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Article

Development of New Leishmanicidal Compounds via Bioconjugation of Antimicrobial Peptides and Antileishmanial Guanidines

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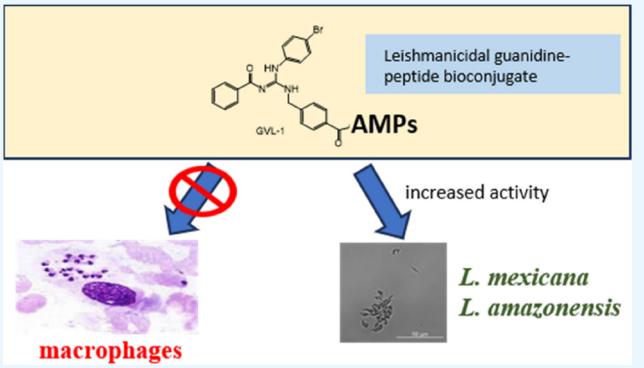
ABSTRACT: Leishmaniasis refers to a collection of diseases caused by protozoa from the *Leishmania* genus. These diseases, along with other parasitic afflictions, pose a significant public health issue, particularly given the escalating number of at-risk patients. This group includes immunocompromised individuals and those residing in impoverished conditions. The treatment of leishmaniasis is crucial, particularly in light of the mortality rate associated with nontreatment, which stands at 20−30,000 deaths per year globally. However, the therapeutic options currently available are limited, often ineffective, and potentially toxic. Consequently, the pursuit of new therapeutic alternatives is warranted. This study aims to design, synthesize, and evaluate the leishmanicidal activity of antimicrobial peptides functionalized with guanidine compounds and identify those with enhanced potency and selectivity against the parasite. Accordingly, three bioconjugates were obtained by using the solid-phase peptide synthesis protocol. Each proved to be more potent against intracellular amastigotes than their respective peptide or guanidine compounds alone and demonstrated higher selectivity to the parasites than to the host cells. Thus, the conjugation strategy employed with these compounds effectively contributes to the development of new molecules with leishmanicidal activity.



INTRODUCTION

Leishmaniasis refers to a collection of diseases caused by protozoa from the *Leishmania* genus. These diseases are prevalent in the American continent, East Africa, North Africa, and Western and Southeast Asia. Over the past two decades, the Pan American Health Organization[1] has reported 1,067,759 cases of cutaneous and mucosal leishmaniasis. Consequently, parasitic diseases pose a major public health challenge, particularly given the increasing number of immunocompromised individuals and those living under impoverished conditions. The therapeutic options for these diseases are limited, and the existing treatments are often ineffective and accompanied by side effects.[2,3] The necessity for leishmaniasis treatment is underscored by the global mortality rate associated with nontreatment, which stands at 20−30,000 deaths per year. Therefore, the pursuit of new, more selective therapeutic alternatives is both justified and essential. In this regard, Antimicrobial Peptides (AMPs) and their bioconjugates present a promising alternative, potentially paving the way for the development of novel, more effective therapies against *Leishmania*.

The primary mechanism of action for AMPs is their capacity to interact with, permeate, and disrupt cell membranes.[4,5] This process is influenced by several factors, including secondary structure, total net charge, size, and amphipathicity.[6] In bacteria, the selectivity of the AMP is primarily determined by the electrostatic attraction between the cationic AMPs and the negatively charged bacterial membrane surface.[7] Bacteria possess a higher negative charge than host cells, such as blood cells. Following the initial interaction, the peptide functions by forming pores (e.g., toroidal or barrel stave) or by disintegrating the lipid bilayer, which leads to micellization (carpet-like).[8]

Most AMPs typically follow a similar mechanism of action to assert their antiparasitic activity, primarily targeting the cell

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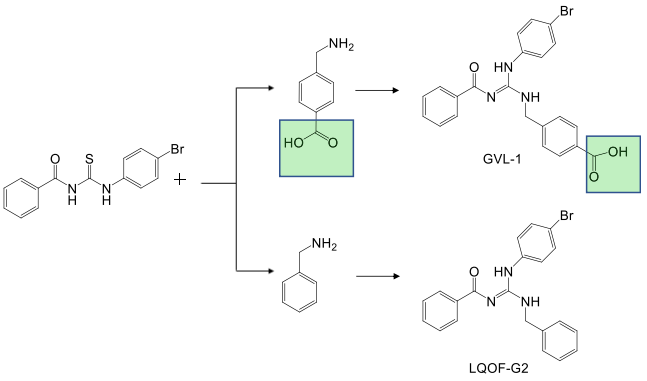
membrane as the main target.[9] The cell membrane of *Leishmania* mainly consists of negatively charged phospholipids, with phosphatidylinositol and phosphatidylserine being the most prevalent. These phospholipids are anchored to the glycocalyx. The composition of this membrane varies depending on the parasite's morphology and developmental stage, and it is more negatively charged than mammalian cell membranes. This difference allows AMPs to act more selectively.[10] In addition to targeting cell membranes, AMPs have been found to employ other mechanisms. Antiparasitic peptides may target intracellular processes; they may induce necrosis and apoptosis-like processes, inhibit mitochondrial ATP synthesis, interfere with cell wall synthesis, disrupt parasite bioenergetics, or inhibit cysteine protease, among others, leading to the parasite's death.[11−13] Despite the great potential of AMPs, several challenges, including potential human toxicity, susceptibility to proteases, production costs, and the rarer bacterial resistance,[14] hinder their general therapeutic applications. To overcome these issues, strategies, such as bioconjugation or dimerization, can be employed to enhance the pharmaceutical properties of AMPs. Numerous studies have demonstrated the effectiveness of this strategy in developing antimicrobial, anticancer, antifungal,[15−17] and antileishmanial[18,19] compounds.

Peptide conjugation is a comprehensive strategy employed to enhance the selectivity and potency of peptides,[20] thereby bolstering the benefits of peptide-based pharmacology with medicinal chemistry.[21] The conjugates utilized in peptide therapeutics may comprise poly(ethylene glycol), ferrocene, lipids, or other molecular types. Our group has leveraged this conjugation to augment the activity of AMP against cancer,[16,17] bacteria, fungus,[18] and viruses.[17,22] Furthermore, peptide−drug conjugates have been instrumental in the development of antiplasmodial compounds.[19,23]

In this study, AMPs were conjugated with a guanidine compound at the N-terminus of the peptides to create more selective and potent compounds. A peptide from the temporins class TSHa was selected for two reasons: (1) it demonstrated anti-*Leishmania infantum (L. infantum)* activity;[24] and (2) its positive charge, which facilitates electrostatic interaction with the *Leishmania* membrane, thereby increasing its selectivity to the parasite. The nonmembranolytic dimeric cationic peptide p-Bt (desCys^{11}/Lys^{12}/Lys^{13}(p-BthTX-I)$_2$K) is an analogue of the BthTX-I peptide[25] from *Bothrops jararaca* venom. This peptide possesses antiviral[26] and antimicrobial activity against both Gram-positive and Gram-negative bacteria, with relatively low toxicity.[27] Its mechanism of action differs from that of TSHa. Furthermore, this dimeric peptide allows for bioconjugation with two guanidines in their N-terminus groups.

The peptides were bioconjugated with guanidine molecules, which possess antiparasitic activity.[28] Guanidines, a broad class of compounds, are found in nature including in plants, microorganisms, and terrestrial animals. They have demonstrated a range of biological activities, such as anti-inflammatory,[29] antibacterial, antifungal,[30] and antiprotozoal.[13,28,31] They have also been studied in the context of diabetes.[32] Their antiparasitic activity, specifically leishmanicidal,[28] has also been reported. The *N*-benzoyl-*N*′-benzyl-*N*″(4-bromophenyl)guanidine LQOF-G2 (Scheme 1)[28] exhibited potent antiamastigote activity ($IC_{50\text{-ama}}$ = 5.6 μM) against *Leishmania amazonensis (L. amazonensis)*, comparable to that of amphotericin B (Amph B, $IC_{50\text{-ama}}$ = 4.9 μM). Further, the guanidine compound demonstrated superior efficacy and

Scheme 1. Synthesis of Guanidines LQOF-G2 and GVL1



selectivity.[28] Guanidines have been reported to act as cysteine protease inhibitors, offering potential for the treatment of Chagas disease.[33] Furthermore, the guanidine derivative (*Z*)-*N*-benzoyl-*N*′-benzyl-*N*″-(4-tertbutylphenyl)guanidine (LQOF-G6) targets the *Leishmania* cysteine protease.[13] Guanidine LQOF-G6 reduced the parasite load in mice infected with *L. amazonensis* and exhibited low toxicity in organs and cells,[13] indicating its potential as a future therapeutic agent.

The objective of this study was to bioconjugate AMPs and guanidine. To achieve this, a carboxyl group was introduced to the LQOF-G2 compound, resulting in the creation of (Z)-4-((2-benzoyl-3-(4-bromophenyl)guanidino)methyl) benzoic acid, also known as GVL1. This was then coupled to the N-terminal end of the TSHa peptide (FLSGIVGMLGKLF) or the dimeric p-Bt peptide [(KKYRYHLKPF)$_2$K-NH$_2$]. The resulting conjugation of GVL1 with these two peptides led to the formation of new bioconjugates, which demonstrated enhanced leishmanicidal properties and selectivity.

RESULTS AND DISCUSSION

Guanidine compounds have been extensively researched, owing to their significant biological potential. They exhibit a broad spectrum of action against several diseases, specifically targeting protozoan parasites, such as *Trypanosoma brucei*, *Trypanosoma cruzi*, *Plasmodium falciparum*, and *Leishmania* spp.[34,35] In this study, LQOF-G2 was altered by incorporating a carboxylic group into its benzyl aromatic structure (Scheme 1). The synthesis process involved the reaction of 4-(aminomethyl)benzoic acid (CAS:56−91−7), in place of *N*-benzylamine, with the appropriate thiourea to produce the final product GVL1. Detailed structural characterization of compound GVL1 can be found in the Supporting Information section.

The compound GVL1 demonstrated leishmanicidal activity against both *L. amazonensis* and *L. Mexicana*, as indicated in Table 1. Existing literature suggests that the guanidine LQOF-G2, owing to the presence of a bromine atom in the aniline moiety, possesses both in vitro and in vivo activity against *L. amazonensis*. Furthermore, LQOF-G2 has been observed to exhibit low toxicity in murine red blood cells and peritoneal macrophages, with a high degree of selectivity for the parasite.[36]

The leishmanicidal activity of GVL1 guanidine decreases in the presence of a carboxyl group. This group, which carries a negative charge at physiological pH, may impede membrane permeation, thereby affecting the guanidine's action on internal

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Table 1. Results for Antileishmanial Assays of TSHa, GVL1, and Bioconjugate GVL1-TSHa against *L. amazonensis*

compound	IC_{50} (μM) promastigote	CC_{50} (μM) peritoneal macrophages	IC_{50} (μM) amastigote	SI
LQOF-G2	19.6 ± 0.2	737.9 ± 1.4	5.6 ± 2.5	131.8
GVL1	34.4 ± 0.1	2000 ± 0.013	8.9 ± 1.41	225
TSHa	8.0 ± 0.1	1000 ± 0.02	6.8 ± 2.82	294
GVL1-TSHa	5.4 ± 0.1	2000 ± 0.02	0.33 ± 0.04	6060
Amph B	0.7 ± 0.2	24 ± 0.4	0.63 ± 0.02	38

targets.[37−39] Conversely, the incorporation of the carboxylic group into the guanidine reduces cytotoxicity (CC_{50} > 2000 μM). Consequently, despite an increase in the $IC_{50\text{-}AMA}$, the selectivity index (SI) of the new compound GVL1 surpasses that of LQOF-G2 (Table 1).

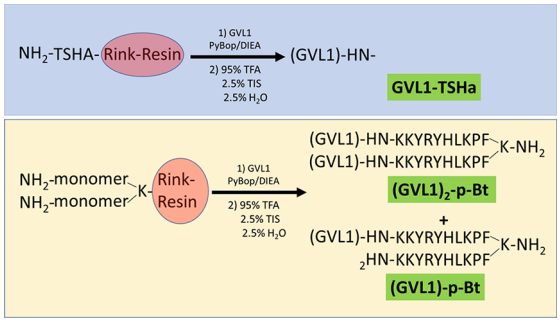
The TSHa demonstrated IC_{50} values of 8.0 and 6.8 μM against *L. amazonensis* in promastigote and amastigote forms, respectively. This value for the promastigote form is comparable to that reported for this peptide by Raja et al.[40] When compared with the control drug Amph B, both GVL1 and TSHa peptides exhibited superior selective indices of 225 and 294, respectively. These values are approximately six times higher than that of Amph B (Table 1). In the case of *L. Mexicana*, both GVL1 and TSHa displayed notable biological activity with IC_{50} values of 51.8 and 6.3 μM, respectively, for the promastigote form and 7.5 and 6.7 μM, for the amastigote form (Table 2).

Table 2. Results for Antileishmanial Assays Based on 48 h of Treatment with TSHa and GVL1 Alone and the Bioconjugate GVL1-TSHa against *L. mexicana*

compound	IC_{50} (μM) promastigote	CC_{50} (μM) peritoneal macrophages	IC_{50} (μM) amastigote	SI
GVL1	51.8 ± 0.10	2000 ± 0.013	7.5 ± 3.53	267
TSHa	6.3 ± 0.85	1000 ± 0.01	6.73 ± 0.1	148
GVL1-TSHa	1.85 ± 0.07	2000 ± 0.02	0.83 ± 0.7	2410
Amph B	0.45 ± 1.2	24 ± 0.02	0.73 ± 0.2	33

The bioconjugate GVL1-TSHa was synthesized by using solid-phase peptide synthesis (SPPS). The protocol employed was straightforward, requiring no additional procedures beyond those used in SPPS. This can be attributed to the stability of GVL1 guanidine in the solvent and reagents used in SPPS with the Fmoc protocol. The GVL1 guanidine was coupled to the TSHa via an amide bond with the N-terminus group (Scheme 2) of the TSHa peptide. Following the synthesis, the bioconjugate was purified and the molecular mass of the desired compound was confirmed by mass spectrometry (Figure 1). The compound GVL1-TSHa demonstrated superior activity against *L. amazonensis*, exceeding that of either the peptide or guanidine alone, with an IC_{50} less than 6.0 μM against promastigotes (Table 1) and 330 nM against amastigotes. This bioconjugate, like GVL1 and TSHa, exhibited low cytotoxicity with a CC_{50} of greater than 2000 μM. GVL1-TSHa was nearly twice as active against the amastigote form of *L. amazonensis* compared to the traditional drug Amph B. Furthermore, the SI of the bioconjugate was much higher (greater than 6060) than that of Amph B, indicating the capacity of the bioconjugation strategy with

Scheme 2. General Representation of the Synthesis of Bioconjugates GVL1-TSHa, (GVL1)-p-Bt and $(GVL1)_2$-p-Bt



guanidine compounds. This potential is due to the increase in leishmanicidal activity coupled with a decrease in toxicity.

The compounds were also evaluated against *L. Mexicana* (Table 2). The bioconjugate demonstrated greater activity against both the promastigote and amastigote forms than either GVL1 or TSHa alone, yielding results comparable to those of Amph B for the amastigote form. Notably, the bioconjugate exhibited high selectivity for the parasite with minimal impact on host cells, as evidenced by the high bioconjugate SI (2410). This data substantiate the exceptional biological activity of bioconjugate.

TSHa, an amphipathic α-helical structured peptide, permeabilizes the membranes of *L. infantum* and *L. amazonensis* and exerts leishmanicidal activity through a membranolytic mechanism.[40] Even at sublytic concentrations, TSHa temporin permeabilizes the parasite membrane, promoting apoptosis. To assess the significance of the peptide's structure and action mechanism in the leishmanicidal activity of the biconjugate, we employed a different peptide in this work. This peptide that lacks a well-defined structure or membranolytic action mechanism[41] was not cytotoxic. Enzymatic inhibition underlies its antiviral activity,[42] also demonstrating its ability to penetrate the cell. This peptide could traverse the parasite membrane and transport the guanidine into the parasite, thereby enhancing the biological activity.

The peptide p-Bt features a single lysine residue at its C-terminal end ($desCys^{11}/Lys^{12}/Lys^{13}$(p-BthTX-I)$_2$K), which forms a dimer with two N-terminal α-amine groups. This structure enables the coupling of two guanidine molecules (Scheme 2). The guanidine coupling process was conducted by using the same methodology employed in the synthesis of GVL1-TSHa. As indicated by the chromatographic profile (Figure 2), the coupling process yielded two compounds, each containing either one ((GVL1)-p-Bt) or two guanidine molecules ($(GVL1)_2$-p-Bt) (Figure 2). The synthesis of a bioconjugate containing a single GVL1 molecule may be attributed to steric hindrance and the lesser excess of GVL1 used in the synthesis. The use of a greater excess of GVL1 resulted solely in the synthesis of ($(GVL1)_2$-p-Bt) (data not shown).

The wild-type dimeric peptide (p-Bt) exhibited no leishmanicidal activity against the promastigote form of the parasite, as shown in Table 3. Similarly, this peptide demonstrated no toxicity against peritoneal macrophages. These findings align with those of Bitencourt et al.,[42] who reported minimal hemolytic activity, below 5%, even at a

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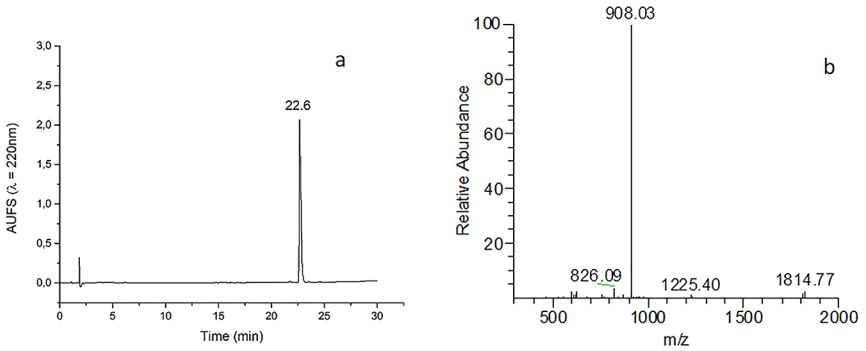

Figure 1. RP-HPLC chromatogram of the pure GVL1-TSHa conjugate (a). Mass spectrum of pure GVL1-TSHa (b). Theoretical MW = 1,813.8 g mol^{-1}, obtained m/z = 1814.8 (Z = 1); 908.0 (Z = 2).

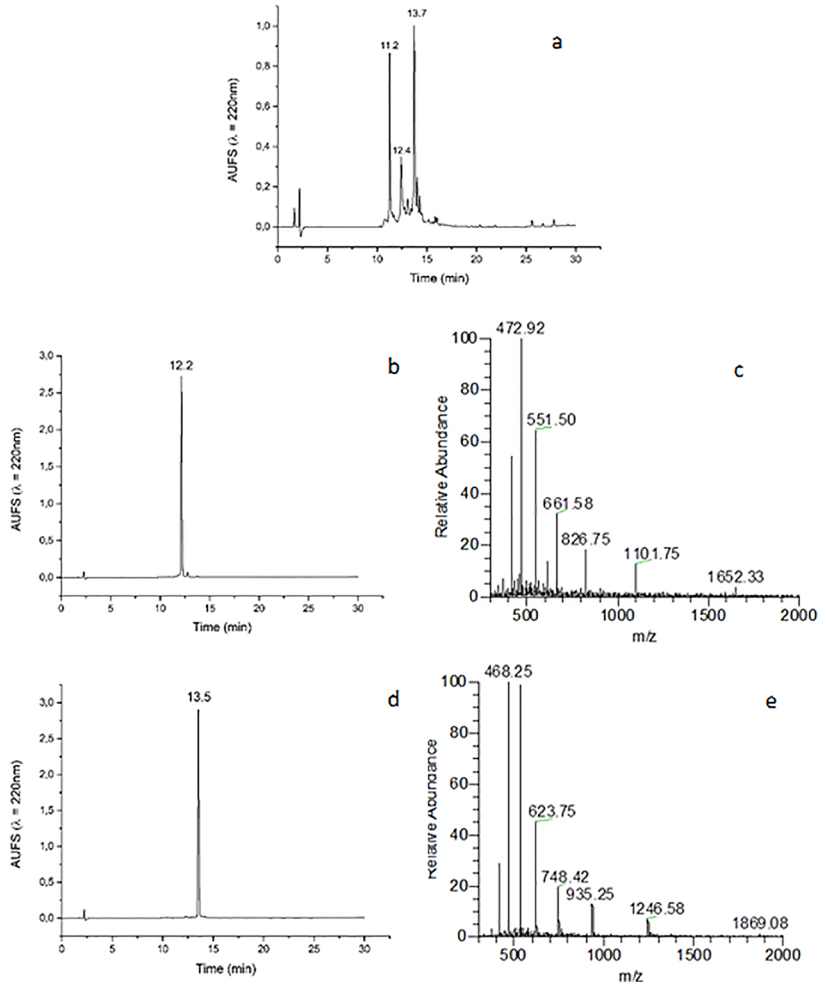

Figure 2. RP-HPLC chromatogram of the crude p-Bt peptide, retention time of 11.2 min (a). RP-HPLC chromatogram of the pure bioconjugate (GVL1)-p-Bt, retention time 12.2 min (b). Mass spectrum of the pure bioconjugate (GVL1)-p-Bt (c). Theoretical MW = 3,301.5 g mol^{-1}, obtained m/z = 413.9 (Z = 8); 472.9 (Z = 7); 551.5 (Z = 6); 661.6 (Z = 5); 826.8 (Z = 4); 1101.8 (Z = 3); and 1652.3 (Z = 2). RP-HPLC chromatogram of the pure bioconjugate $(GVL1)_2$-p-Bt, retention time of 13.5 min (d). Mass spectrum of the pure bioconjugate $(GVL1)_2$-p-Bt (e). Theoretical MW = 3,734.5 g mol^{-1}, obtained m/z = 468.3 (Z = 8); 535.5 (Z = 7); 623.8 (Z = 7); 748.4 (Z = 5); 935.3 (Z = 4); and 1246.6 (Z = 3).

concentration of 512 μg mL^{-1}, suggesting the nontoxic nature of p-Bt. The introduction of guanidine to p-Bt enhanced the antipromastigote activity of GVL1. The IC_{50} of the bioconjugate with one GVL1 ((GVL1)-p-Bt) against promastigotes was nearly 34 times lower (IC_{50} of 34.4−1.1 μM) than that of GVL1 alone. For the amastigote form, the IC_{50} of (GVL1)-p-Bt was eight times lower than that of guanidine alone. Moreover, the selectivity of this bioconjugate was almost 10 times higher than that of GVL1. These findings suggest a great increase in the potency of the bioconjugate with enhanced specificity and selectivity. The $(GVL1)_2$-p-Bt (peptide with two guanidines) also displayed higher leishmanicidal activity against the amastigote form than GVL1 (almost three times) and p-Bt (four times) alone. These results strongly suggest that the p-Bt peptide and their bioconjugates function as cell-penetrating peptides (CPPs),

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Table 3. Results for Antileishmanial Assays of p-Bt Bioconjugates with One or Two Molecules of GVL1 against *L. amazonensis*

compound	IC_{50} (μM) promastigote	CC_{50} (μM) peritoneal macrophages	IC_{50} (μM) amastigote	SI
GVL1	34.4 ± 0.13	2000 ± 0.013	8.9 ± 1.41	225
p-Bt	NA[a]	2000 ± 0.01	7.50 ± 0.15	266.6
(GVL1)-p-Bt	1.10 ± 0.2	550 ± 0.011	0.9 ± 0.07	2222
$(GVL1)_2$-p-Bt	6.25 ± 0.035	2000 ± 0.11	2.25 ± 0.35	889
Amph B	0.7 ± 0.2 (34.3)	24 ± 0.4	0.63 ± 0.02	38

[a]Did not show activity.

selectively penetrating the parasite and enabling the guanidine to act on internal targets, such as enzymes.[43]

A comparison of the activities of the bioconjugate with those of one and two GVL1 molecules revealed that (GVL1)-p-Bt was more active than $(GVL1)_2$-p-Bt. This outcome can be attributed to the significance of the N-terminal charge. It has been established that the N-terminal region plays crucial roles in the mechanism of action of hylin-a1[44] and sticholysins I and II.[45] The presence of a single N-terminal positive charge in one of the chains could enhance the peptide−membrane interaction, facilitating pore formation and, consequently, the transport of guanidine into the macrophages, ultimately targeting the parasite. When compared with the control drug Amph B, (GVL1)-p-Bt, despite having similar potency (IC_{50} = 900 nM), is more selective (SI = 2222) and less toxic in vitro than Amph B against the amastigote form. These findings affirm the potent antileishmanial activity of the peptide−drug conjugates and their potential for the development of new compounds.

The biological data obtained indicate that both GVL1-peptide bioconjugates (TSHa and p-Bt) exhibit higher activity and selectivity than those of isolated guanidine. This can be attributed to the permeability capacity of the molecules and the action of cell penetration peptides, also known as CPPs.[46] When guanidine is coupled with the peptides, the compound's passage into cells through the lipid membrane is likely facilitated by the polarity and charge characteristics of the molecules. This makes the peptide a carrier for guanidine entry, which can act against intracellular targets. Previous studies have shown that guanidines can induce cell death by depolarizing the mitochondrial membrane, increasing reactive oxygen species levels, and enhancing the plasma membrane permeability.[36] The peptides under investigation carry a positive charge, which facilitates interaction with intracellular parasites through electrostatic forces. In other words, the peptides' permeation through the host cell plasma membrane and the parasitophorous vacuole may be facilitated, enabling them to reach their final target, the intracellular parasite.[47] Furthermore, the GVL1 conjugated to the peptides may enhance the peptides' potency, as it can alter the parasite's membrane permeability, potentially triggering cell death.[48−50] In addition to the pore mechanism, conjugated peptides may also redistribute membrane components, such as sterols, form specific phospholipid microdomains, or directly interact with membrane proteins, altering their function.[51]

The infection rate was also evaluated to supplement the above results, specifically for bioconjugates (Figures 3−5). During infection, amastigotes replicate within macrophages and are transmitted to healthy cells, thereby amplifying the infection. This transfer of amastigotes from infected cells to healthy cells[58] is measured by the infection rate. The GVL1-TSHa demonstrated a lower infection rate than those of the control as well as the peptide or guanidine alone against *L.*

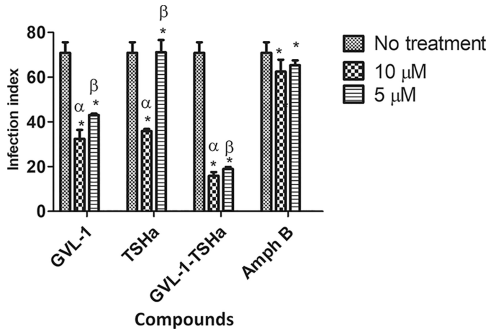

Figure 3. Infection indexes of the GVL1, TSHa, GVL1-TSHa, and Amph B against intracellular amastigotes of *L. amazonensis* after 24 h of treatment at 10 and 5 μM of each compound. The negative control was untreated *L. amazonensis* intracellular amastigotes. The data are expressed as means plus standard deviations (SD) for three independent experiments with two-way ANOVA analysis ($P < 0.05$), where * indicates statistical differences between the tested compounds and the untreated control, and different letters indicate statistical difference between the tested concentrations of each compound.

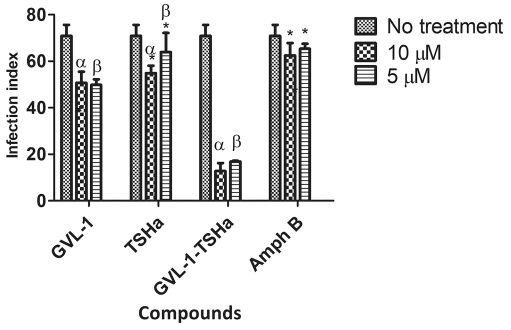

Figure 4. Infection indexes of the GVL1, TSHa, GVL1-TSHa, and Amph B against intracellular amastigotes of *L. mexicana* after 48 h of treatment at 10 and 5 μM of each compound. The negative control was untreated *L. amazonensis* intracellular amastigotes. The data are expressed as means plus standard deviations (SD) for three independent experiments with two-way ANOVA analysis ($P < 0.05$), where * indicates statistical differences between the tested compounds and the untreated control, and different letters indicate statistical difference between the tested concentrations of each compound.

amazonensis and *L. mexicana* (Figures 3 and 4). In *L. amazonensis*, GVL1-TSHa (Figure 4), (GVL1)-p-Bt, and $(GVL1)_2$-p-Bt (Figure 5) exhibited a 3−4-fold lower infection rate at the tested concentrations than did untreated cells and the control drug Amph B. It is suggested that the effects of bioconjugates on amastigotes likely occur within the parasitophorous vacuoles, with the compounds interacting with the cell membranes of macrophages and subsequently with the cell membrane of the parasite. This interaction may cause

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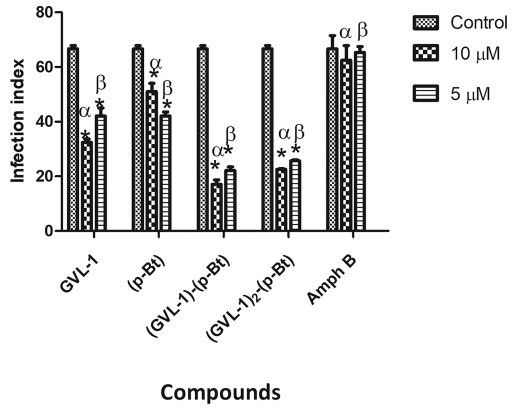

Figure 5. Infection indexes of the GVL1, p-Bt, (GVL1)-p-Bt × $(GVL1)_2$-p-Bt, and Amph B against intracellular amastigotes of *L. amazonensis* after 24 h of treatment at 10 and 5 μM of each compound. The negative control was untreated *L. amazonensis* intracellular amastigotes. The data are expressed as means plus standard deviations (SD) for three independent experiments with two-way ANOVA analysis ($P < 0.05$), where * indicates statistical differences between the tested compounds and the untreated control, and different letters indicate statistical difference between the tested concentrations of each compound.

alterations in the flow of sodium and potassium, leading to an increase in cell volume and rupture of the parasite cell, possibly through pore formation or a carpet-like.[52,53] Literature data indicate that AMPs can reduce the infection rate in *L. amazonensis*. For instance, the peptide Filoseptina-1 (PSN-1) decreased the number of infected cells compared with it did untreated cells.[52] This peptide demonstrated concentration-dependent activity in amastigotes with higher tested concentrations resulting in a greater decrease in the infection rate. Moreover, this peptide had a negligible effect on the NO production and TGF-β release. All bioconjugates evaluated in this study exhibited higher activity than PSN-1. The decrease in the infection rate may be explained by the interference of bioconjugates in the metabolic pathways of the parasite. For example, three magainin analogues (MG-H1, MG-H2, and MG-F5W magainin-2) demonstrated a decrease in ATP production in a dose-dependent manner in *Leishmania donovani* cultures, leading to parasite death.[54]

CONCLUSIONS

We have successfully designed and synthesized selective bioconjugates that incorporate guanidines and AMPs. These bioconjugates demonstrate low micromolar/nanomolar $IC_{50\text{-}AMA}$ values against both *L. Amazonense* and *L. Mexicana* and exhibit reduced toxicity toward peritoneal macrophages. These compounds represent a singular and viable synthesis approach to antileishmanial treatment, holding promise for the advancement of therapeutic development. The enhanced leishmanicidal activity of these bioconjugates, achieved through the coupling of guanidine with AMPs, may be attributed to multiple mechanisms of action, given that guanidine and AMPs both have distinct targets. Studying the action mechanisms of these conjugates could offer valuable insights into the development of new therapeutics against *Leishmania*, a significant global public health concern.

EXPERIMENTAL PROCEDURES

Synthesis of Guanidine Compounds. The guanidine compound was synthesized at the Laboratory of Fine Organic Chemistry, Department of Chemistry and Biochemistry, Faculty of Science and Technology, Unesp Campus of Presidente Prudente. This institution was responsible for the planning, synthesis, and characterization of the precursor. The synthesis was carried out according to the reported procedure.[28] Instead of using *N*-benzylamine, 4-(aminomethyl)benzoic acid (CAS:56-91-7) was employed to react with the selected thiourea, resulting in a functionalized compound with a carboxyl group. Following purification and structural characterization, the compound (Z)-4-((2-benzoyl-3-(4-bromophenyl)guanidino)methyl) benzoic acid was obtained with a purity exceeding 95%, as confirmed by NMR and GC-MS (GVL1).

Peptide Synthesis and Bioconjugation to GVL1 Compound. The peptides were synthesized using SPPS methodology, which is based on the residue-by-residue growth of the peptide chain. This chain is covalently linked by its carboxy-terminal amino acid to the reactive sites on a solid support (resin),[55] following a Fmoc protocol.[56] The experimental conditions mirrored those used by Lorenzón et al. in 2013.[57] The Rink-amide resin[58] was utilized to achieve the peptide in carboxy-amide form after the cleavage step. For the synthesis of the dimeric peptide dimer ($desCys^{11}$/Lys^{12}/Lys^{13}(p-BthTX-I)$_2$K, referred to as p-Bt), one Fmoc-Lys-(Fmoc)−OH was attached to the resin. Following the deprotection of the α- and ε-Fmoc groups with 20% 4-methylpiperidine/dimethylformamide for 2 and 20 min, respectively, the two chains of p-BtTX-I (KKYRYHLKPF) were simultaneously elongated.

The bioconjugation strategy involved coupling the compound GVL1, which was in 1.2-fold excess over the amino component, to the N-terminus of peptidyl-resins containing the TSHa peptide and the dimer (p-BthTX-I)$_2$K. This was achieved using the activator PyBop (benzotriazol-1-yloxytripyrolidinophosphonium hexafluorophosphate) and DIPEA (*N*,*N*-diisopropylethylamine), at 2-fold and 4-fold excess over the amino component, respectively, for a duration of 24 h. The pH was maintained at approximately 9. The resulting products were purified using semipreparative HPLC, employing solvent A (water with 0.045% TFA) and solvent B (acetonitrile with 0.036% TFA). The flow rate was set at 5 mL min^{-1}, with detection at 220 nm, on a C18 reversed-phase column (Jupiter Proteo) measuring 25 cm × 10 mm and containing 5 μm particles. The peptides were purified using solvent concentration gradients, which were determined based on the hydrophobicity of each molecule. The purity of each fraction was ascertained using HPLC in an analytical mode with a Shimadzu spectrometer and a C18 column (25 cm × 10 mm). Detection was set at 220 nm, using a gradient method from 5 to 95% solvent B over 30 min with a flow rate of 1 mL min^{-1}. The molecular weight of the peptides was analyzed using mass spectrometry (Thermo LCQ-fleet, with ESI-IT-MS configuration), with an ion-trap analyzer in positive electrospray mode $(M^+H)^+$, and a range of 200−2000 g mol^{-1}.

Biological Assays. *L. amazonensis* promastigotes (MPRO/BR/1972/M1841-LV-79) were grown in liver infusion tryptose (LIT) medium, while *L. mexicana* (MNYC/BZ/62/M379) were cultivated in Schneider's medium (Sigma), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco/

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Invitrogen).[59] Both were incubated at 28 °C until reaching the mid-log phase of growth. Macrophages were sourced from the peritoneal cavity of Swiss mice, using the methodology outlined in de Almeida-Amaral et al.[59] For antipromastigote assays, *L. mexicana* and *L. amazonensis* cultures in the exponential growth phase were transferred to 96-well plates, achieving a final concentration of 1×10^7 promastigotes mL^{-1}. Subsequently, the tested compounds were added at concentrations ranging from 1.28 to 100 μM to assess antipromastigote activity. After 72 h, viable promastigotes were counted in a Neubauer chamber with the presence of trypan blue.

To determine cytotoxic activity, murine peritoneal macrophages (3×10^5 cells mL^{-1}) were cultured in a complete RPMI-1640 (SIGMA) medium within 96-well plates. Cell adhesion was facilitated at 37 °C in a 5% CO_2 environment for 4 h. The compounds under investigation were tested at concentrations ranging from 1.28 to 2000 μM. The MTT colorimetric method was used, it is based on the determination of the ability of living cells to reduce 3-4(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT).[60] The drug concentration that inhibits 50% of cell growth is denoted as the 50% cytotoxic concentration (CC_{50}). Cytotoxicity for host cells and protozoan species was compared and expressed as the SI, calculated as the ratio of CC_{50} for macrophages to IC_{50} for the protozoan species.

The antiamastigote activity was assessed against macrophages infected with *Leishmania*. Murine peritoneal macrophages (3×10^5 cells mL^{-1}) were cultured in a complete RPMI-1640 medium and distributed onto 13 mm diameter circular coverslips placed in the wells of 24-well plates. The cells were adhered at 37 °C in a 5% CO_2 environment for 4 h. The macrophages were then infected with *L. amazonensis* or *L. mexicana* promastigotes at the stationary phase, using a ratio of 10:1 (promastigotes/cell), and incubated at 37 °C in 5% CO_2 for an additional 18 or 8 h, respectively. Subsequently, nonphagocytosed parasites were removed by washing, and 20 μL of the test compounds were added at varying concentrations from 0.02 to 10 μM. The infected cells were then incubated at 37 °C with 5% CO_2 for 24 h for assays with *L. amazonensis* or 48 h for assays with *L. mexicana*. Amph B was used as a control in all assays. The infection rate and $IC_{50\text{-AMA}}$ were determined through microscopic analysis, following the Giemsa-staining procedure. The infection index (percentage of infected cells × number of intracellular parasites/number of infected cells) was calculated, and the IC_{50} value was determined using nonlinear regression. All assays were performed in duplicate across three independent experiments.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c04878.

Detailed experimental procedures and characterization data for compound GVL1 (PDF)

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N.C.S.C., L.R.A., J.V.M.S., M.C.O.A.B., and V.P.M. carried out the experimental work. E.M.C., E.R.P.G., and M.A.S.G. conceptualized the study and supplied the laboratory resources and infrastructure for the experimental studies. All authors contributed to the writing and review of the manuscript.

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Notes

The authors declare no competing financial interest.

This research did not involve human subjects. Animal experiments were conducted in accordance with the protocol approved by the Institutional Ethics Committee (CEUA), under the Ethics Committee in the Use of Animals (CEUA/FCF/CAr) protocol number 03/2019.

All data are available upon request to the email eduardo.cilli@unesp.br.

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