydrolyze phospholipids from cell membranes. Short peptides derived from PPA2 can cross the membrane showing effective activity against *Leishmania* promastigotes and amastigotes [98,99]. Interestingly, these cationic peptides, rich in lysines, increase their affinity when the lysines are substituted with arginines [100]. Another cationic peptide, tachyplesin, derived from the horseshoe crab (*Tachypleus tridentatus*) has potential against *Leishmania* spp and *T. cruzi* [101–103]. Tachyplesin is a 17mer peptide with a net positive charge that interacts with the parasite membrane, seriously compromising its integrity. Bovine lactoferrin-derived peptides leishmanicidal activity resides in its ability to permeabilize the membrane of promastigotes and axenic amastigotes of *L. donovani* [104].

CPPs can be coupled to cargos and translocated into the cell with high efficiency. Cargos include drugs or biological molecules such as DNA, antibodies, or proteins [105]. Since CPPs can be placed in the membrane of the target cell, they can penetrate into and accumulate inside the intracellular compartments, reaching higher local concentrations and overcoming one of the limitations associated

with common drugs [8,24]. This fact is very interesting, considering the limited concentrations that are possible to attain in plasma with common soluble drugs, which are still more limited in intracellular compartments. A few examples have been reported in this regard. Tachyplesine peptides are also able to transport plasmid EGFP-N1 inside the parasites, becoming fluorescent [103]. Other example is chylal cyclobutanes, which contain cell-penetrating peptides. These peptides are highly selective for *Leishmania donovani* parasites compared with HeLa cells [28]. While *Leishmania* promastigotes are not sensitive to free doxorubicin, the toxicity drops to  $< 1 \mu\text{M}$  when conjugated to these peptides, revealing a potential as vehicle. In addition, when conjugated with TAT (transactivator of transcription), doxorubicin also accumulates inside the parasite, although in lesser extent than with cyclobutane-CPPs [28]. TAT is a positively charged peptide derived from the protein TAT of HIV-1. TAT protein binds and activates RNA polymerase II during infection [106]. TAT is a CPP can pull cargos across membranes in different systems [107]. In *Leishmania*, TAT facilitates internalization and accumulation of the antiparasitic mitelfosine and paromomycin drugs [108–110].

As described, an appealing characteristic of AMPs is the ability to exert microbicidal activity by more than one mechanism. The strong post-transcriptional control of gene expression in trypanosomatids introduces *Leishmania* as a highly sensitive target to foreign RNases. This is the case for the ECP (Eosinophil cationic protein, a human antimicrobial protein), comprising RNase activity. Recently, Abengózar et al. [44] observed that ECP-treated *L. donovani* promastigotes showed a degraded RNA pattern. This is in agreement with the relation between the recruitment of eosinophils into *Leishmania* lesions and a favorable evolution. Just like paromomycin induces protein synthesis inhibition in *L. donovani* promastigotes [62], it has been hypothesized that xenocoumacin acts similarly on *L. tropica* promastigotes [111]. Recently, the modulatory effect of AMPs on *L. major* amastigote gene expression was shown as an additional mechanism [67]. The induction of autophagic cell death in the protozoan pathogen *L. donovani* has been described, as an AMP mode of action, Indolicidin, and two peptides derived from Seminalplasmin (SPK and 27RP), prompt programmed cell death pathways without affecting host cells [112].

In addition, nanodelivery strategies can enhance the activity peptides. Thus, the frog-skin-derived peptide dermaseptin, which has been shown to possess antileishmanial activity [113,114], was encapsulated into sub-micrometer Cry crystal proteins formed naturally by *Bacillus thuringiensis*, enhancing the target to macrophage lysosomes. The encapsulation of dermaseptin in Cry crystal proteins improved the leishmanicidal activity of dermasetin in both in vitro and in vivo infection models [51].

Currently, researchers are focusing their attention on the immunomodulatory ability of AMPs and CPPs. For instance, synthetic peptides derived from *Limulus* anti-LPS factor (LALF), 19-2.5 and 19-4 LF reduced the parasite burden in vivo when topically administered to *L. major* BALB/c-infected mice, by modulating the expression of host genes [67]. Although each peptide displayed its own pattern of cytokine modulatory activity, these peptides caused an increase in Th1 cytokine mRNA levels (*IL-12p35*, *TNF- $\alpha$*  and *iNOS*) in both the skin lesion and the spleen. In addition, in skin lesions from *Leishmania*-infected mice treated with the peptide 19-4LF, a decrease in *IL-4* and *IL-6* gene levels was detected, in agreement with the reduction in parasite burden in those samples [67]. Phylloseptin-1 (PSN-1), a peptide found in the skin secretion of the frog *Phyllomedusa azurea*, showed activity against *L. amazonensis* promastigotes [115] and amastigotes [116]. To understand the molecular changes associated with the leishmanicidal effect of PSN-1 against amastigotes, levels of key cytokines (*TGF- $\beta$* , *TNF- $\alpha$*  and *IL-12*) and the production of reactive species ( $\text{H}_2\text{O}_2$  and  $\text{NO}$ ) were assessed. The increase in *TNF- $\alpha$*  release caused by PSN-1 might have participated in the destruction of the amastigotes inside macrophages. The peptide was also observed to up- and down-modulate *IL-12 p70* production in infected macrophages, in a concentration-dependent manner. Probably, the immunomodulatory effect of the peptide favors the host instead of the parasite, by decreasing the pathogenesis while the peptide kills the parasite [116]. Amphibians are one of the most abundant reservoirs of AMPs in nature, and have also been explored against different species of *Leishmania* parasite. Dermaseptin, which was isolated from frog skin secretions of *Phyllomedusa* genera, has shown activity against *L. major* [101,117], *L. amazonensis* [118], *L. mexicana* [119], *L. panamensis* [101]

and *L. infantum* [120], agents that cause cutaneous or visceral leishmaniosis and are endemic in the New and Old World. Other frog-derived peptides inhibit the growth of parasites, such as bombinins H2 and H4 [121] and temporins [122,123] at micromolar concentrations. Substantial efforts have been invested in cecropin A and melittin, alone or in combination as hybrid molecules (CA-M). In this sense, shortened sequences [124], lipid N-terminal [125], and N-methylated Lys residues [126] have been designed showing relevant activity against *L. donovani* [93] and *L. pifanoi* [125]. In particular, our attention was drawn to interesting results displayed by plant thionins, with IC<sub>50</sub> values < 0.5 μM [127], making them among the most efficient antimicrobial peptides, and which also showed activity against other human pathogens [128,129]. However, one of the probable limitations to some of the included studies is related to antileishmanial activity with respect to the stage/form of the parasite targeted. In general, a high number of studies limited their results to the axenic amastigote (i.e., macrophage-free) or promastigote forms, which are not relevant in human infection. In *Leishmania*, the intracellular amastigote form is consistently more resistant, and growth in the hostile intramacrophagic habitat and membrane surface compositions most likely account for the differences observed upon comparison with promastigotes and axenic amastigotes [104]. In addition, few studies have demonstrated the use of AMPs and CPPs in animal models of infection by *Leishmania* parasites. In this sense, the therapeutic potential of CA-M analogues against canine leishmaniosis has been observed on the basis of infection control through the decrease in parasite burden and the reduction in disease symptoms [75]. The lauric acid conjugated form of brevinin, a defensin isolated from skin secretions, was administered alone and in combination with the CpG motif to treat BABL/c mice previously inoculated in the hind footpad with *L. major* metacyclic promastigotes. It is known that CpG motif application was helpful for inducing a specific immune response in experimental models. Brevinin was subcutaneously administered, whereas the CpG motif was applied via the intraperitoneal route five times over 10 days. In this case, in the fifth week after challenge, the group receiving lauric-acid-conjugated brevinine, as well as those treated with its combination with the CpG motif, showed a significant increase in footpad swelling compared to the group of mice treated with the reference drug Ambisome®, which was notably able to control the footpad swelling [130]. However, parasite load decreased in the popliteal lymph nodes adjacent to the infection site after peptide administration, more significantly in groups treated with the combination brevinin-CpG. Although the production of cytokines in the spleen of mice treated with brevinin did not coincide with parasite replication control results, since it was not, as expected, favorable for Th1 response, which is traditionally accepted as necessary for cutaneous leishmaniosis healing to occur [130]. Along the same lines, the frog-skin-derived peptide dermaseptin, with leishmanicidal activity, was encapsulated into crystal proteins and tested against a mouse cutaneous model of infection caused by *L. amazonensis*. This encapsulation strategy enhanced the peptide efficacy in the in vitro and in vivo infection models. Parasites were inoculated in the hind footpad and the formulated peptide was intralesionally administered a total of six times, every four days. Repeated injections efficiently inhibited the lesion growth compared to the free peptide administration. Similarly, encapsulated dermaseptin decreased the parasite burden in the footpads, whereas free peptide was unable to reduce the number of amastigotes in the lesions [51].

**Table 1.** AMP and CPPs with anti-protozoal activity against intracellular parasites *T. cruzi* or/and *Leishmania* spp.

| Peptide molecule               | Source                                     | Antiprotozoal Activity                   | Reference |
|--------------------------------|--------------------------------------------|------------------------------------------|-----------|
| Andropin                       | Synthetic                                  | <i>L. panamensis</i><br><i>L. major</i>  | [101]     |
| Anti-lipopolysaccharide factor | <i>Penaeus monodon</i> (Marine crustacean) | <i>L. braziliensis</i>                   | [102]     |
| BatxC                          | <i>Bothrops atrox</i> (Snake)              | <i>T. cruzi</i> (Y strain)               | [131]     |
| Bombinins H2 and H4            | <i>Bombina variegata</i> (Frog)            | <i>L. donovani</i>                       | [121]     |
| Cathelicidins (SMAP 29, PG-1)  | Synthetic                                  | <i>L. major</i><br><i>L. amazonensis</i> | [132]     |

|                                                                |                                                              |                                                                              |                |
|----------------------------------------------------------------|--------------------------------------------------------------|------------------------------------------------------------------------------|----------------|
| Cecropin A, D                                                  | <i>Drosophila</i><br><i>Hyalaphora cecropia</i>              | <i>L. aethiopica</i><br><i>L. panamensis</i>                                 | [67,92]        |
| Cecropin A- melittin                                           | Hybrid peptide                                               | <i>L. donovani</i><br><i>L. pifanoi</i>                                      | [93,125]       |
| Cecropin A, B and P1                                           | Synthetic                                                    | <i>L. panamensis</i><br><i>L. major</i><br><i>T. cruzi</i> (Tulahuen strain) | [101]<br>[133] |
| Chyral cyclobutanes                                            | Synthetic                                                    | <i>L. donovani</i>                                                           | [28]           |
| Clavanin A                                                     | <i>Styela clava</i> (Sea squirt)                             | <i>L. braziliensis</i>                                                       | [102]          |
| CM11 (cecropin-melittin hybrid)                                | Synthetic                                                    | <i>L. major</i>                                                              | [47]           |
| Cryptdin-1 and -4                                              | Rhesus macaque small bowel                                   | <i>L. major</i><br><i>L. amazonensis</i>                                     | [132]          |
| Ctn                                                            | <i>Crotalus durissus terrificus</i> (Rattlesnake)            | <i>T. cruzi</i> (Y strain)                                                   | [134]          |
| Defensin                                                       | <i>Phlebotomus duboscqi</i> (Sandfly)                        | <i>L. major</i><br><i>L. amazonensis</i>                                     | [67,135]       |
| Defensin $\alpha$ 1                                            | Human                                                        | <i>T. cruzi</i> (Tulahuen strain)                                            | [136,137]      |
| Defensin (fragments D, P, B, Q & E)                            | <i>Mytilus galloprovincialis</i> (Mussel)                    | <i>L. major</i>                                                              | [138]          |
| Dermaseptin                                                    | <i>Phyllomedusa sauvagii</i> (Frog)                          | <i>L. mexicana</i><br><i>L. panamensis</i><br><i>L. major</i>                | [101,119]      |
| Dermaseptin 01                                                 | Synthetic                                                    | <i>L. infantum</i>                                                           | [120]          |
| Dermaseptin-01, 02, 03, 04, 06 and 07                          | <i>Phyllomedusa hypochondrialis</i> (Frog)                   | <i>L. amazonensis</i>                                                        | [118]          |
| Dermaseptin S1 analogues                                       | Synthetic                                                    | <i>L. major</i>                                                              | [117]          |
| Dhvar4 (histatin 5 analog)                                     | Synthetic                                                    | <i>L. donovani</i>                                                           | [139]          |
| DS 01                                                          | Frog<br><i>Phyllomedusa oreades</i>                          | <i>T. cruzi</i> (Y strain)                                                   | [140]          |
| Enterocin AS-48                                                | <i>Enterococcus faecalis</i>                                 | <i>L. pifanoi</i>                                                            | [141]          |
| Enterocin AS-48 homologs                                       | Synthetic                                                    | <i>L. donovani</i>                                                           | [45]           |
| Eumenitin                                                      | <i>Eumenes rubronotatus</i> (wasp venom)                     | <i>L. major</i>                                                              | [67]           |
| Gomesin                                                        | <i>Acanthoscurria gomesiana</i> (Tarantula)                  | <i>L. amazonensis</i>                                                        | [142]          |
| Histatin 5 (L and d-enantiomers)                               | Synthetic                                                    | <i>L. donovani</i><br><i>L. pifanoi</i>                                      | [139]          |
| Hmc364-382                                                     | <i>Dpenaeus monodon</i> (shrimp)                             | <i>T. cruzi</i> (Y strain)                                                   | [143]          |
| Indolicidin                                                    | Synthetic                                                    | <i>L. donovani</i>                                                           | [112]          |
| Lactoferricin (17-30)<br>Lactoferrampin (265-284)<br>LFchimera | Bovine milk lactoferrin (domain N1)                          | <i>L. pifanoi</i><br><i>L. donovani</i>                                      | [104]          |
| LTP2 $\alpha$ -1                                               | <i>Hordeum vulgare</i> (Barley)                              | <i>L. donovani</i>                                                           | [127]          |
| M-PONTX-Dq3a[1-15]<br>/ [Lys] <sup>3</sup> -M-PONTX-Dq3a[1-15] | <i>Dinoponera quadricaps</i> (Ant)<br>Synthetic modification | <i>T. cruzi</i> (Y strain)                                                   | [144,145]      |
| Magainin                                                       | <i>Xenopus laevis</i> (Frog)                                 | <i>L. braziliensis</i><br><i>L. major</i>                                    | [67,102]       |
| Magainin analogues (MG-H1 / H2) & F5W-magainin 2               | Synthetic                                                    | <i>L. donovani</i><br><i>L. amazonensis</i>                                  | [146]          |
| Melittin                                                       | Bee venom<br><i>Apis mellifera</i>                           | <i>L. donovani</i><br><i>L. infantum</i><br><i>L. panamensis</i>             | [67,101,147]   |

|                                                                    |                                                                     |                                                                                                                       |                   |
|--------------------------------------------------------------------|---------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------|-------------------|
|                                                                    |                                                                     | <i>L. major</i><br><i>T. cruzi</i> (CL Brener strain)                                                                 |                   |
| Mylitin A                                                          | Mussel<br><i>Mytilus edulis</i>                                     | <i>L. braziliensis</i>                                                                                                | [102]             |
| NK2                                                                | Synthetic                                                           | <i>T. cruzi</i> (Tehuantepec strain)                                                                                  | [148]             |
| Ovispirin                                                          | Synthetic                                                           | <i>L. major</i><br><i>L. amazonensis</i>                                                                              | [132]             |
| p-Acl and analogue p-AclR7                                         | Synthetic cationic peptides                                         | <i>L. amazonensis</i><br><i>L. infantum</i>                                                                           | [99]              |
| Penaeidian-3                                                       | Whiteleg shrimp<br><i>Litopenaeus vannamei</i>                      | <i>L. braziliensis</i>                                                                                                | [102]             |
| Rhesus                                                             | Synthetic                                                           | <i>L. major</i><br><i>L. amazonensis</i>                                                                              | [132]             |
| Phylloseptin-1                                                     | Synthetic                                                           | <i>L. amazonensis</i>                                                                                                 | [115]             |
| Polybia-CP                                                         | <i>Polybia paulista</i> (wasp)                                      | <i>T. cruzi</i> (Y strain)                                                                                            | [149]             |
| PTH-1                                                              | <i>Solanum tuberosum</i> (Potato)                                   | <i>L. donovani</i>                                                                                                    | [127]             |
| Pr-1, 2 and 3                                                      | Synthetic                                                           | <i>L. panamensis</i><br><i>L. major</i>                                                                               | [101]             |
| Pylloseptin 7                                                      | <i>Phyllomedusa nordestina</i> (Frog)                               | <i>T. cruzi</i> (Y strain)                                                                                            | [150]             |
| SALPs                                                              | Synthetic                                                           | <i>L. major</i>                                                                                                       | [67]              |
| Snakin-1                                                           | <i>Solanum tuberosum</i> (Potato)                                   | <i>L. donovani</i>                                                                                                    | [127]             |
| Seminalplasmin (SPK & 27RP)                                        | Synthetic                                                           | <i>L. donovani</i>                                                                                                    | [112]             |
| StigA25                                                            | <i>Tityus stigmurus</i> (Scorpion)-<br>Synthetic                    | <i>T. cruzi</i> (Y strain)                                                                                            | [151]             |
| Tachyplesin                                                        | <i>Tachyplesus tridentatus</i><br>(Horseshoe crab)                  | <i>L. panamensis</i><br><i>L. major</i><br><i>L. braziliensis</i><br><i>L. donovani</i><br><i>T. cruzi</i> (Y strain) | [101–<br>103,152] |
| TAT (48-57) peptide<br>TAT (48-60) peptide<br>TAT and polyarginine | TAT (transactivator of<br>transcription) protein from HIV-1         | <i>L. donovani</i><br><i>L. infantum</i>                                                                              | [28,108,109<br>]  |
| Temporins A and B                                                  | <i>Rana temporaria</i> (Frog)                                       | <i>L. donovani</i><br><i>L. pifanoi</i>                                                                               | [67,123]          |
| Temporin-1Sa, 1Sb and 1Sc                                          | <i>Pelophylax saharica</i> (Frog)                                   | <i>L. infantum</i>                                                                                                    | [122]             |
| Temporizin-1                                                       | Synthetic                                                           | <i>T. cruzi</i> (Y strain)                                                                                            | [153]             |
| Thionin $\alpha$ -1, $\alpha$ -2 and $\beta$ type I                | <i>Triticum aestivum</i> (Wheat)<br><i>Hordeum vulgare</i> (Barley) | <i>L. donovani</i>                                                                                                    | [127]             |
| [Arg] <sup>11</sup> -VmCT1                                         | <i>Vaejovis mexicanus</i> (Scorpion)                                | <i>T. cruzi</i> (Y strain)                                                                                            | [154]             |

### 5.3. AMPs and CPPs to Combat *T. cruzi* Infection

We carried out an exhaustive bibliographical review of those studies that describe the anti-*T. cruzi* activity of AMPs and CPPs. It should be noted that in our search, as in recent reviews by other authors [80], we did not find studies that involved the analysis of these peptides from plants with activity against *T. cruzi*. Additionally, despite the wide variety of studies of peptides with in vitro antiprotozoal potential, there is currently no evidence to support the existence of AMPs or CPPs with in vivo anti-*T. cruzi* activity. This fact demonstrates once again the difficulty of obtaining a definitive treatment against this neglected zoonosis. The development of effective treatments for *T. cruzi* infection remains a great challenge, and more research is needed to identify new therapies. The first report of the effects of an AMP against *T. cruzi* was described in 1988. In that year, Jaynes et al. [133] demonstrated that two analogues of cecropin B, were 50% and 100% effective, respectively, in killing *T. cruzi* trypomastigotes in vitro. These analogues varied only slightly from cecropin B in amino acid sequence homology, but the charge distribution, amphipathic, and hydrophobic properties of the

natural molecule were conserved. Since then, many natural or synthetic AMPs have shown activity against *T. cruzi*.

*Bothrops atrox* is a snake of great medical importance in the Amazon. Its venom is specialized at killing prey in nature, but it is also a source of peptides with antiprotozoal potential. Indeed, Batroxidin (BatxC) is a CPP extracted from its venom with trypanocidal activity [131]. The peptide was more active against all three morphological forms of the *T. cruzi* Y strain than benznidazole, as a control drug, and with very high selectivity. After 24 hours of incubation, BatxC was able to significantly reduce the number of amastigotes at an  $IC_{50}$  of 0.44  $\mu$ M, (Bz  $IC_{50}$ =282  $\mu$ M). As a proposed mechanism of parasite killing, BatxC can kill epimastigotes by ROS generation, pore formation, and cell membrane degradation, inducing necrosis. Crotalicidin (Ctn) is an antiprotozoal peptide obtained from *Crotalus durissus terrificus* rattlesnake's venom gland. The peptide exhibited activity against all morphological forms of *T. cruzi* Y strain showing a high selectivity index (>200) [134]. Therefore, *T. cruzi* trypomastigote and amastigote forms appear to be more susceptible to Ctn than epimastigotes. This special susceptibility may be related to the prevalence of negatively charged molecules in the parasite's cell membrane, especially in trypomastigotes. The studies carried out reveal that the mechanism of cell death induced by Ctn seems to be necrosis and late apoptosis. Furthermore, the peptide showed higher selectivity for the parasite compared to Bz. The results make Ctn a promising lead for the development of new peptide-based drugs to treat Chagas disease. Two fragments of the AMP M-PONTX-Dq3a, isolated from the *Dinoponera quadricaps* ant venom are M-PONTX-Dq3a[1-15] and [Lys]<sup>3</sup>-M-PONTX-Dq3a[1-15]. Both fragments have been demonstrated to possess trypanocidal activities similar to those of the parent peptide against all three forms of *T. cruzi* Y strain but with lower toxicity, better bioavailability and lower cost of production [144,145]. Due to their reduced peptide length, both fragments would have a better chance of reaching clinical application. Mechanism studies on the form of epimastigotes have revealed the necrotic pathway of the new peptides, through plasma membrane disruption and mitochondrial DNA fragmentation. Stigmurin (StigA25) is an AMP obtained from the venom gland of the scorpion *Tityus stigmurus*. StigA25 was synthesized by replacing the uncharged, polar Ser and Gly residues of the native peptide with positively charged Lys residues to increase the positive charge of the StigA25 [151]. The antiparasitic activity of StigA25 was evaluated in the epimastigote form of *T. cruzi* Y strain and the peptide induced 90% parasite death after 24 h at concentrations below 12.5  $\mu$ M. The same antiparasitic pattern has been seen against the trypomastigote form, reaching 100% death at 25  $\mu$ M. StigA25 showed higher antiparasitic activity in the lowest concentrations and shorter incubation time tested compared to Bz. Although the possible antiparasitic mechanism of action of the peptide has not been reported up to now, StigA25 is stable to variations in pH and temperature, characteristics that make the peptide a good candidate as an anti-chagas drug. Another antiprotozoal peptide is [Arg]<sup>11</sup>-VmCT1, which is isolated from the venom of the scorpion *Vaejovis mexicanus* [154]. The Arg-substitution at position 11 improved the potency and selectivity of natural type AMP VmCT1 against the three developmental forms of *T. cruzi*, maintaining its necrotic mechanism of action. The peptide, at concentrations lower than 1  $\mu$ M, was able to reduce the number of *T. cruzi* amastigotes by around 50% after 24 h of incubation. The results of in vitro efficacy and low cytotoxicity revealed that [Arg]<sup>11</sup>-VmCT1 is a promising candidate for the development of new anti-Chagas therapies. One more marine CPP with anti-*T. cruzi* activity is tachyplesin-I. It is a host defense peptide from the horseshoe crab *Tachyplesus tridentatus*, whose antileishmanial activity we have already mentioned, and even possesses anticancer properties [102]. Tachyplesin-I was more potent against trypomastigote than epimastigote forms of *T. cruzi*, since the former were completely killed by a much lower peptide concentration (12.5  $\mu$ M,  $LD_{50}$ = 9.3  $\mu$ M after 7 h incubation) than the latter (100  $\mu$ M), and in a shorter time. Moreover, tachyplesin-I did not show any cytotoxic effect against Vero cells at concentrations > 40  $\mu$ M. Again, these differences, might be explained by the distinct surface composition of both parasite forms. According to Souto-Pradón [152], the epimastigote forms have the least negative surface charge among the different developmental stages of *T. cruzi*, whereas trypomastigotes have the most negative surface. In any case, the antiparasitic mechanism has not yet been described. A promising antichagasic hemocyanin fragment obtained from the *Penaeus monodon* shrimp is Hmc364-

282 [143]. The peptide showed good effect and high selectivity against the epimastigote, trypomastigote and amastigote forms of *T. cruzi* Y strain, and was clearly more active and less cytotoxic than Bz (Hmc364-382 was 77 times more active and 45 times less cytotoxic). The studies carried out on the type of cell death produced by the peptide reveal necrosis as the mechanism of action. Polybia-CP is a wasp venom AMP that has been reported as a potent trypanocidal agent [149]. The peptide was able to inhibit the main developmental forms of *T. cruzi* with higher efficacy and less cytotoxicity than the standard Bz. The great efficacy of Polybia-CP against intracellular amastigotes confirms its high penetrability into the parasite (decrease in the number of amastigotes by 38% after 24 h of incubation). The mechanism of action by which Polybia-CP exerted its antichagasic activity was via an apoptosis-like process. The peptide did not damage the membrane of the parasite, even at higher concentrations than its EC<sub>50</sub>. The mentioned characteristics make Polybia-CP an interesting scaffold for the development of novel anti-chagas therapies. Melittin is the main toxic component in *Apis mellifera* venom. In vitro assays demonstrated that the AMP melittin affects all of *T. cruzi* (CL Brener clone) developmental forms at low concentrations (up to 1 µg/ml) with low toxicity in mammalian cells [147]. It has been suggested that the mechanism of action of Melittin depends on the parasite form. According to this, the main mechanism of cell death in epimastigotes and amastigotes would be autophagy. Conversely, for trypomastigote form, melittin could produce cell death via apoptosis. In any case, melittin does not appear to affect the plasma membrane of the trypanosome. The in vitro activity and the different mechanisms of action confirm the great potential of melittin for the development of new therapies against neglected diseases, such as Chagas disease. Dermaseptin 01(DS 01) is a 29-residue-long peptide isolated from the skin secretion of the frog *Phyllomedusa oreades*. Bioassays revealed that DS 01 is a potent anti-*T. cruzi* Y strain agent (reduction of epimastigotes and trypomastigotes to an undetectable level at 6 µM after 2 h of incubation). The peptide induced the death of the parasites most likely by membrane disruption and cell leakage. The results point to the trypanocidal activity of this AMP [140]. Pylloseptin 7 is a natural AMP isolated from *Phyllomedusa nordestina* frog secretion with antitrypanosomal activity of 1296-fold higher than Bz [150]. The peptide targets the plasma membrane of *T. cruzi* leading cellular death by permeabilization, with an IC<sub>50</sub> of 0.34 µM against *T. cruzi* trypomastigotes. Pylloseptin 7 is a promising scaffold for the design of new antichagasic drugs. Defensin-α1 is a biologically active human AMP with demonstrated in vitro trypanocidal effect. Reported assays indicate that this human peptide kills *T. cruzi* Tulahuen strain tripomastigotes and amastigotes in a peptide-concentration-dependent and saturable manner, with amastigotes being more susceptible [136,137]. It appears that the toxicity of human Defensin-α1 against *T. cruzi* is mediated by the formation of membrane pores and the induction of nuclear and mitochondrial DNA fragmentation, leading to trypanosome destruction. It seems that the peptide is inserted into the trypanosome membrane, forming pores that facilitate the entry of the peptide that causes the death of the parasite. These results suggest that Defensin-α1 plays a beneficial role in reducing *T. cruzi* infection of human cells. Therefore, the development of Defensin-1 derivatives could be beneficial for the discovery of new anti-*T. cruzi* agents. NK-2 is a shortened synthetic peptide, formed by the cationic core region comprising residues 39 to 65 of porcine NK-lysine. Although both natural NK-lysine and NK-2 (IC<sub>50</sub>= 2.5 µM) have been shown to be capable of killing trypomastigotes (Tehuantepec strain), NK-2 demonstrated greater safety for human cells [148]. In addition, NK-2 also inhibits the replication of intracellular amastigotes. Despite studies having been carried out, the possible mechanism of action of NK remains unclear, although the peptide quickly permeabilizes the parasite's plasma membrane within minutes. This indicates that the parasite's plasma membrane is targeted by NK-2, making the peptide a potential trypanocidal drug. Temporizin 1 is a synthetic hybrid peptide, containing the N-terminal region of Temporin A (produced by *Rana temporaria*), the pore-forming region of Gramicidin, and a C-terminus consisting of alternating leucine and lysine [153]. Temporizin-1 is an improved version of Temporicin, created by shortening the four residues related to the gramicidin ionic channel pore, the origin of the unique mode of action of the peptide. The trypanocidal effect of Temporicin-1 was studied in *T. cruzi* Y strain epimastigotes and was dose-dependent with EC<sub>50</sub> = 817.3 ng/ml at 1.5 hours of incubation. Additionally, at concentrations up to 100 µg/ml, the peptide showed low toxicity.

According to this, temporizin-1 improves the antitrypanosomal activity of temporizine and gramicidin, in addition to showing less cytotoxicity. Regarding the mode of action, Temporizin-1 seems to produce alterations in mitochondria and nuclear DNA, albeit, curiously, with no alterations in the plasma membrane. Its toxicity is based on the different composition of mammalian cell membranes and that of trypanosomes. Temporizin-1 appears to form ion channels in mammalian cell membranes, generating low toxicity. However, its toxicity towards trypanosomes seems to be attributed to an intracellular effect rather than pore formation. This peptide has the desired characteristics to be a good candidate for the development of new antitrypanosomal drugs.

## 6. Biological Models to Evaluate the Activity of AMPs and CPPs

The development of safe, effective, and efficient therapies and vaccines depends on the selection of appropriate biological models and a proper understanding of the advantages and limitations of these models, since a well-designed biological model provides a solid foundation for supporting good science and ensuring the most beneficial use of resources. Biological models include: *in vitro*, *ex vivo*, and *in vivo* animal models that, in the case of infectious disease research, are intended to emulate the biological phenomenon of interest for a disease occurring in a human or animal. Biological models of infectious diseases, such as leishmaniosis and Chagas disease, allow in-depth research of the molecular mechanisms of the pathology, high-throughput studies of new drugs and genetic targets, and visualization of the specific effects of new molecules on these microorganisms. Therefore, these models allow scientists to obtain a complete overview of these diseases and to perform detailed and efficient studies of possible diagnostic methods and new therapeutic alternatives.

### 6.1. Cell Lines and Primary Cell Cultures for Cytotoxicity Assays

Over the years, several types of cell line and primary cell culture have been used to determine the cytotoxic activity of new molecules *in vitro*. Among the most widely used are *Cercopithecus aethiops* (African green monkey) normal epithelial kidney cells VERO (ATCC® CCL-81™); *Maccaca mulata* (rhesus monkey) normal epithelial kidney LLC-MK2 (ATCC® CCL-7™); *Mus musculus* (mouse) embryo fibroblast NIH/3T3 (ATCC® CRL-1658™); *Mus musculus* (mouse) macrophages from reticulum cell sarcoma J774A.1 (ATCC® TIB-67™); *Rattus norvegicus* myoblast H9c2(2-1) (ATCC® CRL 1446™); *Canis familiaris* canine macrophage DH82 cell line; *Homo sapiens* normal tissue lung fibroblast MRC-5 (ATCC® CCL-171™); *Homo sapiens* epithelial bone osteosarcoma U-2 OS (ATCC® HTB-96™); and *Homo sapiens* tissue monocytes U-937 (ATCC® CRL-1593.2™) [155–158].

Nonetheless, considering the biology of the *Leishmania* spp. and *T. cruzi* parasites, the most recommended cell types are those isolated from humans, such as U-937 tissue monocytes for both parasite species, because these cells meet the parasites at the site of the insect vector's bite. Additionally, for *T. cruzi*, other recommended cells include *Homo sapiens* aorta smooth muscle fibroblast-like T/G HA-VSMC (ATCC® CRL 199™), *Homo sapiens* normal esophagus epithelial cells Het-1A (ATCC® CRL-2692™) and *Homo sapiens* normal colon epithelial cells FHC (ATCC® CRL-1831™), because these are tissues for which the different strains of *T. cruzi* exhibit tropism.

In addition, for *in vitro* cytotoxicity tests, it is also advisable to carry out an initial screening for liver and kidney toxicity, for which the most recommended cells would be the *Homo sapiens* liver epithelial-like cell Hep G2 (ATCC® HB-8065™) and *Homo sapiens* kidney epithelial tissue 293T (ATCC® CRL-3216™). Most of these cells are maintained in culture at 37°C and 5% CO<sub>2</sub> and >95% humidity atmosphere in DMEM or RPMI medium (except for the FHC cells which are cultured in DMEM: F12 medium) supplemented with 5%–10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin or 50 µg/mL of gentamycin. Because some cell lines may require other specific nutrients, it is recommended to follow the American Type Culture Collection (ATCC) instructions (<https://www.atcc.org/>) for the handling of each cell line. Primary culture cells include splenocytes, peritoneal macrophages, and bone marrow-derived macrophages or dendritic cells obtained from BALB/c mice and hamsters cultivated in RPMI-1640 medium supplemented with 10% of FBS and 50 µg/mL of gentamycin or 100 U/ml penicillin and 0.1 mg/ml streptomycin.

Cytotoxic activity is expressed as the concentration of AMPs and CPP compounds capable of reducing the cell viability of cells by 50% after 48 h of treatment, expressed as median cytotoxic concentration (CC<sub>50</sub>) or median lethal concentration (LC<sub>50</sub>).

### 6.2. Antiparasitic Activity

In vitro antileishmanial activity is routinely evaluated using *Leishmania* promastigotes grown at 26 °C in Schneider's insect medium, supplemented with 10% (v/v) heat-inactivated fetal bovine serum, plus the antibiotic mixture of penicillin and streptomycin. Like cells, many parasite species/strains are used freely to evaluate the antiparasitic activities of new molecules. In the case of *Leishmania* spp., due to the large number of described species that are pathogenic for humans, in cutaneous leishmaniasis, the species that are more prevalent in specific regions are used, such as *L. braziliensis*, *L. panamensis*, *L. amazonensis* or *L. mexicana* in Central and South America, or *L. major* and *L. tropica* in Europe and Asia, while for visceral leishmaniasis, *L. infantum* are used in America and *L. donovani* are used in Europe, Asia and Africa. For some of the different *Leishmania* species, there are strains expressing the green fluorescent protein (GFP) gene as well as the luciferase (LUC) gene.

Similarly, to evaluate the trypanocidal activity of a compound, in vitro assays are traditionally used with forms of *T. cruzi* epimastigotes cultured in LIT hepatic tryptose infusion at 28 °C plus 10% fetal bovine serum (FBS), 10 U/mL penicillin, and streptomycin. A 10 µg/mL concentration is usually incubated, at pH 7.2, in the presence of the candidate compound to be evaluated, and collected during the exponential growth phase. For *T. cruzi*, the most frequently used strains are Tulahuen strain Discrete Typing Unit (DTU) VI clone C2 and C4, expressing beta-galactosidase gene [155,157,159–163] or luciferase gene Luc2-Tulahuen [164], Y strain DTU II wild type [165,166] or expressing the firefly luciferase gene [159], Sylvio X10 strain DTU I [156], Dm28c strain DTU I [163], RA strain DTU VI [167–169] and VD strain DTU VI [170].

In both types of assay, the corresponding parasite forms are seeded and incubated with different concentrations of the candidate compound whose activity is to be evaluated [171,172]. The EC<sub>50</sub> is calculated based on the mean percentage reduction of parasites compared to the untreated controls. The index of selectivity is calculated by dividing the LC<sub>50</sub> or CC<sub>50</sub> by the EC<sub>50</sub> (IS = LC<sub>50</sub> or CC<sub>50</sub> /EC<sub>50</sub>).

### 6.3. In Vivo Models

Experimental animal models are models in which the scientist induces a disease or condition in animals [173]. In this case, these models can be perfectly adapted to evaluate the efficacy of AMPs and CPPs in combatting neglected zoonoses. The One Health approach extends beyond conventional zoonotic disease control models to consider the interactions of human and animal health systems within their shared environment and the broader social and economic context. Thus, these study models are an essential part of the fight against neglected zoonoses [174], which are the subject of this review. In addition, it is necessary to carefully adjust to the international guidelines of the Care and Use of Laboratory Animals to achieve scientific progress, always considering the “Four Rs principles” (Reduction, Refinement, Replacement and Responsibility) and other fundamental ethical considerations. Consequently, the use of experimental animals should be avoided in those research areas where alternative in vitro or in silico methods are available. The choice of the most suitable animal model must be undertaken on the basis of a process of careful analysis in order to combine animal welfare with scientific progress [175].

There are no standardized protocols for the evaluation of compounds in animal models, and researchers have described different protocols. The species most widely used as animal models of the main forms of leishmaniasis and Chagas diseases are the Syrian golden hamster (*Mesocricetus auratus*), BALB/c, C57BL/6, and albino Swiss mice [38,176–180]. The use of dogs (the main reservoirs of zoonotic visceral leishmaniasis VL caused by *L. infantum* in the Mediterranean area, Middle East, Asian countries, and Latin America) as experimental models has led to great advances in the development of vaccines and immunotherapy against VL [181]. Remarkably, the dog is a good model for chemotherapy studies not only for leishmaniasis, but also for Chagas disease, and may be indicated for preclinical trials of new treatments [182]. Dogs mimic human disease, as they are able

to reproduce the clinical and immunological findings described in chagasic patients and can develop cardiomyopathy [180,183]. In contrast to rodent models of *T. cruzi* infection, all clinical stages of Chagas disease can be consistently reproduced in Syrian hamsters, including the chronic phase, with cardiomyopathy [184].

## 7. Challenges to Overcome Regarding the Current Limitations of AMPs and CPPs

The numerous advantages of peptide molecules are evident; however, several drawbacks remain to be resolved for their use as therapeutic agents. Considering their peptide nature, AMP and CPP molecules generally have low stability. Stability issues can be addressed by using specialized delivery methods, such as nanoparticles or liposomes, to enable them to reach their target sites in the body. However, this requires an increase in the complexity and cost of treatment, which is something to consider in the case of neglected diseases, thus compromising patients' adherence to the therapy [15,185]. Peptide molecules can be susceptible to degradation by enzymes (proteases) in the body, which can limit their effectiveness as therapeutic agents [186]. Furthermore, because of their low stability, peptide molecules are expected to have a short half-life in vivo, which means that they may need to be administered frequently and in high doses to maintain therapeutic levels in the body. This can increase the risk of toxicity, although it seems that these peptides are non-toxic to human cells and have minimal side effects. Furthermore, AMPs with clinical application generally bind at low doses; they are part of the innate immune system, and should therefore be well tolerated by the body [187]. However, some AMPs may also be recognized as foreign by the immune system, leading to an immune response that could reduce their effectiveness as therapeutic agents [188]. As mentioned above, a handicap for CPPs entering by endocytosis is their need to be equipped with molecules capable of lysing the endosome and reaching their target [18], thus overcoming the drawback of endosomal entrapment.

## 8. Emerging Biotechnological Tools: Future Directions

To face the challenges presented by the current limitations of AMPs and CPPs, different research teams have worked on reducing the size of drug carriers that have dimensions on the nanometric scale and, consequently, are capable of being more easily internalized in cells, reaching specific intracellular locations. Among the different systems that allow the encapsulation of AMPs and CPPs are liposomes, dendrimers, solid-core nanoparticles, carbon, and DNA nanotubes. These systems can be functionalized with different biomolecules (antibodies, cell penetrating peptides, carbohydrates, and aptamers) that provide binding specificity to different cell types or locations. Of these, aptamers are coming to be of great interest due to their properties and characteristics [189]. Aptamers are single-stranded folded nucleic acids (RNA or ssDNA) that are able to specifically recognize target molecules with high affinity. The term aptamer, derived from the Latin word "aptus", which means to fit, was first introduced by the Nobel laureate J.W. Szostak and A.D. Ellington [190], when they described the in vitro selection of RNA molecules that bind specifically to a variety of organic dyes. Aptamers are selected through an "in vitro" process called SELEX (Systematic Evolution of Ligands by Exponential enrichment), developed by L. Gold and C. Tuerk [191].

The selection process begins with the synthesis of an oligonucleotide library consisting of a central region with random sequence flanked by constant 5' and 3' ends that serve as primers (Fig. 1). Every member of the library is a linear oligonucleotide with a unique sequence that acquires a three-dimensional structure on the basis of the experimental conditions (pH, ionic strength, temperature, etc.) or the presence of a ligand [192]. These highly structured aptamers are capable of binding to the target with high affinity and specificity. The diversity of an oligonucleotide library is dependent on the number of random nucleotides containing each oligonucleotide molecule. Thus, an oligonucleotide library whose molecules contain a random sequence of 40 nucleotides ( $4^{40}$ ) would be represented by  $1.2 \times 10^{24}$  different sequences. However, in practical terms, the complexity of a combinatorial library of oligonucleotides is limited to between  $10^{12}$  and  $10^{18}$  different individual sequences [193].

After the SELEX process, the selected aptamer population may act like polyclonal antibodies. Subsequently, individual aptamers may mimic monoclonal antibodies. When compared to antibodies, aptamers possess various advantages: (i) they can be obtained against non-immunogenic proteins; (ii) they can be regenerated; (iii) they are stable under a wide range of environmental conditions; (iv) they can be chemically modified; (v) there is no need for cell culture or experimentation animals to produce them; (vi) they can be produced with high reproducibility; and (vii) they can be labeled with a great variety of fluorochromes.

During the last 20 years, many aptamers targeting parasite proteins have been described that could eventually be used for the diagnosis and treatment of different infections, representing a viable and promising strategy that could compete with the development of other currently used drugs. On the other hand, application of AMPs and CPPs in the treatment of intracellular pathogens has been limited by their in vivo instability and low penetrating ability into mammalian cells. In this sense, aptamers have shown enormous potential as tools for targeting other drugs, something that could be of great interest in the case of AMPs and CPPs.

In fact, several articles have already been published in which aptamers are used for this purpose. Thus, Lee et al. demonstrated that an AMP, HPA3PHis, loaded in a conjugate of gold nanoparticles and DNA aptamers is an effective therapeutic tool against *V. vulnificus* infection in vivo in mice, observing a complete inhibition of the colonization of *V. vulnificus* in different organs in the murine model, leading to a 100% survival rate among treated mice, whereas all control mice died within 40 hours of infection [194,195]. Similarly, Pourhajibagher et al. investigated the antimicrobial effects of dermcidin-derived DCD-1L peptide loaded on aptamer-functionalized emodin nanoparticles (Apt@EmoNp-DCD-1L) against *Enterococcus faecalis* as one of the most common bacteria implicated in recurrent root canal treatment failures. The results showed that the cell viability of *E. faecalis* exposed to Apt@EmoNp-DCD-1L was significantly reduced compared to the control group ( $P < 0.05$ ), and that Apt@EmoNp-DCD-1L in combination with a blue laser light was able to enhance the antibiofilm activity of aPDT against *E. faecalis* biofilm. Data obtained from qRT-PCR analysis showed a significant reduction in the expression level of genes involved in bacterial biofilm formation after exposure to aPDT ( $P < 0.05$ ) [196]. On the other hand, Yeom et al. reported that gold nanoparticles conjugated with DNA aptamer (AuNP-Apt) efficiently delivered AMPs into mammalian living systems with enhanced stability of the AMPs. Thus, the authors showed that C-terminally 6XHIS-tagged A3-APO AMPs loaded onto AuNPs conjugated with His-tag DNA aptamer (AuNP-Apt (His)) was delivered into *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*)-infected HeLa cells, resulting in the increased viability of host cells due to the elimination of intracellular *S. Typhimurium* cells. Furthermore, the intravenous injection of AuNP-Apt (His) loaded with A3-APO(His) into *S. Typhimurium*-infected mice resulted in a complete inhibition of *S. Typhimurium* colonization in the mouse organs, leading to 100% survival of the mice. Therefore, AuNP-Apt (His) can serve as an innovative platform for AMP therapeutics to treat intracellular bacterial infections in mammals [197].

Conversely, Macleod et al. showed that LL-37, an important immunomodulatory protein that is upregulated in several inflammatory skin diseases, such as psoriasis, rosacea and eczema, forms complexes with the IL-17A-recognizing RNA aptamer (Apt 21-2). In contrast to free Apt 21-2, Apt 21-2 complexed with LL-37 was found to effectively penetrate both keratinocytes and fibroblasts, and remained immunologically inert in keratinocytes, fibroblasts, and peripheral blood mononuclear cells, including dendritic cells and infiltrating monocytes. The results of this study suggest that RNA aptamers administered in LL-37-rich inflammatory medium can form complexes and subsequently be internalized by the surrounding skin cells [198].

In recent years, several papers have been published in which CPPs and aptamers were used for the development of cancer therapeutic systems. In fact, the first article referring to the use of CPPs and aptamers for cancer treatment was published by Sakai et al. in 2014 [199]. In this article, the authors identified specific aptamers against the Ca (2+)-ATPase 2a (SERCA2a)- phospholamban (PLN) from the sarcoplasmic reticulum, which plays a key role in regulating the intracellular Ca (2+) cycle in ventricular cardiomyocytes. One of these optimized aptamers (RNA-Apt30) showed high affinity for the cytoplasmic region of PLN, but did not bind to the phosphorylated form of PLN or to a

phosphomimetic mutant. Conjugation of the RNA-Apt30 aptamer to a cell-penetrating peptide allowed its delivery into adult rat cardiomyocytes, where it improved both Ca (2+) transients and contractile function. These effects of the aptamer were also evident in the presence of the  $\beta$ -adrenergic receptor antagonist propranolol. This cell-penetrating PLN aptamer may provide a basis for the development of novel HF therapeutics without the need for gene transfer or a change in endogenous protein expression. Diao et al. addressed the specificity and efficiency of SURVIVIN-siRNA delivery by constructing a therapeutic complex expressing an STD fusion protein, consisting of streptavidin, a cell-penetrating peptide called Trans-Activator of Transcription (TAT), and a domain of double-stranded RNA binding, to which biotinylated prostate-specific membrane antigen (PSMA)-specific A10 aptamer and SURVIVIN-siRNA bound. This complex specifically targeted PSMA (+) tumor cells, demonstrating highly efficient siRNA delivery to target cells and increased apoptosis. Following systemic administration, this complex also showed significant efficacy at suppressing tumor growth in nude mice. The authors concluded that this therapeutic complex could specifically and efficiently deliver SURVIVIN-siRNA to target cells and suppress tumor growth in vivo, indicating its potential for use as a new strategy in prostate cancer therapy [200]. In another interesting paper, Liu et al. used nanoparticles containing a histidine-modified stearylized polyarginine stearginine (H3R5) peptide, an aptamer with specificity for hepatocellular carcinoma cells fused with a cell-penetrating peptide (ST21) conjugated to the H3R5 peptide as a targeting probe. miRNA-195 (miR195) is a potent genetic drug for inhibiting VEGF and fasudil for the suppression of vasculogenic mimicry by blocking ROCK2. In vitro and in vivo experiments confirmed that ST21-modified nanoparticles showed significantly higher cellular uptake and therapeutic efficacy in tumor cells or tumor tissues than their unmodified counterparts. The authors concluded that the aptamer-conjugated peptides hold great promise for the simultaneous administration of chemical and genetic drugs to combat hepatocellular carcinoma [201]. Another very interesting application of CPPs and aptamers is the functionalization of nanopores for the recognition of tumor cells. To this end, Guo et al. described a unique molecule double conjugated with an aptamer and a cell-penetrating peptide to identify and recognize Ramos cells. While the aptamer sequence provided specificity for the Ramos cell, the CPP helped the array of nanopores to reach the surface of the cell membrane and enter the cells [202]. In another interesting paper, Yamada et al. reported the development of a dual ligand liposomal system composed of octaarginine (R8) that enhances cellular uptake and an RP aptamer for mitochondrial targeting to allow a nanocarrier to be delivered efficiently to mitochondria. Surprisingly, cellular uptake of the R8-modified nanocarrier was facilitated by conjugation with the RP aptamer. In a confocal laser scanning microscopy analysis, the dual ligand-modified nanocarrier was shown to result in effective mitochondrial targeting via an ATP-dependent pathway, and was much more effective than a single ligand R8-modified nanocarrier. This is the first report of the regulation of intracellular trafficking by a nanocarrier system modified with mitochondrial RNA aptamers [203].

As described in the articles mentioned in this section, a lot of effort has been directed towards the development of CPPs in combination with aptamers for their potential application in the treatment of bacterial infections or cancer. However, its potential use in the treatment of parasitic diseases has not yet been shown. In this sense, the use of specific aptamers against *Leishmania* [204–207] or *Trypanosoma* proteins [208–211] could occur in conjunction with AMPs and CPPs to treat these neglected zoonosis, similar to what has been described in cancer models.

## 9. Conclusions

Considering the priorities established by the WHO in relation to the need to achieve affordable, safe, and efficient treatments for two of the most relevant neglected zoonoses, leishmaniasis and Chagas disease, AMPs and CPPs represent a potent alternative to conventional antibiotics. Several studies have demonstrated the activity of these natural peptides against intracellular trypanosomatids [8,13,79,80,212]. Among the distinguishing features with respect to conventional therapies, it is worth mentioning that AMPs and CPPs present little or no toxicity to mammalian cells and exert few anti-inflammatory effects [6,13]. The high biomedical potential of these peptides is being demonstrated. Several AMPs have been approved for antibacterial treatment (polymyxins and

daptomycin) by the FDA, and many other AMPs are in clinical development. However, to date, no CPPs or CPP–drug conjugates have been approved by the FDA due to some drawbacks (toxicity, endosomal entrapment, immunogenicity, and in vivo stability issues) that, as we have mentioned, are currently being resolved [6,77].

Therefore, the optimization of AMPs and CPPs through the use of emerging biotechnology, or the achievement of conventional therapies combined with the appropriate properties of AMPs and CPPs allows us to increase the potential of AMPs and CPPs by reducing toxicity and adverse effects and preventing the appearance of multiresistance. The effort being made by scientists around the world in the field of innovative therapies is commendable. The alternative based on antimicrobial peptide molecules against neglected zoonoses will be a reality in a short period of time.

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